ISSN: 2158-284X Volume 10, Number 3, March 2019

Scientific Research Publishing

International Journal of Clinical Medicine

ISSN: 2158-284X
9 772158 284007 03

www.scirp.org/journal/ijcm

Journal Editorial Board

ISSN: 2158-284X (Print) ISSN: 2158-2882 (Online)
http://www.scirp.org/journal/ijcm

Scientific Research Publishing

International Journal of Clinical Medicine, 2019, 10, 101-249
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Table of Contents

Volume 10 Number 3 March 2019

International Journal of Clinical Medicine (IJCM)
Journal Information

SUBSCRIPTIONS

The *International Journal of Clinical Medicine* (Online at Scientific Research Publishing, www.SciRP.org) is published monthly by Scientific Research Publishing, Inc., USA.

Subscription rates:
Print: $79 per issue.
To subscribe, please contact Journals Subscriptions Department, E-mail: sub@scirp.org

SERVICES

Advertisements
Advertisement Sales Department, E-mail: service@scirp.org

Reprints (minimum quantity 100 copies)
Reprints Co-ordinator, Scientific Research Publishing, Inc., USA.
E-mail: sub@scirp.org

COPYRIGHT

PRODUCTION INFORMATION

For manuscripts that have been accepted for publication, please contact:
E-mail: ijcm@scirp.org

International Journal of Clinical Medicine, 2019, 10, 101-108
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

The Application and Operation-Effect Analysis for Complex Tibial Plateau Fractures with 3D Printing Technique

Changjin Guo[1], Yubo Zhang[2], Li Yang[1], Qiaofeng Zhu[1], Sanming Zou[2*]

[1]Medical College, Wuhan University of Science and Technology, Wuhan, China
[2]Department of Orthopaedics, Xiaogan Central Hospital, Wuhan University of Science and Technology, Xiaogan, China
Email: 765312069@qq.com, *xgzsmys@163.com

How to cite this paper: Guo, C.J., Zhang, Y.B., Yang, L., Zhu, Q.F. and Zou, S.M. (2019) The Application and Operation-Effect Analysis for Complex Tibial Plateau Fractures with 3D Printing Technique. *International Journal of Clinical Medicine*, **10**, 101-108.
https://doi.org/10.4236/ijcm.2019.103010

Received: January 30, 2019
Accepted: March 2, 2019
Published: March 5, 2019

Abstract

Objective: To investigate the value of 3D printing techniques in the treatment of complex tibial plateau fractures. **Methods:** From September 2016 to September 2018, 28 patients with complex tibial plateau fractures were treated in our hospital. According to the odevity of hospitalized order, the patients were divided into two groups. Group A used 3D reconstruction, virtually reduction, 3D printing and demonstration of individual fracture model before operation while group B only received conventional process by use X-rays or CT image. Comparison between the two groups was made in operation time, operative blood loss, radiation frequency, surgery instrument cost and knee function score. **Results:** The follow-up was 14.4 months on average (ranged 6 to 22 months). There was no statistical difference of the surgery instrument cost between the 2 groups ($P > 0.05$). The operation time of group A was significantly shorter than that of group B ($P < 0.05$). Group A also performed better than group B in comparison of operative blood loss and radiation frequency. The excellent good rate of HSS score in group A was 92.86%; it was higher than that 85.71% in group B. There was no statistical difference between the 2 groups ($\chi^2 = 0.373$, $P = 0.54$). **Conclusion:** 3D printing techniques can improve surgery effect in complex tibial plateau fractures.

Keywords

Tibial Plateau Fracture, 3D Printing Technique, Individualized Treatment

1. Introduction

Complex tibial plateau fracture is one of the most common and complex frac-

tures in limb trauma, in which the knee joint suffers from inversion or vertical compression violence, resulting in unicondylar or bicondylar fractures of the medial and lateral tibial plateau condyles. Large data analysis shows that tibial plateau fractures account for 1% - 2% of the total body fractures and 8% of the elderly fractures [1]. With the increasing incidence of accidents and traffic accidents in recent years, the incidence of various complex injuries has gradually increased, and the incidence of tibial plateau fractures has also increased. Surgical treatment of complex tibial plateau fractures requires a high standard of surgeons. Since it is a complex intra-articular fracture with high energy, it is often accompanied by severe articular surface collapse, ligament injury around the knee joint and poor soft tissue condition. Therefore, the incidence of early complications such as skin ischemic necrosis, infection, poor wound healing, bone and internal fixation exposure, as well as late complications such as long-term pain, postoperative dysfunction, and traumatic osteoarthritis is higher than other fractures. The way to restore the smooth joint surface as much as possible, and at the same time to achieve the minimum damage to the soft tissue, is the primary problem in front of the surgeon. In recent years, the rapid development of digital software and 3D printing technique in China has provided a rare opportunity to solve the above problems. Orthopaedics surgeons can design and simulate the operation on the 3D printing model in advance, so as to improve the surgical treatment effect. In 2014, the team of Professor Wang Liao and Professor Dai Kerong had introduced the individualized orthopaedic treatment and 3D printing technique in detail [2], bringing digital orthopaedic surgery into the vision of domestic orthopaedic physicians. Therefore, the application of 3D printing technique in complex tibial plateau fracture surgery is an effective means to realize the individualization and precision of orthopaedic surgery.

2. Materials and Methods

2.1. General Materials

28 patients admitted to and treated in our hospital with complex tibial plateau fractures from September 2016 to September 2018 were enrolled in the clinical trial, including 17 males and 11 females, aged from 19 to 62 years, with an average age of 38.8 years. Unified inclusion and exclusion criteria were adopted for the study objects. All patients were with complicated tibial plateau fractures and elective surgery, with informed consent. Patients with pathological fractures, multiple fractures of the same limb, combined with nerve and blood vessel injuries, dislocation of knee joint and intolerance for the surgery were excluded. Preoperative MRI examination was performed to exclude patients with meniscus or ligament injury. According to Schatzker classification: There were 11 cases of type II, 7 cases of type IV, 8 cases of type V and 2 cases of type VI. All the surgeries were performed by the same surgeon and the corresponding team. The patients were divided into groups according to the order of admission. Patients with odd number of admission orders were classified into group A, and patients

with even number were classified into group B. There were 14 cases in group A, 9 males and 5 females; aged from 19 to 58 years, with an average age of 38.13 years. For the fracture classification: 5 cases of type II, 3 cases of type IV, 5 cases of type V and 1 case of type VI. There were 14 cases in group B, 8 males and 6 females; aged from 21 to 62 years, with an average age of 38.47 years. For the fracture classification: 6 cases of type II, 4 cases of type IV, 3 cases of type V and 1 case of type VI. Inclusion criteria: patients meeting the diagnostic criteria for complex tibial plateau fractures; patients with severe complications and open fractures were excluded. All the patients are able to tolerate surgery and anesthesia, and informed consent to the treatment.

2.2. Grouping Data Processing and 3D Reconstruction Software Application Analysis

All patients in group A underwent anteroposterior and lateral films of knee joint, CT plain scan of knee joint and 3D reconstruction before surgery. Professionals imported CT data into Mimics software and convert them into 3D models. The situation of fracture blocks were observed, the pixel set of each bone block was separated to establish a 3D model of each bone block, and each bone block was marked with different colors for fracture classification. All patients in group B underwent anteroposterior and lateral films of knee joint, CT plain scan of knee joint and 3D reconstruction before surgery.

2.3. Preoperative Virtually Reduction and 3D Printing Model

For the reconstructed 3D model of bone, according to the actual needs of fracture surgery, the virtually reduction of the main fracture directly related to surgery was completed, and the articular surface of tibial plateau was reconstructed to obtain the 3D model after reduction. The virtual reduction process was observed to fully understand the size and shape of the fracture block, displacement, reduction mode, movement distance and rotation angle, and determine the individualized surgical plan: the choice of operative approach, the position of the steel plate and the length and direction of the screw. After that, the models of each bone block were integrated and the 1:1 model was printed out hierarchically. After observing the printed fracture model, the surgical team carried out pre-operation process, including understanding the match of the fracture block, simulating the placement of anatomical steel plate, understanding the relationship between the fracture line and the position of the steel plate after reduction, and the design of the position and angle of the cancellous bone screw on the inner and outer platform.

2.4. Evaluation of efficacy

Hospital for special surgery (HSS) knee score [3] was used to evaluate the recovery of knee joint function. HSS has a relatively high accuracy in comparing the

recovery of joint function before and after surgery, especially in the evaluation of early postoperative period, which can comprehensively evaluate the movement of the patellofemoral joint and the femoral condyle.

2.5. Statistical Method

Data were analyzed by the statistical software of SPSS 13.0. The operation time and operative blood loss, radiation frequency, surgery instrument cost were expressed by mean (+ standard deviation) ($x \pm s$) and t-test was adopted. While the knee function score after operation was tested by χ^2 test, the difference was considered statistically significant with $P < 0.05$.

3. Results

3.1. Comparison of Surgical Effects

There was no significant difference in the surgery instrument cost between group A and group B ($t = 1.661$, $P > 0.05$), indicating that the application of 3D printing technique had no significant impact on the use of surgery instrument in traditional surgery. While, there were statistically significant differences in operation time, operative blood loss and radiation frequency ($P < 0.05$), indicating that compared with traditional surgery, the application of 3D printing technique reduced the operation time and intraoperative fluoroscopy times, thus improving the operation efficiency and safety. See Table 1.

Table 1. Comparison of key intraoperative parameters between group A and group B for complex tibial plateau fractures.

Group	Case	Operation time min	Operative blood loss ml	Perspective times time	Internal fixation consumables cost Ten thousand yuan
Control group	14	122.20 ± 3.26	447.90 ± 31.08	4.71 ± 0.62	3.04 ± 0.23
Observation group	14	105.10 ± 4.14	314.30 ± 37.04	2.71 ± 0.40	2.54 ± 0.18
t		t = 3.237	t = 2.762	t = 2.730	t = 1.661
P		<0.05	<0.05	<0.05	>0.05

3.2. Comparison of Functional Recovery after Operation

Six months after operation, the HSS score was used. In group A, 11 cases were excellent, 2 cases were good, 1 case was fair, the excellent and good rate was 92.86%. In group B, 5 cases were excellent, 7 cases were good, 2 cases were fair, the excellent and good rate was 85.71%. There was no significant difference between the two groups ($\chi^2 = 0.373$, $P = 0.54$) for the excellent and good rate, while the difference of the excellent rate between the two groups was statistically significant ($\chi^2 = 5.250$, $P < 0.05$), indicating that the application of 3D printing technique can achieve better long-term postoperative functional recovery and better patient satisfaction. See Table 2, Figure 1 and Figure 2.

Table 2. Comparison of functional recovery of knee joint between group A and group B after operation for complex tibial plateau fractures.

Group	Excellent (case)	Good (case)	Fair (case)	Total cases	Excellent and good rate (%)	Excellent rate (%)
Control group	5	7	2	14	85.71	35.71
Observation group	11	2	1	14	92.86	78.57
χ^2					$\chi^2 = 0.373$	$\chi^2 = 5.250$
P					0.54	<0.05

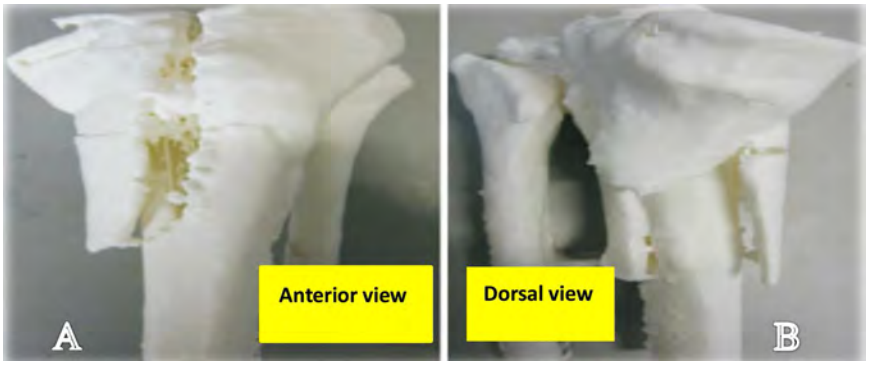


Figure 1. The Anterior view and Dorsal view of 3D printing model.

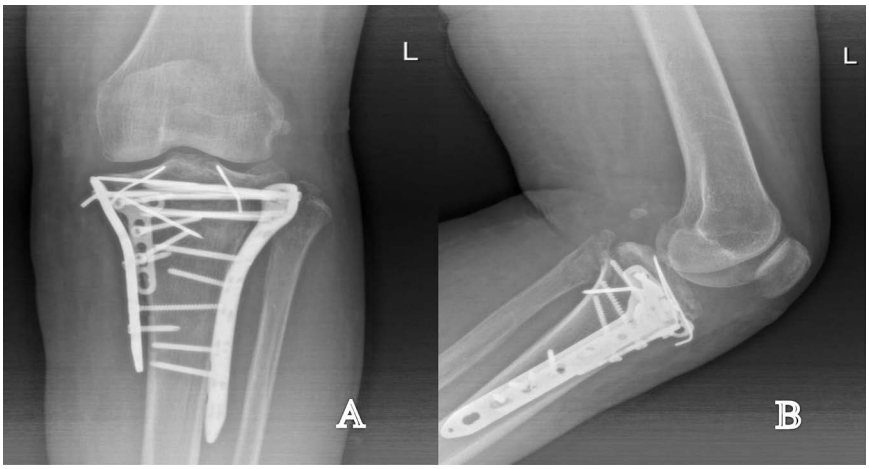


Figure 2. X-rays was reexamined one month after surgery.

4. Discussion

4.1. The Characteristics of Tibial Plateau Fractures and the Commonly Used Treatment Methods in China and Abroad

Tibial plateau fracture is a complex intra-articular fracture. Schatzker proposed in 1979 to classify tibial plateau fracture into six types [4], which has been used up to now. However, it was found in clinical practice that fractures combined with the posterior bone block of the tibial plateau were not well applicable to the Schatzker classification. Based on this, Luo Congfeng *et al.* [5] proposed a Three-

column Classification of tibial plateau fractures based on CT scan results in 2012. The tibial plateau was divided into lateral column, medial column and posterior column. Cortical rupture was defined as column fracture, and the collapse of tibial plateau articular surface without cortical rupture was defined as zero column fracture. Three-column Classification can better guide orthopaedics to choose surgical approaches and internal fixation methods, so as to obtain better reduction and more stable fixation, which is conducive to the recovery of knee joint function, and significantly improve the therapeutic effect. In recent years, Zhang Yingze *et al.* [6] have proposed that the current classification cannot include the special type of tibial plateau fracture combined with fibular head fracture, so a new 6-type comprehensive classification method is proposed to make the surgical treatment of tibial plateau fracture more integrated. According to different types of fractures, the treatment methods are also different, and individualized surgical plans should be made according to different types of fractures. With the development of modern orthopaedics and the deepening of research on tibial plateau fractures, the concept of treatment is constantly updated, and the treatment methods are gradually improved and perfected. Limited incision, minimally invasive technique, indirect reduction by prying technique, application of external fixator and biological fixation are the future development directions of tibial plateau fracture treatment [7].

4.2. Advantages of 3D Printing Technique over Other Methods

4.2.1. Preoperative Planning of Sham Operated

Preoperative design of complex tibial plateau fracture is formulated by using 3D printing technique to simulate the operation of virtually reduction and internal fixation of complex tibial plateau fracture before operation [8]. In this way the fracture can be accurately and effectively reduced and fixed during operation, the operative blood loss can be reduced, the success rate of one-time placement of implants and devices can be increased, and the accuracy of surgery can be improved, it is considered as a relatively simple and practical clinical tool [9].

4.2.2. Accuracy of Intraoperative Navigation

The application of navigation template for 3D printing technique can help to accurately insert the bone fracture plate and screw, reduce the penetration rate and direction error rate of screw placement [10] [11], and avoid iatrogenic osteoporosis and internal fixation loosening caused by repeated screw placement.

4.2.3. Clinical Teaching and Surgical Training

Through the intuitive understanding of 3D printing model, medical students and young doctors can not only diagnose and classify accurately, but also improve the understanding of complex fractures, which is conducive to the growth of young doctors and shorten the learning curve [12] [13].

4.2.4. Facilitate Doctor-Patient Communication and Understanding

The application of 3D printing technique can effectively promote the doctor-pa-

tient communication, in conversation with the patient, using 3D printing technique to make the model vividly and describe the disease condition to patients and their families and inform them of the process during the operation, so that patients can truly feel "clear" treatment, effectively alleviate the tension between patients, with significant clinical benefits [14]. Therefore, it is an effective tool to help doctors and patients communicate [15].

4.3. Problems in the Promotion and Application of 3D Printing Technique

Although preliminary studies have shown that 3D printing technique can improve the therapeutic effect of fracture surgery and functional recovery after surgery. However, in clinic, there are still many difficulties in making 3D printing model and applying and popularizing virtually reduction technology [16]. 1) Increase the total cost of surgery, increase the financial burden of patients seeking medical treatment. 2) Printing of individualized prostheses is still in the scientific research stage, and commercial promotion is still limited by the application of biomaterials, whether it can adapt to long-term high-intensity use is still unknown. 3) The relevant clinical guidelines and laws and regulations are faultiness: 3D printing technique involves many fields such as intellectual property rights, human ethics, dangerous goods manufacturing and so on. At present, there is no clear related policies and laws to complement with it. 4) Low timeliness: From the establishment of imaging data to the printing of physical models and the manufacture of individualized prostheses and internal plants, the whole process takes hours to days, so it is difficult to be used in emergency surgery.

5. Prospect of 3D Printing for Tibial Plateau Fractures

The development and application of 3D printing technique has indeed improved the therapeutic effect and functional recovery of traditional tibial plateau fracture surgery, and made individualized and precise medical treatment possible. However, how to further control the cost, research and development of biomaterials and gradually popularize the application are still worth considering. With the rapid development of digital medicine and the deepening of research, the application of computer software-assisted surgery, surgical robots, complex tibial plateau fractures and other complex trauma diagnosis and treatment will be more rapid and effective.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Gardner, M.J. and Schmidt, A.H. (2014) Tibial Plateau Fractures. *Journal of Knee Surgery*, **27**, 3-4. https://doi.org/10.1055/s-0033-1363854

[2] Wang, L. and Dai, K.R. (2014) Individualized Treatment of Orthopaedics and 3D Printing Technology. *Journal of Medical Biomechanics*, **29**, 193-199.

[3] Cho, W.S., Park, S.S., Kim, D.H., *et al.* (2000) The Reliability of HSS Knee Rating System.

[4] Schatzker, J., Mcbroom, R., Bruce, D., *et al.* (1979) The Tibial Plateau Fracture. The Toronto experience 1968-1975. *Clinical Orthopaedics*, **138**, 94-104.

[5] Luo, C.F., Hu, C.F., Gao, H., *et al.* (2009) Three-Column Classification for Tibial Plateau Fractures. *Chinese Journal of Orthopaedic Trauma*, **11**, 201-205.

[6] Chang, Z.L., Chang, H.R., Zhang, Y.Z., *et al.* (2018) Preliminary Study on Comprehensive Classification of Tibial Platform Fractures. *Journal of Hebei Medical University*, **39**, 1354-1355.

[7] Editorial Committee of Chin J Orthop Trauma (2015) Expert Consensus on Diagnosis and Treatment of Tibial Plateau Fractures. *Chinese Journal of Orthopaedic Trauma*, **17**, 1671-7600.

[8] Meng, G.L., Liu, J., Hu, Y.Y., *et al.* (2011) Rapid Prototyping Used in the Operation Design for Complex Fractures of Tibial Plateau. *Chinese Journal of Orthopaedic Trauma*, **13**, 1135-1138.

[9] Dong, L.L., Guo, P.M., Zuo, Q., *et al.* (2016) Application of 3D Printed Fracture Model in Schatzker Classification of Tibial Platform Fractures. *Chinese Journal of Bone and Joint Injury*, **31**, 860-861.

[10] Lu, S., Zhang, Y.Z., Wang, Z., *et al.* (2012) Accuracy and Efficacy of Thoracic Pedicle Screws in Scoliosis with Patient-Specific Drill Template. *Medical & Biological Engineering & Computing*, **7**, 751-758. https://doi.org/10.1007/s11517-012-0900-1

[11] Kawaguchi, Y., Nakano, M., Yasuda, T., *et al.* (2012) Development of a New Technique for Pedicle Screw and Magerl Screw Insertion Using a 3-Dimensional Image Guide. *Spine*, **23**, 1983-1988.

[12] Sodian, R., Schmauss, D., Schmitz, C., *et al.* (2009) 3-Dimensianal Printing of Medels to Create Custom-Made Devices for Coil Embolization of an Anastomotic Leak after Aortic Arch Replacement. *The Annals of Thoracic Surgery*, **88**, 974-978. https://doi.org/10.1016/j.athoracsur.2009.03.014

[13] Kim, G.B., Lee, S., Kim, H., *et al.* (2016) Three-Dimensional Printing: Basic Principles and Applications in Medicine and Radiology. *Korean Journal of Radiology*, **17**, 182-197.

[14] Yi, C.R., Luo, J.S., Wang, W.J., *et al.* (2015) Progress of 3D Printing Technology in Individualized Treatment of Bone Tissue Repair. *Medical Science Journal of Central South China*, **43**, 330-333.

[15] Yang, L., Shang, X.W., Fan, J.N., *et al.* (2016) Application of 3D Printing in the Surgical Planning of Trimalleolar Fracture and Doctor-Patient Communication. *BioMed Research International*, **2016**, 2482086.

[16] Huo, L.F. and Ni, H.J. (2015) Application and Prospects of Digital Orthopedics: More Precise, Individual and Intuitional Outlook. *Chinese Journal of Tissue Engineering Research*, **19**, 1457-1462.

International Journal of Clinical Medicine, 2019, 10, 109-121
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Impact of Therapeutic Education on the Viral Load of HIV Infected Children and Adolescents on Antiretroviral Therapy at the Douala Laquintinie Hospital, Cameroon

Calixte Ida Penda[1,2*], Anne-Cécile Zoung-Kanyi Bissek[3,4,5], Serge Clotaire Bilong[3,5,6], Loic-Ardin Boupda[2], Cécile Okala[7,8], Francis Atéba Ndongo[4,9], Grace Dallé Ngondi[2], Else C. Moukoko Eboumbou[7], Louis Richard Njock[2,9], Olivier Koki Ndombo[3,10]

[1]Clinical Sciences Department, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Douala, Cameroon
[2]Laquintinie Hospital Douala, Douala, Cameroon
[3]Faculty of Biomedical Sciences, University of Yaoundé 1, Yaoundé, Cameroon
[4]Division of Operational Research in Health, Ministry of Public Health of Cameroon, Yaoundé, Cameroon
[5]National HIV Drug Resistance, Surveillance and Prevention Working Group, Yaoundé, Cameroon
[6]Groupe Technique Central—CNLS, Yaoundé, Cameroon
[7]Biological Sciences Department, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Douala, Cameroon
[8]Clinical Biology Laboratory, General Hospital, Douala, Cameroon
[9]Chirurgical Department, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Douala, Cameroon
[10]Chantal Biya Foundation, Mother-Child Centre, Yaoundé, Cameroon
Email: *idapenda@yahoo.fr

How to cite this paper: Penda, C.I., Bissek, A.-C.Z.-K., Bilong, S.C., Boupda, L.-A., Okala, C., Ndongo, F.A., Ngondi, G.D., Eboumbou, E.C.M., Njock, L.R. and Ndombo, O.K. (2019) Impact of Therapeutic Education on the Viral Load of HIV Infected Children and Adolescents on Antiretroviral Therapy at the Douala Laquintinie Hospital, Cameroon. *International Journal of Clinical Medicine*, **10**, 109-121.
https://doi.org/10.4236/ijcm.2019.103011

Received: January 31, 2019
Accepted: March 4, 2019
Published: March 7, 2019

Abstract

Introduction: One of the biggest challenges for HIV-infected adolescents on antiretroviral therapy (ART) is the long-term maintenance of viral suppression, which is the third 90% goal of UNAIDS. Therapeutic Education (TE), process of acquiring abilities and skills that help the patient to live optimally with his illness is one of the strategies that contribute to the achievement of viral suppression through the therapeutic adhesion contract and the follow-up of the patient. The aim of this study was to evaluate the impact of TE on the virologic response of children and adolescents aged 8 - 19 under ART and followed up at the Laquintinie Hospital of Douala (LHD). **Method:** A cross-sectional study was conducted at the Pediatric Unit of the HIV/AIDS Accredited Treatment Center (ATC) at LHD from February to May 2016. Children and adolescents aged 8 to 19 years on ART, followed in ATC/LHD whose parents had agreed to participate in the study, and who had achieved at least one viral load before and after initiation of TE, were recruited consecutively during routine medical follow-up. Data were collected from patients' medical

records and questionnaires administered to study participants. **Results**: A total of 198 children and adolescents were included in this study with an average age of 14 years (±3). In this study population, 86.1% of children aged 8 - 10 years had acquired knowledge of the importance of taking medications, 95.4% and 97.3% of adolescents aged 11 - 14 years and 15 - 19 years had knowledge of medication schedules respectively. Among children and adolescents with undetectable viral load prior to initiation of TE, 76.5% maintained an undetectable viral load after initiation of TE. In addition, 72.3% of those whose viral load was detectable before initiation of TE had acquired an undetectable or decreasing viral load after initiation of TE. The only exposure factor significantly associated with maintaining undetectable viral load after initiation of TE was having less than 10 TE sessions ($p = 0.02$). **Conclusion**: The virologic response appears to be better in subjects who acquire skills faster through TE and therefore require fewer learning sessions to adapt. In addition, TE effectively contributes to achieving the third 90% goal of UNAIDS.

Keywords

HIV, Children, Adolescents, Therapeutic Education, Cameroon

1. Introduction

Infection with pediatric Human Immunodeficiency Virus (HIV) remains a public health priority. Of 1.8 million children under the age of 15 living with HIV worldwide in 2016, almost 90% lived in sub-Saharan Africa [1]. With antiretroviral therapy, an increasing number of children infected with HIV through vertical transmission in Africa are living longer and reaching adolescence [2] [3] [4] [5]. One of the biggest challenges in this adolescent population is the long-term maintenance of therapeutic compliance to achieve viral suppression.

Cameroon has adhered to the strategic Objectives 90-90-90 of United Nations Program on HIV/AIDS (UNAIDS) [6], which aims to increase HIV testing to 90%, achieve ART coverage of 90% and 90% of people on antiretroviral therapy (ART) achieve viral suppression by 2020. Therapeutic Education (TE), process of acquiring abilities and skills that help the patient to live optimally with his illness, is one of the strategies proposed by achieve viral suppression [7]. For HIV infected children and adolescents, it consists in transmitting to the patient on ART the skills that help him to live optimally with his illness and to improve the therapeutic observance. It integrates the process of announcing HIV status in children and adolescents [7]. In children, the disclosure of HIV status is a progressive and delicate process considering their cognitive level and their emotional state [8]. The beneficial effects of TE on mental health and adherence [9] [10] may help to mitigate the low retention of care seen in children and adolescents on ART [11] [12]. However, few studies have been conducted to evaluate the efficacy of TE on the therapeutic response of children and adolescents on ART in

Cameroon.

Since 2011, "the therapeutic school" has been set up at the Accredited HIV/AIDS Treatment Center (ATC) of LHD to transmit therapeutic self-management skills to children and adolescents on ART. The aim of this study was to evaluate the impact of TE on the virologic response of children and adolescents aged 8 - 19 under ART and monitored at the ATC/LHD.

2. Material and Methods

2.1. Study Design

A cross-sectional study was conducted at the pediatric unit of the ATC/LHD during the period from February to May 2016 (4 months). It is a center of excellence for pediatric HIV care covering an active line of 452 children and adolescents at the time of the study.

2.2. Study Population

Children and adolescents aged 8 to 19 on ART, followed at ATC/LHD whose parents accepted the study and had at least one viral load before and after the start of participation in the study. Therapeutic School were recruited consecutively during routine medical consultations.

2.3. Establishment of the Therapeutic School at Laquintinie Hospital in Douala

Therapeutic classes have been in place at ATC/LHD since March 2011 to improve the disclosure of HIV status, viral load control and the quality of life of HIV infected children and adolescents. They include individual interviews on one hand and group meetings on the other hand for children and adolescents aged 8 to 19 (organized into three age groups (8 - 10, 11 - 14 and 15 - 19 years). They are facilitated by a multidisciplinary team of trained health care providers (educators, psychologists, nurses, doctors). The content of the therapeutic classes is adapted to the age group as follows: 1) from the 8 - 10 age group: destructive action of the virus (unnamed virus) in the body, the importance of taking the drug, the effect of falling asleep by the drug and the means of transmitting the virus; 2) from 11 - 14 years of age: destructive action of the virus (virus named) in the body, the importance of taking the drug at the given times, management of the secrecy related to HIV status in those who already benefited from the full disclosure of HIV status; 3) from the 15 - 19 age group: interpretation of CD4 count and viral load.

2.4. Data Collection Tools

Data were collected from patients' medical records and questionnaires administered to study participants. Data collected from patients' medical records included sociodemographic characteristics, clinical and laboratory characteristics (viral load) before and after initiation of TE sessions, and HIV status announce-

ment level of study participants. Questionnaires were used to describe the type (individual interview or group meeting) and the number of TE sessions received by participants, and to categorize the skills acquired by the participants in the therapeutic classes considering the age group according to a "bad", "average" or "good" scale.

2.5. Definition of Operational Terms

The assessment of the scale of knowledge for the acquisition of the notions transmitted to the therapeutic education was defined according to the age based on a score graduated from 0 to 10.

Was considered as patient with a good knowledge, patients who obtained:

- a score of 8 to 10 for children aged 8 to 10;
- a score of 11 to 15 for children aged 11 to 14;
- a score of 16 and 20 for children aged 15 to 19.

For average knowledge:

- a score of 5 to 7 for children aged 8 to 10;
- a score of 7 to 10 for children aged 11 to 14;
- a score of 11 and 15 for children aged 15 to 19.

For bad knowledge:

- a score of 0 to 4 for children aged 8 to 10;
- a score of 0 to 6 for children aged 11 to 14;
- a score of 0 and 10 for children aged 15 to 19.

2.6. Data Collection Procedure

An informed consent form was provided to the parent or legal guardian of eligible children and teens for the study, as well as a consent form for adolescents over the age of 14 in the waiting room for consultations, medical or pharmaceutical, during the dispensing of drugs. For unaccompanied children and adolescents, an informed consent form was given to them to give to their parent or legal guardian. In addition, a telephone conversation was sometimes necessary to provide them with additional information for a better understanding of the study. After obtaining the parent's consent and, if applicable, the adolescent's consent, an age-appropriate assessment skills questionnaire was provided to the participant at a time. Monthly session of the therapeutic school or in the waiting room for medical consultations or during the dispensing of medicines from the pharmacy. The participant completed the questionnaire alone for a maximum of 15 minutes. At the same time, the other study data were collected in the participant's medical record.

2.7. Variables

The main variable of interest was the change in viral load (VL), measured before and after the initiation of TE sessions, defined as a binary qualitative variable of modalities. VL detectable after initiation of the TE or VL undetectable after init-

iation of TE if the participant belonged to the group with an undetectable viral load prior to initiation of TE (group 1). It was defined by the terms "stationary or augmented VL after initiation of TE" or "regression or undetectable VL after initiation of TE" if the participant belonged to the group with a detectable viral load prior to initiation of TE (group 2).

The main explanatory variables included the type of TE (individual-only, or individual and collective) and the number of TE sessions administered (≤10 sessions or >10 sessions).

Secondary explanatory variables related to characteristics of the child or adolescent (age (≤14 years or >14 years), degree of HIV status announcement (partial or complete), level of education (illiterate/primary or secondary/superior)) and the caregiver of the child or adolescent (type of relationship with the child or adolescent (father/mother or guardian), HIV status (negative or positive)).

2.8. Statistical Analysis

The descriptive analysis consisted in estimating the size of the categories of the qualitative variables and the average of the quantitative variables associated with the standard deviation.

The association between the explanatory variables and the evolution of the viral load in children or adolescents who initiated TE was studied by univariate and multivariate analyzes using logistic regression models. These analyses were performed separately in groups 1 (undetectable viral load before initiation of TE) and 2 (detectable viral load after initiation of TE). Odds ratios between the categories of exposure variables were compared using the Score test. The level of education, the degree of announcement of HIV status, the type and number of therapeutic education sessions administered were included in all multivariate models (a priori exposure factors). Other exposure variables, with a significance level (p) ≤ 0.20 in univariate analysis, were also included in the initial multivariate model. The final multivariate model, constructed by top-down explanatory variables, included a priori exposure factors and other exposure variables with p ≤ 0.20.

The collected data was captured on the SPSS software and analyzed using the Stata © 13.0 software (Stata Corp, College Station, TX). The threshold of significance was $p < 0.05$.

2.9. Ethics Approval and Consent to Participate

This study was conducted in accordance with ethics regulations for research on humans in Cameroon. An ethical clearance was obtained from the Institutional review Board of the University of Douala prior to the start of the study (N˚/CEI-UDo/740/01/2017/T) and administrative authorization (N˚0010/AR/MINSANTE/DHL/CM) was obtained from the LHD. Before enrolment and the administration of questionnaire, parents or legal guardians were informed on the purpose and process of the investigation (background, goals, methodology,

study constraints, respect of privacy and data confidentiality, and rights to opt out from the study), and a signed informed assent was obtained from all parents or legal guardians for inclusion and use of anonymous data of their children in publication and conference presentations. Participation was voluntary, anonymous and without compensation.

3. Results

3.1. Study Population

A total of 198 children and adolescents were included in this study at an average age of 14 years (±3). The 8 - 10, 11 - 14 and 15 - 19 age groups accounted for 17.2%, 44.9% and 37.9% of the study population respectively (Table 1). Of these children and adolescents, just under half (48.0%) were male, the vast majority (91.9%) resided in the city of Douala, almost two-thirds (69.7%) were at a level of secondary education. More than half (56.1%) had already received the full announcement of their HIV status. Children with both parents living accounted for 37.9% and 40.4% were fatherless or motherless. The person in charge of child or adolescent care was the mother in 48.5% of the cases and her HIV status was positive in (54.5%) of the cases.

3.2. Skills Acquired during TE Sessions

At the time of this study, the knowledge of the importance of taking medicines was acquired by 86.1% of children 8 - 10 years and the effect of drowsiness of the virus by taking the drug by 75.0% (Table 2). Respectively 95.4% and 97.3% of adolescents aged 11 - 14 years and 15 - 19 respectively knew the importance of taking medication schedules. About three-quarters (74.7%) of adolescents aged 15 - 19 were able to interpret the result of their viral load while a smaller proportion of them (58.7%) knew how to interpret the result of the CD4 count (Table 2).

3.3. Evolution of Viral Load after Initiation of TE

Prior to initiation of TE, 133 (67.2%) children and adolescents in this study had an undetectable viral load (group 1) (Table 3) while 65 (32.8%) had a detectable viral load (group 2) (Table 4). Among children in group 1, 101 (76.5%) maintained an undetectable viral load after initiation of TE while 33 (23.5%) had detectable viral load (Table 3). In group 2, 47 (72.3%) acquired an undetectable or decreasing viral load after initiation of TE, whereas in 18 (27.7%) viral load had not regressed (Table 4).

3.4. Significant Association between the Evolution of Viral Load and the Lower Number of FTE Learning Sessions

In group 1 (undetectable viral load prior to TE initiation), the only exposure factor significantly associated with maintaining undetectable viral load after initiation of TE in the univariate analysis was receiving less than 10 TE sessions (OR = 2.6 (1.1-5.9), p = 0.02) (Table 3). In multivariate analysis, having less than

Table 1. Sociodemographic characteristics of children and adolescents before therapeutic education (LHD, Cameroon, February-May 2016).

Feature	Total, n (%) N = 198
Age, in years	
8 - 10	34 (17.2)
11 - 14	89 (44.9)
15 - 19	75 (37.9)
Mean ± standard deviation	14 ± 3
Sex,	
Male	95 (48.0)
Residence	
Douala	182 (91.9)
Littoral region, out of Douala	10 (5.1)
Another region	6 (3.0)
HIV status disclosure	
Complete	111 (56.1)
Partial	87 (43.9)
Level of education	
Primary	54 (27.3)
Secondary	138 (69.7)
University	3 (1.5)
Illiterate	3 (1.5)
Vital status of parents	
Father and mother alive	75 (37.9)
Orphan of mother	29 (14.6)
Orphan of father	51 (25.8)
Orphan of both parents	43 (21.7)
Person in charge of the child or adolescent's care	
Mother	96 (48.5)
Father	23 (11.6)
Guardian	80 (39.9)
HIV status of person in charge of the child or adolescent's care	
HIV−	90 (45.5)
HIV+	108 (54.5)
Nature of the relationship between the child or adolescent and the guardian. if applicable	
Uncle or Aunt	52 (65.8)
Grandparent	21 (26.6)
Step mother or step father	3 (3.8)
Brother or Sister	2 (2.5)
Institution	1 (1.3)

LHD: Laquintinie Hospital of Douala; **n:** Number of children or adolescents in the approved category of the variable; **%:** percentage; **N:** Total number of children/adolescents.

Table 2. Knowledge and skills acquired by children and adolescents during TE sessions (LHD, Cameroon, February-May 2016).

Nature of knowledge or skill	Age range		
	8 - 10 years, n (%) n = 34	11 - 14 years, n (%) n = 87	14 - 19 years, n (%) n = 75
Destructive mechanism of virus on the child's organism	16 (45.7)		
Knowledge on the importance of taking medications	31 (86.1)		
Effect of quarantine of virus by the drug	27 (75.0)	47 (54.0)	51 (68.0)
Knowledge on life treatment and chronic disease		69 (79.3)	70 (93.3)
Knowledge on name of the drug		57 (65.5)	62 (82.7)
Knowledge on time to take medication		83 (95.4)	73 (97.3)
Interpretation of viral load			56 (74.7)
Interpretation of CD4 cell count			44 (58.7)

TE: Therapeutic Education; **LHD:** Laquintinie Hospital of Douala; **n:** Number of children/adolescents in the approved category of variable; **%:** percentage; **N:** Total number of children/adolescents.

Table 3. Evolution of viral load among children and adolescents with undetectable viral load before initiation of therapeutic education (LHD, Cameroon, February-May 2016), N = 133.

Factor	Evolution of viral load after initiation of therapeutic education					
	Univariate analysis				Multivariate analysis	
	Detectable viral load after initiation of TE (Group 1) n = 32 n (%)	Undetectable viral load after initiation of TE (Group 2) n = 101 n (%)	OR (95% CI)	p	Ajusted OR (95% CI)	p
Type of TE						
Individual	14 (43.8)	39 (38.6)	1	0.27		
Individual and collective	18 (56.2)	62 (61.4)	0.8 (0.4 - 1.8)			
Number of TEsessions						
≤10	11 (34.4)	58 (52.4)	1	0.02	1	0.03
>10	21 (65.6)	43 (48.6)	2.6 (1.1 - 5.9)		3.1 (1.1 - 8.7)	
HIV status of the person in charge of child or adolescent care						
HIV+	13 (40.6)	57 (56.4)	1	0.12		
HIV−	19 (59.4)	44 (43.6)	1.9 (0.8 - 4.3)			
Type of person in charge of child or adolescent care						
Father or mother	16 (50.0)	65 (64.4)	1	0.15	1	0.07
Guardian	16 (50.0)	36 (35.6)	1.8 (0.8 - 4.0)		2.2 (0.9 - 5.2)	
Age of child or adolescent						
≤14 years	18 (63.2)	66 (65.4)	1	0.35		
>14 years	14 (36.8)	35 (34.6)	1.5 (0.7 - 3.3)			
Level of education						
Secondary or higher	23 (71.9)	75 (74.3)	1	0.79	1	0.26

Continued

Illiterate or primary	9 (28.1)	26 (25.7)	1.1 (0.5 - 2.8)		1.9 (0.6 - 6.1)	
HIV disclosure status						
Partial	10 (31.3)	48 (47.5)	1	0.11	1	0.18
Complete	22 (68.7)	53 (52.5)	2.0 (0.9 - 4.6)		2.2 (0.7 - 6.7)	

TE: Therapeutic Education, **LHD:** Laquintinie Hospital of Douala; **Group 1:** Children and adolescents with undetectable viral load before TE; **Group 2:** Children and adolescents with detectable viral load before TE; **VL:** Viral load; **n:** Number of children or adolescents belong to the category considered of the variable; **%:** percentage; **N:** Total number of children or adolescents; **OR:** Odds Ratio; **95% CI**: 95% Confidence Interval; **p:** Chi-2 test.

Table 4. Evolution of viral load amongst chidren or adolescents with detectable viral load before initiation of therapeutic education (LHD, Cameroon, February-May 2016), N = 65.

Factor	Evolution of viral load after initiation of TE					
	Univariate analysis				Multivariate analysis	
	VL stationary or increased after initiation of TE n = 18 n (%)	VL declining or undetectable after initiation of TE n = 47 n (%)	Crude OR (95% CI)	p	Ajusted OR (95% CI)	p
Type of TE						
Individual only	4 (22.2)	12 (25.5)	1	0.78	1	0.66
Individual and collective	14 (77.8)	35 (74.5)	1.2 (0.3 - 4.3)		1.4 (0.3 - 5.6)	
Number of TE sessions						
≤10	7 (38.9)	26 (55.3)	1	0.24	1	0.26
>10	11 (61.1)	21 (44.7)	2.0 (0.6 - 6.0)		2.0 (0.6 - 6.8)	
HIV status of person in charge of care						
HIV+	13 (72.2)	25 (53.2)	1	0.17		
HIV−	5 (27.8)	22 (46.8)	0.4 (0.1 - 1.4)			
Type of person in charge of adolescent or child care						
Father or mother	14 (77.8)	26 (59.6)	1	0.17	1	0.12
Guardian	4 (22.2)	19 (40.4)	0.4 (0.1 - 1.7)		0.3 (1.0 - 1.3)	
Age of child or adolescent						
≤14 years	9 (50.0)	30 (63.8)	1	0.31		
>14 years	9 (50.0)	17 (36.2)	1.8 (0.6 - 5.3)			
Level of education						
Secondary or higher	13 (72.2)	25 (53.2)	1	0.17	1	0.98
Illiterate or primary	5 (27.8)	22 (46.8)	0.4 (0.1 - 1.4)		1.0 (0.2 - 5.5)	
HIV disclosure status						
Partial	7 (38.9)	22 (46.8)	1	0.57	1	0.85
Complete	11 (61.1)	25 (53.2)	1.4 (0.5 - 4.2)		1.2 (0.2 - 5.9)	

TE: Therapeutic Education; **LHD:** Laquintinie Hospital of Douala; **Group 1:** Children and adolescents with undetectable viral load before TE; **Group 2:** Children and adolescents with detectable viral load before TE; **VL:** Viral load**; n:** Number of children or adolescents belong to the category considered of the variable; **%:** percentage; **N:** Total number of children or adolescents; **OR:** Odds Ratio; **95% CI:** 95% Confidence Interval; **p:** Chi-2 test.

10 sessions of TE remained significantly associated with maintaining an undetectable viral load after initiation of TE (OR = 3.1 (1.1 - 8.7), p = 0.03) while a non-significant association was noted in children and adolescents living with their father or mother (OR = 2.2 (0.9 - 5.2), p = 0.07). There was no association between the maintenance of an undetectable viral load after initiation of TE and the HIV status of the person in charge of child or adolescent care, the type of TE administered, the level of education, the degree of announcement of HIV status and the age of the child or adolescent.

In group 2 (detectable viral load prior to TE initiation), no exposure factor was associated with changes in viral load after initiation of TE (**Table 4**).

4. Discussion

This study evaluated the impact of therapeutic education on the virologic response in 198 children and adolescents aged 8 to 19 years on ART and monitored at LHD. In this study population, 86.1% of children aged 8 - 10 years had acquired knowledge of the importance of taking medications, 95.4% and 97.3% of adolescents in the 11 - 14-year-old and 15 - 19-year-old age groups respectively had knowledge of medication schedules. These results are comparable to those of another study conducted among Cameroonian children [13]. Among children and adolescents with undetectable viral load prior to initiation of TE, 76.5% maintained an undetectable viral load after initiation of TE. In addition, 72.3% of those whose viral load was detectable before initiation of TE had acquired an undetectable or decreasing viral load after initiation of TE. These results show the beneficial effect of TE on achieving or maintaining an undetectable viral load. Indeed, therapeutic education had positively influenced therapeutic compliance in a cohort of Romanian children and adolescents [10].

The only exposure factor significantly associated with maintaining undetectable viral load after initiation of TE was having less than 10 TE sessions. This result seems to reflect a better virologic response in subjects who acquire skills faster through TE and therefore require fewer learning sessions to adapt. In addition, the nonsignificant association between maintaining undetectable viral load and living with his father or mother may be due to a lack of power in our study. A larger population of children with undetectable viral loads prior to TE initiation would result in a statistically significant association. In a Ugandan study [14], motherless children did not have adequate physical, mental, and social support at home. This situation may hinder the effectiveness of TE in children living with a guardian. In addition, the death of the parents during the TE process may promote an alteration of therapeutic compliance and partially justify the proportion of subjects who were unable to maintain their viral load at an undetectable level after initiation of the treatment. In this study, parental mortality was not described throughout the TE process.

In our study, the degree of disclosure was not associated with changes in viral load, contrasting with a Romanian study [10] that showed a significant associa-

tion between the lack of disclosure of HIV status and the increase in viral load in HIV infected children on ART. The difference in these results could be explained by the fact that in our study population, all subjects had already received a partial or total announcement of their status according to their level of cognitive maturity and their emotional state.

The lack of association between undetectable viral load after initiation of TE and different risk factors in the group of children and adolescents with detectable viral load prior to initiation of TE could be explained by a lack of statistical power. Indeed, the size of this group (N = 65) was relatively small.

5. Conclusion

The level of acquisition of skills and knowledge through TE was satisfactory among children and adolescents on antiretroviral therapy in this study. In addition, the TE had a favorable influence on maintaining or achieving undetectable viral load. As a result, TE effectively contributes to achieving the third 90% goal of UNAIDS and to improving pediatric HIV care in Cameroon.

Contributions of Authors

- CIP, ACZKB, SCB, OKN and LRN concepted and designed the study.
- CIP, LAB, CO, FAN, GDN and CEEM collected, analyzed and interpreted the data.
- CIP, GDN, FAN drafted the manuscript.
- All authors approved of the final version.

Acknowledgements

We are very grateful to the children and adolescents and their families who have agreed to participate in this study. We express our gratitude to the ATC/LHD management team and its educators for their dedication and to the CBCHB and EPPAF partners in pediatric HIV care.

Conflicts of Interest

The authors declare that they have no competing interests.

References

[1] UNAIDS. Fact Sheet-World Aids Day 2018. 2017 Global HIV Statistics. http://www.unaids.org/sites/default/files/media_asset/UNAIDS_FactSheet_en.pdf

[2] UNAIDS. 2015 Progress Report on the Global Plan towards the Elimination of New HIV Infections among Children and Keeping Their Mothers Alive. http://www.unaids.org/en/resources/documents/2015/JC2774_2015ProgressReport_GlobalPlan

[3] Foster, C. and Fidler, S. (2010) Optimizing Antiretroviral Therapy in Adolescents with Perinatally Acquired HIV-1 Infection. *Expert Review of Anti-Infective Therapy*, **8**, 1403-1416. https://doi.org/10.1586/eri.10.129

[4] Vaz, L.M.E., Eng, E., Maman, S., Tshikandu, T. and Behets, F. (2010) Telling Child-

ren They Have HIV: Lessons Learned from Findings of a Qualitative Study in Sub-Saharan Africa. *AIDS Patient Care STDs*, **24**, 247-256.
https://doi.org/10.1089/apc.2009.0217

[5] National AIDS Committee, UNAIDS. Report 2015 Estimates and Projections on HIV and AIDS in CAMEROON 2010-2020.
http://cnls.cm/sites/default/files/estimation_et_projections_sur_le_vih_et_le_sida_au_cameroun_2010-2020_rapport_2015.pdf

[6] Bain, L.E., Nkoke, C. and Noubiap, J.J.N. (2017) UNAIDS 90-90-90 Targets to End the AIDS Epidemic by 2020 Are Not Realistic: Comment on "Can the UNAIDS 90-90-90 Target Beachieved? A Systematic Analysis of National HIV Treatment Cascades". *BMJ GlobHealth*, **2**, e000227.
https://doi.org/10.1136/bmjgh-2016-000227

[7] World Health Organization. Regional O. (1998) Therapeutic Patient Education: Continuing Education Program for Health Care Providers in the Field of Prevention of Chronic Diseases: Report of a WHO Working Group. *Report No. EUR/ICP/QCPH* 01 01 03 *Rev.*2, WHO Regional Office for Europe, Copenhagen.
http://apps.who.int/iris/handle/10665/108151

[8] World Health Organization (2011) Guideline on HIV Disclosure Counselling for Children up to 12 Years of Age. World Health Organization, Geneva.
http://www.ncbi.nlm.nih.gov/books/NBK304307/

[9] Menon, A., Glazebrook, C., Campain, N. and Ngoma, M. (1999) Mental Health and Disclosure of HIV Status in Zambian Adolescents with HIV Infection: Implications for Peer-Support Programs. *Journal of Acquired Immune Deficiency Syndromes*, **46**, 349-354.

[10] Ferris, M., Burau, K., Schweitzer, A.M., Mihale, S., Murray, N., Preda, A., *et al.* (2007) The Influence of Disclosure of HIV Diagnosis on Time to Disease Progression in a Cohort of Romanian Children and Teens. *AIDS Care*, **19**, 1088-1094.
https://doi.org/10.1080/09540120701367124

[11] McNairy, M.L., Lamb, M.R., Carter, R.J., Fayorsey, R., Tene, G., Mutabazi, V., *et al.* (2013) Retention of HIV-Infected Children on Antiretroviral Treatment in HIV Care and Treatment Programs in Kenya, Mozambique, Rwanda, and Tanzania. *Journal of Acquired Immune Deficiency Syndromes*, **62**, e70-e81.

[12] Billong, S.C., Fokam, J., Penda, C.I., Amadou, S., Kob, D.S., Billong, E.-J., *et al.* (2016) Predictors of Poor Retention on Antiretroviral Therapy as a Major HIV Drug Resistance Early Warning Indicator in Cameroon: Results from a Nationwide Systematic Random Sampling. *BMC Infectious Diseases*, **16**, 678.
https://doi.org/10.1186/s12879-016-1991-3

[13] Nlend, A.E.N., Lyeb, A.S., Moyo, S. and Nsangou, D. (2016) Therapeutic Patient Education and Disclosure of Status of HIV Infected Children in Yaounde, Cameroon Achievements and Competence. *Médecine Santé Trop*, **3**, 308-311.

[14] Rwenyonyi, C.M., Kutesa, A., Muwazi, L., Okullo, I., Kasangaki, A. and Kekitinwa, A. (2011) Oral Manifestations in HIV/AIDS-Infected Children. *European Journal of Dentistry*, **5**, 291-298.

Abbreviations

AIDS: Acquired Immunodeficiency Syndrome;
ART: Antiretroviral Therapy;
ATC: Accredited Treatment Centre;
LHD: Laquintinie Hospital of Douala;
TE: Therapeutic Education;
RLS: Resource-Limited Settings;
UNAIDS: Joint United Nations Program on HIV/AIDS;
VL: Viral Load;
WHO: World Health Organization.

International Journal of Clinical Medicine, 2019, 10, 122-127
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Analysis of the Causes of Chronic Pain after Inguinal Hernia Repair without Tension and Its Prevention and Treatment

Hao Wu[1], Weimin Li[2*]

[1]School of Medicine, Wuhan University of Science and Technology, Wuhan, China
[2]Xiaogan Hospital Affiliated to Wuhan University of Science and Technology, Wuhan University of Science and Technology, Xiaogan, China
Email: 502559545@qq.com, *xglwm1962@163.com

How to cite this paper: Wu, H. and Li, W.M. (2019) Analysis of the Causes of Chronic Pain after Inguinal Hernia Repair without Tension and Its Prevention and Treatment. *International Journal of Clinical Medicine*, **10**, 122-127.
https://doi.org/10.4236/ijcm.2019.103012

Received: January 30, 2019
Accepted: March 4, 2019
Published: March 7, 2019

Abstract

Purpose: Analyzing the causes of pain after tension-free repair in the inguinal hernia, and propose corresponding treatment strategies. **Results:** The patients in this group were followed up for 3 - 12 months. A total of 5 patients still had pain relief within 3 months after surgery. Further treatment was needed, and the incidence rate was 5%. **Conclusion:** Skilled surgical procedures are performed in patients with local anesthesia, and some of the absorbable repair materials can be used. The improved patch fixation and extra peritoneal repair can reduce the pain after inguinal hernia repair without tension.

Keywords

Inguinal Hernia, Tension-Free Hernia Repair, Chronic Pain, The Reason, Treatment

1. Introduction

Inguinal hernia is a common disease in general surgery and can be cured by surgery. Inguinal hernia repair is one of the most common surgical procedures [1]. Worldwide, approximately 20 million patients undergo inguinal hernia repair each year [2]. According to reports [3], in patients undergoing inguinal hernia repair, 16% to 62% have postoperative chronic groin pain. Although this pain is usually mild in nature, studies on the quality of life of patients with chronic pain in the inguinal hernia indicate that the severity of chronic pain can seriously affect normal daily activities. After extensive patch repair, the recurrence rate after inguinal hernia surgery has been accepted, and the focus of the

study has shifted from recurrence to chronic pain. Although pain can be controlled with analgesics, postoperative chronic pain remains a major clinical concern and significantly affects the patient's quality of life [4].

Definition of Postoperative Chronic Pain

Persistent pain: defined as at least the presence of pain, cannot be ignored, and interferes with daily activities of the past week Persistent pain: defined as at least the presence of pain, cannot be ignored, and interferes with daily activities of the past week [5]. Since the publication of Chronic postsurgical pain (CPSP), the current postoperative chronic pain has gradually received the attention of clinicians. However, CPSP does not have a complete and definitive definition. It generally refers to persistent pain that occurs after surgery. A large number of literatures are based on postoperative pain for 3 months. It is also believed that CPSP refers to a situation in which postoperative pain is unlikely to be relieved, difficult to handle, and pain is still exceeded beyond normal recovery time. Zhou Jianping [6] believes that although the International Association for the Study of Pain has determined that symptoms last for more than 3 months as chronic pain, in actual sputum surgery, the chronic inflammatory response lasts for more than 3 months due to the implantation of foreign bodies such as patches. Therefore, some people have pain symptoms for 6 months or longer as the standard for postoperative chronic pain. Zhou Jianping [7] said in his case report that chronic pain after tension-free hernia repair persisted for more than 3 months after surgery, and the pain range exceeded the pain in the affected area. It is a common complication after hernia repair. The incidence rate varies widely from literature to literature, ranging from about 5% to 35%. Since the inflammatory reaction of foreign substances such as patches can last for several months, the pain is currently more than 6 months as the standard for postoperative chronic pain. Therefore, the author believes that the tracking time of postoperative chronic pain should be 3 months or 6 months after surgery, because the incidence of chronic pain will decrease correspondingly with the extension of time, so the selected postoperative study The time points are different, so the incidence of chronic pain is also different.

2. Pain Mechanism

The pathophysiological mechanisms of chronic pain are complex and depend on the type of pain, including somatic, neurological, non-neuro (inflammatory non-neural pain) and visceral pain [8]. Somatic pain is sometimes referred to as periostitis and is confined to the pubic tuberosity, usually due to damage to the periosteum of the pubic tuberosity. Neuralgia is thought to be caused by damage to the inguinal nerve, causing pain in the sensory distribution of the affected nerve. The most common are the genital branches of the inguinal hernia, the inferior epigastric nerve, and the genital femoral nerve. For laparoscopic surgery, the femoral or femoral cutaneous nerves of the inferior femoral artery may also

be involved. Nerve damage can occur during or after surgery, and the mechanisms include indirect or direct structural damage and nerve crush injuries, caused by sutures or fixation devices, folded mesh, or inflammation and scarring around the nerves. Non-neuropathic pain is the result of tissue damage and local inflammatory response, caused by endogenous inflammatory mediators acting on pain receptors. Visceral pain is pain that is felt in the gut, spermatic cord, or other structures surrounding the urethra. Zhou Jianping believes that pain may be related to the following three aspects: one is the direct injury to the nerve in the surgical area during surgery; the other is the deformation and dysfunction of the nerve structure caused by scar contraction and deformation during postoperative tissue healing; Psychological factors, patients pay too much attention to this local discomfort, leading to serious psychological reactions, may be related to the reduction of pain threshold caused by nerve remodeling [6]. The most important of these is nerve damage. The mechanism may be caused by sensory and motor neurological disorders in the groin area. The nerves associated with pain are mainly the inferior tibia, the inguinal hernia, and the reproductive nerve [7]. Nerve ligature or suture, mesh scar tissue compression nerve, neuroma formation, etc. are the main causes of chronic pain after inguinal hernia [7].

3. Materials and Methods

3.1. Capital Information

100 patients with inguinal hernia who underwent tension-free repair between 2015 and June 2017 were selected as males. The age ranged from 21 to 79 years, with an average of 64.2 years. Among them, 75 cases were inguinal hernia, 22 cases were inguinal hernia, and 3 cases were compound hernia. Among them, 10 cases were bilateral inguinal hernia, and 8 cases recurred after inguinal hernia.

3.2. Method

3.2.1. Surgical Methods

Forty-five patients were treated with a tapered ankle ring filler and patch (manufactured by Bard, USA), 22 patients were treated with a clinical conical ankle ring filler (for domestic phase III), and 23 patients were treated with partially absorbable materials (Produced by Johnson & Johnson, USA). Twenty patients underwent local anesthesia and 80 patients underwent continuous epidural anesthesia or spinal anesthesia. Twenty-six patients underwent plain patch repair, 70 underwent iliac ring-filled tension-free repair, and 4 underwent plain laparoscopic repair.

3.2.2. Pain Management Strategy

First, patients should be given local physiotherapy such as infrared light and acupuncture. Analgesic drugs should be applied to patients with unresolved pain, mainly non-steroidal drugs. For patients with acute neuropathic pain, nerve block therapy can be applied. Dexamethasone and 0.5 can be used. % li-

docaine was treated with local block injection, once/week for 3 - 5 weeks. The inguinal, inguinal, and femoral nerves were injected around the femoral nerve. The site of tenderness was often used. Surgical treatment is feasible after non-surgical treatment of pain.

4. Result

The patients were followed up for 3 - 13 months. A total of 5 patients still had pain relief at 3 months after surgery. Further treatment was needed, with a 5% incidence. Among them, 1 case of skin redness/local pain was considered to be a chronic inflammatory reaction caused by materials. Symptoms disappeared after 5 days of treatment with antibiotics and hormones, swelling and discomfort occurred around 2 incisions, tenderness at the upper end of the incision, symptoms after drug treatment and physiotherapy Relieved or disappeared, 2 cases of pubic nodules to the upper part of the scrotum and upper thigh skin pain, symptoms disappeared after 3 - 5 times of nerve block treatment.

5. Discussion

The use of tension-free hernia repair for inguinal hernia has the advantages of short recovery time, low recurrence rate and few complications. At present, clinicians mainly use this surgical method to treat inguinal hernia, but the incidence of postoperative pain is higher. About 5% to 35%, in addition to the recurrence of inguinal hernia, another major problem that seriously plagues them. This article summarizes the causes of postoperative pain as follows.

5.1. Repair Material Factor

Polypropylene mesh and filler are more repair materials used at the present stage. Although their tissue compatibility is good, the surface is rough, the texture is hard, and it is easy to form fiber adhesion or scar with the surrounding tissue, while the tapered filler is often A fibrous mass is formed that compresses the surrounding tissues and nerves, producing a distinct foreign body sensation and pain. Chronic rejection and inflammatory responses to the material can also cause pain.

5.2. Fixed Factor of Material

When a good patch fixation is not performed, the patch is displaced, twisted, or shrunk, and the surrounding blood vessels and nerves are compressed to cause pain. If the patch is fixed by a method of fixing the thread by one turn, the patient may cause foreign body repulsion, which may cause infection, and the patch may be tied to the nerve when the patch is excessively fixed, so that postoperative pain occurs. In addition to the appeal factors, the compression and separation of the spermatic vessels in the surgery is too extensive, the formation of hematoma in the patient's incision and related psychological factors often cause postoperative pain in patients.

According to the cause of postoperative pain, the following aspects should be noted during surgery: 1) Surgery should be performed by a physician with clinical experience in the Department of Surgery. Sharp separation should be used during the operation. Care should be taken to protect the lower abdomen, the groin and the reproductive femoral nerve. When the cremaster muscle is cut and the inner ring is dissected, the reproductive nerve is avoided. The reproductive branch causes damage. Avoid deep sutures at the pubic nodules to prevent periosteal traction. Preperitoneal repair of plain films can reduce the rate of nerve damage, thereby reducing the incidence of postoperative pain. In this study, 4 patients were treated with preperitoneal repair, and no pain occurred after operation. 2) Individualized solutions should be used when using materials. If the patient is thin or has a small ankle ring, the plain film can be used alone. If the patient's financial situation is available, some absorbable materials can be used. Because the material is soft, the surrounding area can be lightened. The inflammatory response of the tissue is better. In this study, 23 patients with partial absorbable patches did not have postoperative pain. 3) When the repair material is fixed, the hospital uses absorbable sutures, which can significantly reduce the foreign body rejection of the suture and the infection caused by the suture, and can avoid the postoperative pain. In recent years, medical adhesives have been used to fix the patch, so that the pain caused by the occlusion of the nerve has been avoided, and the effect is good, and the patient has no pain after the operation. 4) During the operation, the spermatic cord should be protected and the blood should be carefully stopped to prevent hematoma from forming. If the patient's isolated wound is large, a drainage tube should be placed to reduce the occurrence of scrotal swelling. In this study, 3 of the 5 patients with pain were recurrent sputum. Considering the psychological factors of the patients, they could be given psychological intervention.

5.3. Neurological Factor

Neurological factors are the main cause of postoperative pain and are considered as a result of groin movement and sensory neurological disorders. The genital nerve of the inferior tibia, the inguinal hernia and the genital tract are the main nerves. Because of the large variation, the recurrence after the operation of the sputum can lead to anatomical unclear, which is easy to cause damage to the nerve during surgery and form a neuroma. There is severe pain that cannot be alleviated. And the pubic symphysis periosteum is rich in nerve fibers. When the patch is fixed, it often causes severe pain after the needle is inserted in the periosteum, which causes the patient to have a more obvious tingling sensation when walking the leg after the operation.

In the treatment of inguinal hernia after tension-free repair, the hospital believes that targeted treatment should be taken according to the actual situation of the patient. If the patient's symptoms are mild and intermittent pain, then the drug treatment and physiotherapy can be used to receive significant therapeutic

effects. The drugs mainly use weak opioid analgesics and non-steroidal drugs；If the patient has an early rejection or chronic inflammatory response, antibiotics and hormonal drugs should be used; If the patient has neurological involvement, local nerve block should be performed. Local anesthetic plus steroid mixture should be used for treatment. The main nerves around the main inguinal region such as the inguinal, groin and reproductive femoral nerve are the main injection sites. The patient with obvious trigger point can be injected at the trigger point; If the patient's pain cannot be alleviated after taking the above measures, the patch material should be taken out.

6. Conclusion

In summary, skilled surgical procedures in patients with local anesthesia, the use of partial absorbable repair materials, improved patch fixation and extraperitoneal repair can make pain in the inguinal hernia without tension repair cut back.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Kingsnorth, A. and Leblanc, K. (2003) Hernias: Inguinal and Incisional. *Lancet*, **362**, 1561-1571. https://doi.org/10.1016/S0140-6736(03)14746-0

[2] Öberg, S., Andresen, K., Klausen, T.W., *et al.* Chronic Pain after Mesh versus Non-mesh Repair of Inguinal Hernias: A Systematic Review and a Network Metaanalysis of Randomized Controlled Trials. *Surgery*.

[3] Jeroukhimov, I., Wiser, I., Karasic, E., *et al.* (2014) Reduced Postoperative Chronic Pain after Tension-Free Inguinal Hernia Repair Using Absorbable Sutures: A Single-blind Randomized Clinical Trial. *Journal of the American College of Surgeons*, **218**, 102-107. https://doi.org/10.1016/j.jamcollsurg.2013.09.010

[4] Nikkolo, C. and Lepner, U. (2015) Chronic Pain after Open Inguinal Hernia Repair. *Postgraduate Medicine*, **128**, 69. https://doi.org/10.1080/00325481.2016.1121090

[5] Lundström, K.J., Holmberg, H., Montgomery, A., *et al.* (2017) Patient-Reported Rates of Chronic Pain and Recurrence after Groin Hernia Repair. *British Journal of Surgery*, **105**. https://doi.org/10.1002/bjs.10652

[6] Zhou, J.P. and Shu, G.S. (2009) Chronic Pain after Inguinal Hernia Repair. *Chinese Journal of Abdominal and Abdominal Surgery* (*Electronic Edition*), **3**, 371-375.

[7] Ren, F. and Zhou, J.P. (2011) Treatment of Chronic Pain after Tension-Free Repair of Inguinal Hernia with Nerve Ablation and Mesh Removal: Report of One Case. *Chinese Journal of Modern Surgery*, **15**, 441-442.

[8] Hu, Q.L. and Chen, D.C. (2018) Approach to the Patient with Chronic Groin Pain. *Surgical Clinics of North America*. https://doi.org/10.1016/j.suc.2018.02.002

International Journal of Clinical Medicine, 2019, 10, 128-134
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Comparison of Severe Complications after Acute Stanford Type B Aortic Dissection under Different Surgical Timing

Jie Wan, Jianhui Xu, Peng Li, Rui Li*

Department of Cardiology, Xiaogan Hospital, Wuhan University of Science and Technology, Xiaogan, China
Email: *xgyyxnk@163.com

How to cite this paper: Wan, J., Xu, J.H., Li, P. and Li, R. (2019) Comparison of Severe Complications after Acute Stanford Type B Aortic Dissection under Different Surgical Timing. *International Journal of Clinical Medicine*, **10**, 128-134.
https://doi.org/10.4236/ijcm.2019.103013

Received: February 5, 2019
Accepted: March 5, 2019
Published: March 8, 2019

Abstract

Objective: To investigate the relationship between early intervention timing and complications of acute Stanford type B aortic dissection. **Methods:** The clinical data of 146 patients with acute stanford type B aortic dissection treated with transseptal stent for aortic endovascular repair (TEVAR) from January 2012 to October 2017 in Xiaogan Central Hospital were analyzed. The time was divided into 3 groups, including the onset to TEVAR time ≤ 48 h group (41 cases in group A), the onset to TEVAR time 48 h - 7 d group (56 cases in group B), the onset to TEVAR time 7 d - 14 d group (49 cases in group C)). The clinical baseline data, the incidence of different complications during perioperative period, and the mortality rate at 30 days were compared between the three groups. **Results:** There were no significant differences in age, gender and comorbidities between the three groups (all $P > 0.05$). Group A had a clearer indication of immediate intervention compared with group B and group C ($P < 0.05$). The overall incidence of severe complications in group C was significantly lower than that in group A and group B, and the difference was statistically significant ($P < 0.05$). There was no significant difference in reoperation rate and 30-day mortality between the 3 groups (all $P > 0.05$). **Conclusion:** Early intervention of acute TBAD may increase the risk of serious complications after surgery, and the incidence of serious complications will gradually decrease over time; the reduction of severe complications after early grouping is not accompanied by Early mortality and reoperation rates were significantly reduced, and TEVAR treatment in some patients with dissection did not prevent dissection progression and rupture.

Keywords

Aortic Dissection, Thoracic Endovascular Aortic Repair, Surgical Timing, Serious Complications

1. Introduction

Aortic dissection (AD) is one of the common high-risk chest pains in clinical work. In recent years, with the development of imaging techniques and clinical testing techniques, clinicians can quickly identify life-threatening causes of chest pain, especially with pulmonary embolism and the identification of acute coronary syndromes. The treatment of AD from Dake *et al.* [1] reported that the stent graft has been rapidly developed in the treatment of aortic dissection for several decades. Many recent mid-term follow-up results at home and abroad [2] [3] [4] also confirmed the success rate of TEVAR surgery. High, severe complication rate, and low trauma, endovascular isolation of the stanford type B aortic dissection (TBAD) has gradually replaced surgery as the first choice [5]. Although the evidence for the efficacy of T-type aortic dissection for TEVER treatment has accumulated, there is not much information available on the timing of early intervention for acute TBAD and the occurrence of postoperative serious complications. Foreign data [6] show that early intervention of acute TBAD may increase perioperative complications, and the occurrence of postoperative serious complications is related to the timing of intervention. There is currently no uniform understanding of how to perform early interventions to reduce early complications and reduce mortality in patients with acute complex TBAD or TBAD without clear complications. This study was a single-center retrospective study to investigate the relationship between the timing of early intervention in acute Stanford type B aortic dissection and postoperative serious complications.

2. Methods

2.1. Patients

Clinical data of 146 patients with acute TBAD who underwent aortic endovascular repair with a stent graft from January 2012 to October 2017 in Xiaogan Central Hospital. According to the time from onset to TEVAR, the patients were divided into the following three groups: the onset to TEVAR time ≤ 48 h group (41 cases in group A), the onset to TEVAR time 48 h - 7 d group (group B 56), the onset to TEVAR time 7 - 14 d group (C Group of 49 cases). Inclusion criteria: 1) patients with acute TABD who were admitted to our hospital from January 2012 to October 2017; 2) treated with TEVAR during hospitalization; 3) complete aortic computed tomography angiography and related clinical data. Exclusion criteria: 1) history of surgical treatment of aortic disease; 2) type A aortic dissection and chronic type B aortic dissection; 3) intra-aortic hematoma (IMH); 4) Aortic atherosclerotic penetrating ulcer (PAU).

2.2. Research Methods

1) Systematic examination of medical records, radiographic data and clinical follow-up information of patients during hospitalization; 2) All patients with TBAD underwent TEVAR on the basis of standard medical therapy. The stan-

dard of treatment success was that the proximal endometrial rupture was closed. No graft displacement and no residual endoleak, no significant branching vessels were closed and severe ischemic consequences; 3) Postoperative follow-up: Six months of follow-up for eligible patients, assessment of perioperative complications, reoperation rate, 30 d mortality, etc.

2.3. Statistical Analysis

Data analysis was performed using SPSS 19.0 statistical software. The measurement data were expressed as x ± s, and the comparison between groups was analyzed by ANOVA. The comparison between the two groups was performed by LSD method; the count data was expressed as a percentage, and the comparison between groups was performed by chi-square test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Comparison of Baseline Data of Three Groups of Patients

There were no significant differences in age, sex ratio and incidence of comorbidities between group A, group B and group C ($P > 0.05$); Group A had a clearer indication of immediate intervention compared with group B and group C ($P < 0.05$), See Table 1 for details.

3.2. Comparison of Postoperative Complications and Recent Survival Rate

There was no significant difference between the reoperation rate and the 30 d

Table 1. Patient demographics and cause of TEVAR intervention.

	Group A (N = 41)		Group B (N = 56)		Group C (N = 49)		*P* value
	%	n	%	n	%	n	
Patient demographics							
Age (y, $\bar{x} \pm s$)	49.76 ± 11.67		51.75 ± 9.77		47.24 ± 8.15		0.068
Sex (male, %)	35	85.4	49	87.5	41	83.7	0.855
Hypertension	36	87.8	40	71.4	36	73.5	0.136
Diabetes	3	7.3	5	8.9	5	10.2	0.892
CHD	2	4.9	4	7.1	5	10.2	0.628
Preoperative indication							
Diameter > 45 mm	16	39.0	13	23.2	10	20.4	0.104
Aura rupture	6	14.6	3	5.4	2	4.1	0.123
Repeated pain	7	17.1	8	14.3	4	8.2	0.428
Progression of dissection	6	14.6	5	8.9	3	6.1	0.385
Insufficient perfusion of vital organs	8	19.5	9	16.1	5	10.2	0.453
other	7	17.1	21	37.5	26	53.1	0.002

mortality rate in group A compared with group B and group C ($P > 0.05$). The overall incidence of severe complications in group C was significantly lower than that in group A and group B, and the difference was statistically significant ($P < 0.05$). See Table 2 for details.

4. Discussion

Aortic dissection (AD) often leads to immediate life threats due to rapid disease progression. It is highly valued in clinical diagnosis and treatment. The untreated acute AD 24 h mortality rate is about 33%, and the mortality rate within 48 hours is as high as 50% [7] [8]. Usually, the age of onset of AD is 48 - 67 years old, and the ratio of male to female is (2 - 5):1 [9]. Hypertension is the most common cause of AD [10]. This study also showed that there were significant gender differences in 146 patients with stanford type B aortic dissection. The overall male to female ratio was approximately 6:1. More than 70% of patients had hypertension, and 40 to 65 years of age were the prevalence of TBAD. segment.

TEVAR has been widely accepted for the treatment of Stanford B-type dissection. Compared with surgical open surgery and standard medical treatment, its comprehensive advantages have become increasingly prominent. In 2014, the European Society of Cardiology's guidelines for the diagnosis and treatment of aortic diseases used TEVAR as the first choice for complex TBAD. Treatment (I, C), non-complex TBAD may also consider TEVAR treatment (IIa, B). However, the INSTEAD trial [11] and the ADSORB study [12] demonstrated that patients with acute noncomplexity TBAD had better aortic remodeling with TEVAR than with drug therapy alone, and patients who underwent early TEVAR intervention in the INSTEAD trial were 5 years later. The rate of aortic-related mortality and dissection was much lower than that of the drug-only group. Complex TBAD should be considered when aortic aura rupture (blood thoracic, mediastinal hematoma, etc.), major organ hypoperfusion, recurrent pain, refractory

Table 2. Major complications.

	Group A (N = 41)		Group B (N = 56)		Group C (N = 49)		*P* value
Major complications	%	n	%	n	%	n	
New kidney failure	12.2	5	7.1	4	2.0	1	0.164
Retrograde sandwich	4.9	2	0.0	0	0.0	0	0.075
Paraplegia	4.9	2	1.8	1	2.0	1	0.611
Brain infarction	2.4	1	1.8	1	0.0	0	0.577
Lower limb ischemia	4.9	2	1.8	1	0.0	0	0.263
Gastrointestinal ischemia	7.3	3	3.6	2	0.0	0	0.164
total	36.6	15	16.1	9	4.1	2	0.0002
Reoperation rate	12.2	5	7.1	4	2.0	1	0.164
30 d mortality	14.6	6	5.4	3	4.1	2	0.123

hypertension, early dissection of the dissection, and persistent progression of the lesion. In this study, the proportion of acute complex TBAD in group A was significantly higher than that in group B and C ($P < 0.05$), indicating that the clinical situation of patients with early TEVAR intervention was more complicated, but it was caused by complex clinical conditions and varied. There is no very effective risk stratification tool and assessment tool for aortic anatomy. Studies [13] have shown that when TBAD patients have the above clinical manifestations, the mortality rate is significantly increased. Therefore, early intervention of TEVAR on the basis of standard drug therapy is particularly important for such patient populations.

Regarding severe postoperative complications, the overall incidence of severe complications in group C was significantly lower than that in group A and group B ($P < 0.05$). The overall incidence of severe complications in group B was lower than that in group. Group A ($P < 0.05$), group C was lower than group B ($P < 0.05$), indicating that the overall postoperative serious complication rate of patients with acute TBAD undergoing TEVAR treatment gradually decreased over time. The probable cause is that the proportion of acute complex TBAD in the earliest stage of intervention is higher, followed by acute vascular inflammation and edema, aortic intima fragile and easy to tear, and TEVAR is more prone to endoleak, new breach formation, and dissection. Postoperative complications such as retrograde tearing and aortic rupture [14]. However, we found that with the reduction of the incidence of serious complications, the reoperation rate and early mortality of the corresponding patients did not decrease significantly ($P > 0.05$), indicating that patients with acute TABD are a diverse group, simple staging and Early intervention problems in some patients with acute TABD who are facing immediate life threats cannot be resolved. Complex clinical conditions and variable aortic anatomy suggest the need for individualized treatment.

At the follow-up, we found that the risk factors for postoperative complications of aortic dissection were mainly age, underlying disease, and coverage of the left subclavian artery. Postoperative ischemic stroke is closely related to preoperative and postoperative hemodynamic changes; endoleak is the result of the combined effects of dissection lesions, vascular conditions, and artificial vascular stent grafts. Two cases of postoperative ischemic stroke were caused by atherosclerotic plaques in the internal carotid artery and subclavian artery, plus surgical procedures and hemodynamic changes. These cases provide a reference for our future work. Postoperative type II and III endoleaks generally require close follow-up without special treatment. For patients with TBAD with stable disease, TEVAR should be treated as soon as possible after 1 week (**Figure 1**). Special attention should be paid to the preoperative renal function of the patient, because the application of intraoperative contrast agent will further aggravate renal insufficiency or failure. Detailed evaluation and formulation of the plan before surgery, intraoperative standard operation, good stent selection, and strict follow-up after surgery are beneficial to reduce or reduce the occurrence of postoperative complications.

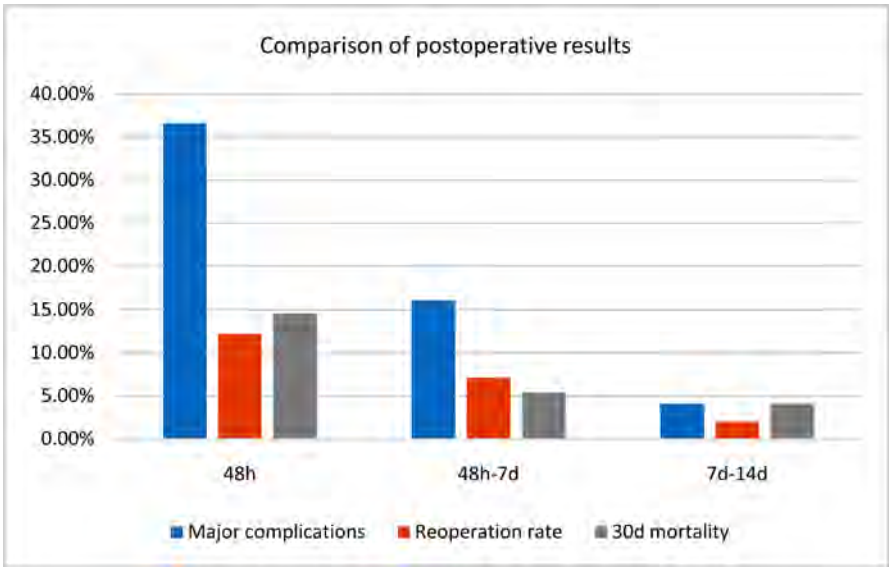


Figure 1. Short-term prognosis.

5. Conclusion

In summary, early intervention of acute TBAD will increase the risk of serious complications after surgery, and the incidence of serious complications will gradually decrease over time. The reduction in postoperative severe complications in the early grouping was not associated with a significant reduction in early mortality and reoperation rates. TEVAR treatment in some patients with dissection did not prevent dissection progression and rupture. At present, it is difficult to classify and effectively classify patients with acute TBAD. Early intervention also faces the complex clinical environment and the test of a variety of aortic anatomy. Early intervention strategies for patients with acute TBAD have yet to be refined in a multicenter, prospective, randomized controlled trial.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Dake, M.D., Kato, N., Mitchell, R.S., *et al.* (1999) Endovascular Stent-Graft Placement for the Treatment of Acute Aortic Dissection. *The New England Journal of Medicine*, **340**, 1546-1552. https://doi.org/10.1056/NEJM199905203402004

[2] Pang, D., Hildebrand, D. and Bachoo, P. (2015) Thoracic Endovascular Repair (TEVAR) versus Open Surgery for Blunt Traumatic Thoracic Aortic Injury. The Cochrane Library, John Wiley & Sons, CD006642.

[3] Lee, H.C., Joo, H.C., Lee, S.H., *et al.* (2015) Endovascular Repair versus Open Repair for Isolated Descending Thoracic Aorticaneursm. *Yonsei Medical Journal*, **56**, 904-912. https://doi.org/10.3349/ymj.2015.56.4.904

[4] Li, D.L., Zhang, H.K., Chen, X.D., *et al.* (2016) Thoracic Endovascular Aortic Repair for Type B Aortic Dissection: Analysis among Acute, Subacute and Chronic Pa-

tients. *Journal of the American College of Cardiology*, **67**, 1255-1257. https://doi.org/10.1016/j.jacc.2015.12.044

[5] Writing Committee, Riambau, V., Bockler, D., Brunkwall, J., *et al.* (2017) Editor's Choice e Management of Descending Thoracic Aorta Diseases: Clinical Practice Guidelines of the European Society for Vascular Surgery (ESVS). *European Journal of Vascular and Endovascular Surgery*, **53**, 4-52. https://doi.org/10.1016/j.ejvs.2016.06.005

[6] Miyairi, T., Miyata, H., Chiba, K., *et al.* for the Japan Adult Cardiovascular Database Organization (2018) Influence of Timing after Thoracic Endovascular Aortic Repair for Acute Type B Aortic Dissection. *The Annals of Thoracic Surgery*. https://doi.org/10.1016/j.athoracsur.2017.11.054

[7] Moon, M.R. (2009) Approach to the Treatment of Aortic Dissection. *Surgical Clinics of North America*, **89**, 869-893. https://doi.org/10.1016/j.suc.2009.05.003

[8] Ohlmann, P., Faure, A., Morel, O., *et al.* (2006) Diagnostic and Prognostic Value of Circulating D-Dimers in Patients with Acute Aortic Dissection. *Critical Care Medicine*, **34**, 1358-1364. https://doi.org/10.1097/01.CCM.0000216686.72457.EC

[9] Lo, R.C., Bensley, R.P., Hamdan, A.D., *et al.* (2013) Gender Difference in Abdominal Aortic Aneurysm Presentation, Repair, and Mortality in the Vascular Study Group of NewEngland. *Journal of Vascular Surgery*, **57**, 1261-1268. https://doi.org/10.1016/j.jvs.2012.11.039

[10] Fukuda, S., Watanabe, H., Iwakura, K., *et al.* (2015) Multicenter Investigations of the Prevalene of Abdominal Aortic Aneurysm in Elderly Japanese Patents with Hypertension. *Circulation Journal*, **79**, 524-529.

[11] Nienaber, C.A., Rousseau, H., Eggebrecht, H., *et al.* (2009) Randomized Comparison of Strategies for Type B Aortic Dissection: The Investigation of Stent Grafts in Aortic Dissection (INSTEAD) Trial. *Circulation*, **120**, 2519-2528. https://doi.org/10.1161/CIRCULATIONAHA.109.886408

[12] Brunkwall, J., Kasprzak, P., Verhoeven, E., *et al.* (2014) Endovascular Repair of Acute Uncomplicated Aortic Type B Dissection Promotes Aortic Remodelling: 1 Year Results of the ADSORB Trial. *European Journal of Vascular and Endovascular Surgery*, **48**, 285-291. https://doi.org/10.1016/j.ejvs.2014.05.012

[13] Tolenaar, J.L., Froehlich, W., Jonker, F.H., *et al.* (2014) Predicting In-Hospital Mortality in Acute Type B Aortic Dissection: Evidence from International Registry of Acute Aortic Dissection. *Circulation*, **130**, 45-50. https://doi.org/10.1161/CIRCULATIONAHA.113.007117

[14] Mehta, M., Paty, P. and Bhaghtwar, P. (2015) False Lumen Embolization during TEVAR for Complicated Aortic Dissections Is Associated with a Lower 30-Day Mortality and Improved Longterm Survival. *Journal of Vascular Surgery*, **62**, 817. https://doi.org/10.1016/j.jvs.2015.06.128

International Journal of Clinical Medicine, 2019, 10, 135-149
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Combination of Nonwoven Filters and Mesenchymal Stem Cells Reduced Glomerulosclerotic Lesions in Rat Chronic Kidney Disease Models

Hideo Hori[1*], Masanori Shinzato[2], Yoshiyuki Hiki[1], Shigeru Nakai[1], Gen Niimi[3], Shizuko Nagao[4], Nobuya Kitaguchi[1]

[1]Faculty of Clinical Engineering, School of Health Sciences, Fujita Health University, Toyoake, Japan
[2]Faculty of Medical Management & Information Science, School of Health Sciences, Fujita Health University, Toyoake, Japan
[3]Joint Research Laboratory Center for Research Promotion and Support, Fujita Health University, Toyoake, Japan
[4]Education and Research Facility of Animal Models for Human Diseases Center for Research Promotion and Support, Fujita Health University, Toyoake, Japan
Email: *hori@fujita-hu.ac.jp

How to cite this paper: Hori, H., Shinzato, M., Hiki, Y., Nakai, S., Niimi, G., Nagao, S. and Kitaguchi, N. (2019) Combination of Nonwoven Filters and Mesenchymal Stem Cells Reduced Glomerulosclerotic Lesions in Rat Chronic Kidney Disease Models. *International Journal of Clinical Medicine*, **10**, 135-149.
https://doi.org/10.4236/ijcm.2019.103014

Received: February 14, 2019
Accepted: March 10, 2019
Published: March 13, 2019

Abstract

Background and Objectives: The increasing incidence of patients requiring hemodialysis has become a medical and economic problem globally; it is necessary to maintain renal glomerulus functionality to prevent the progression of chronic kidney disease. As a therapeutic tool for preventing the progression of chronic kidney disease, mesenchymal stem cells (MSCs) are a promising source of both growth factors and cells for regeneration. However, the escape of MSCs from injection sites, as well as the insufficient production of growth factors, is issues that remain unresolved. In the present study, a complex of cells and a nonwoven filter was localized in an injured kidney to provide growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). **Methods and Results**: Nonwoven biodegradable polylactic acid (PLA) filters were used to capture rat bone marrow stem cells (r-BMSCs). The capture rates of r-BMSCs on five PLA filter disks were over 85%. The production of HGF by r-BMSCs on PLA filters was markedly enhanced through interactions between the cells and the nonwoven filter. Conversely, the production of VEGF by r-BMSC on PLA filters was unchanged. Complexes of nonwoven filters and cells were implanted onto the surfaces of kidneys of 5/6-nephrectomized rats, which are characterized by progressive glomerulosclerosis. Within the r-BMSC/PLA complexes, deleterious changes in serum creatinine levels were not attenuated. However, PLA filters with r-BMSCs, which enhanced HGF production over 4 weeks of cul-

ture, significantly (P = 0.03) decreased urinary protein levels at 4 weeks after implantation compared to untreated nephrectomized rats. Further histopathological studies revealed that glomerulosclerotic lesions were significantly (P = 0.008) reduced by treatment with the r-BMSC/PLA complex. **Conclusion**: Devices made of PLA nonwoven filters and stem cells are potentially useful for the prevention and treatment of chronic kidney disease.

Keywords

Nonwoven Filter, Mesenchymal Stem Cell, Hepatocyte Growth Factor, Regenerative Medicine, Chronic Kidney Disease

1. Introduction

Over 26 million people worldwide have undergone renal replacement therapy, including hemodialysis; this increase in hemodialysis patients has become a medical and economic problem worldwide [1] [2]. During the progression of kidney diseases, focal and segmental sclerotic changes in the glomeruli have been observed due to glomerular hypertension, promoting renal damage. This finding has been shown to be independent of underlying diseases and is known to be a common pathway. Thus, in order to prevent the progression to end-stage kidney disease, it is important to suppress the pathway leading to glomerulosclerosis [3].

Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) have been reported to be important for kidney regeneration [4]. Previous studies have revealed that injections of VEGF can prevent glomerulosclerotic injury in rats [5], and that injections of HGF can ameliorate chronic kidney disease (CKD) in rodents [3] [6]. However, the delivery of the appropriate concentrations and combinations of growth factors to the kidney at the appropriate times is difficult when using exogenous treatments. Furthermore, the short half-life of HGF precludes the ability to maintain high blood concentrations of this factor [7]. Several studies have shown that the intravenous administration of mesenchymal stem cells (MSCs) can ameliorate CKD or acute kidney injury [8] [9] [10] [11]. However, intravenously injected MSCs were ineffective, as MSCs did not successfully reach and remain in the injured site [12]. Therefore, the injection of extrinsic MSCs and growth factors may not be the most adequate method to deliver MSCs and growth factors to an injured kidney.

In this study, we focused on nonwoven filters as a tool for the localization of MSCs near the lesion area. Nonwoven filters are widely used to remove leukocytes from blood for two purposes. They are either used for the removal of leukocytes from donated blood in order to prevent post-transfusion graft versus host disease, or to treat ulcerative colitis by removing leucocytes from systemic circulated blood. Therefore, nonwoven filters may be suitable for the capture of MSCs and circulating mononuclear cells (MNCs), as well as their subsequent

applications in regenerative medicine. We previously created novel devices with nonwoven filters for regenerative medicine using these filters as a tool to localize the cells to the injured site. In our previous studies, nonwoven filter disks composed of polylactic acid (PLA) fibers with fiber diameters of 1.8 μm effectively captured MSCs and peripheral blood cells and enhanced the production of several growth factors. Specifically, human peripheral blood cells captured on nonwoven biodegradable PLA filters enhanced the production of VEGF, platelet-derived growth factor-AB, and transforming growth factor (TGF)-β1; furthermore, human MSCs captured on nonwoven biodegradable PLA filters enhanced the production of VEGF [13]. Further, as a therapeutic device, nonwoven filters that captured cells were used to enhance wound healing [14]. Briefly, mouse peripheral blood cells (m-PBCs), including MNCs, were captured on nonwoven filters in an appropriate housing. Then, the filters were removed from the housing and embedded into wounded skin areas so that the cells were located very close to the target site. These complexes of PLA nonwoven filters and m-PBCs promoted the healing of skin wounds in db/db mice, possibly as a result of the enhanced production of fibroblast growth factor-7 and/or TGF-β1 [14]. The cells captured on the filters provide a localized scaffold and may interact with the wound site to produce a suitable composition of growth factors and cytokines at the appropriate times.

In the present study, we adapted this technology to use nonwoven filters and cells for the treatment of CKD, as shown in **Figure 1**. Cells were captured on filters and subsequently placed near injured kidneys to provide a local supply of growth factors. Our basic strategy for renal regeneration was as follows: 1) appropriate cells could be localized around the kidney using nonwoven filters, 2) nonwoven filters could stimulate and enhance the production of growth factors

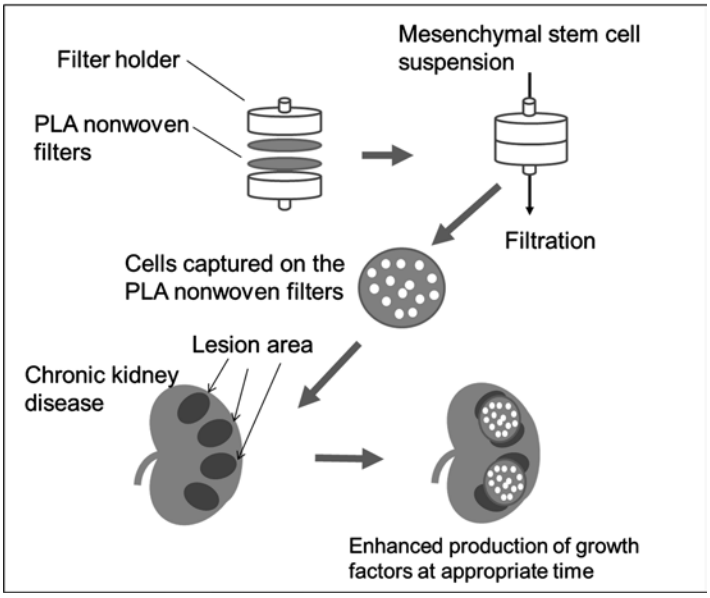


Figure 1. Schema of the strategy for renal regeneration using cell-capture devices with nonwoven filters.

and cytokines, and 3) cells and growth factors/cytokines could be provided from *in situ* cell-laden nonwoven filters at the appropriate times and concentrations.

2. Materials and Methods

2.1. Animal Ethics

This study was performed in accordance with the Regulations for the Management of Laboratory Animals at Fujita Health University, and all protocols were approved by the Institutional Animal Care and Use Committee at the Education and Research Facility of Animal Models for Human Diseases Center of Fujita Health University (Toyoake, Japan).

2.2. Nonwoven Filter Devices for Capturing Cells

Nonwoven filters made of PLA (fiber diameter, 1.8 μm; fiber density, 33 g/m^2) were provided by Asahi Kasei Fibers Corporation (Tokyo, Japan). Rat bone marrow mesenchymal stem cells (r-BMSCs) were purchased from Lonza Japan (Tokyo, Japan) and cultured according to the manufacturer's recommendations. Nonwoven filter disks of 13-mm in diameter were used for in vitro studies of growth factor production as previously reported [13]. For in vivo therapeutic studies of CKD rats, 25-mm filters were used for covering wider areas of the kidney surface. Devices with nonwoven filters for capturing cells were prepared as previously described [13]. Briefly, bundles of 13- or 25-mm nonwoven-filter disks were placed in Swinnex 13 or Swinnex 25 Filter Holders (Millipore, Billerica, MA, USA), respectively, and used to filter r-BMSCs suspensions in injection syringes.

The cell capture rate was defined as follows:

$$\text{Capture rate}(\%) = 100 \times \left(1 - \frac{C_a}{C_b}\right)$$

C_a, Cell count after filtration. C_b, Cell count before filtration.

2.3. Electron Microscopy

Electron microscopy was performed as described previously [13]. Briefly, filter disks with captured cells were fixed with 2.5% glutaraldehyde/0.05 M sodium phosphate (pH 7.4) for at least 24 h at 4˚C. Subsequently, filter disks were dehydrated in a graded ethanol series, followed by 100% t-butyl alcohol, and then freeze-dried at −5˚C using a freeze dryer (JFD-310, JEOL, Tokyo, Japan) and coated with gold using an ion sputtering device (JFC-1500, JEOL). Samples were then examined using a scanning electron microscope (S-2600N, Hitachi, Tokyo, Japan).

2.4. Measurements of Growth Factor Production by Captured Cells on Filters

To collect r-BMSCs, 13-mm diameter disks were placed in a Swinnex 13 Filter

Holder and washed with phosphate buffered saline (PBS); subsequently, 1-ml suspensions of 8×10^4 cells in culture media were filtered using the cell-capture devices. Nonwoven filters with captured r-BMSCs (r-BMSC/PLA group) were placed in 6-well plates, and 200-μl aliquots of 0.3 mg/ml type I collagen (Cellmatrix Type I-A, Nitta Gelatin, Osaka, Japan) were added.

Subsequently, complexes of r-BMSCs and nonwoven filters were cultured for 4 weeks in 3-ml aliquots of MSCGM Bullet Kit medium (Lonza) in a humidified atmosphere containing 5% CO_2 at 37˚C. The medium was changed every 3 - 4 days, and conditioned media were collected and combined each week. Growth factor concentrations were measured in conditioned media at 1 (0 - 7 days), 2 (7 - 14 days), 3 (14 - 21 days), and 4 (21 - 28 days) weeks after the start of culture. Control media were collected from cultures of r-BMSCs without nonwoven filters (control group) on plates coated with 200-μl aliquots of 0.3-mg/ml type I collagen. The concentrations of HGF and VEGF in conditioned media were measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.5. Rat CKD Model

Twelve-week-old male 5/6-nephrectomized rats (Weight 396 ± 30.5 g) with the right kidney removed, as well as the lower and upper one-third of the left kidney removed, were used as rat CKD models. Ten-week-old male 5/6-nephrectomized SD rats were purchased from Charles River Laboratories (Yokohama, Japan).

2.6. Implantation of Nonwoven Filters with Captured Cells into Rats with CKD

To collect r-BMSCs, 25-mm diameter disks were placed in a Swinnex 25 Filter Holder and washed with saline. Subsequently, 5-ml suspensions of 3×10^5 r-BMSCs in culture medium were filtered through the devices. Next, a ventrotomy was performed in 12-week-old 5/6-nephrectomized rats under isoflurane anesthesia. Nonwoven filters with captured cells (r-BMSC/PLA group), nonwoven filters without cells (PLA group), and cells without nonwoven filters (r-BMSC group) were then placed onto kidneys after gently removing the renicapsule. Filters were then fixed to adipose tissues around the kidneys using surgical sutures, and 500-μl aliquots of 0.3 mg/ml type I collagen were dropped onto the nonwoven filters. The control (untreated) group was comprised of untreated 5/6-nephrectomized rats.

2.7. Implantation of Nonwoven Filters with Captured Cells into Rats with CKD

To evaluate the extent of kidney injury, blood and urine samples were collected every 4 weeks after implantation surgery. Blood samples were collected from the caudal vein under anesthesia, and serum creatinine concentrations were determined using LabAssay Creatinine kits (Wako, Osaka, Japan). To determine urinary protein content, rats were placed gently in metabolic cages and urine sam-

ples were collected over 24 h in sample cups. Subsequently, urine samples were mixed thoroughly and assayed using the Quick Start Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Histopathological Evaluations of Kidneys

Eight weeks after implantation surgery, kidneys were resected from rats and fixed in 10% formalin. Next, paraffin-embedded sections stained with Periodic Acid-Schiff reaction (PAS) and Hematoxylin & Eosin (H&E) staining were used for microscopic analyses. The severity of glomerulosclerotic changes was determined by a renal pathology expert in a blinded manner. The evaluation was performed in all glomeruli in a single cross-section of the coronal plane of kidney in each rat. The incidence of glomerulosclerotic lesions was defined as follows:

$$\text{Incidence of glomerulosclerotic lesions}(\%) = 100 \times \frac{N_a}{N_b}$$

N_a, Number of glomeruli with segmental sclerotic changes.

N_b, Total number of observed glomeruli in a single section.

2.9. Statistical Analysis

All data are expressed as the mean ± standard deviation (SD). The differences in nonparametric continuous variables were identified using Wilcoxon's rank sum test using JMP10 software (SAS Institute, Inc., Cary, NC, USA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Growth Factors Produced by r-BMSCs on Nonwoven-Filter Disks

Five PLA nonwoven-filter disks captured 85.8% ± 11.9% of r-BMSCs (n = 3). Electron micrographs of nonwoven PLA filters without cells (**Figure 2(a)**) and filters with r-BMSCs (**Figure 2(b)**) were then analyzed. **Figure 2(b)** shows that r-BMSCs (white arrows) were captured mainly through adherence to fiber surfaces, rather than by filtration.

HGF and VEGF production by r-BMSCs on nonwoven PLA filters was measured over 4 weeks of culture. HGF production from r-BMSC/PLA complexes gradually increased during this period, and was significantly higher than in controls at 2, 3, and 4 weeks. At 4 weeks, HGF production was almost unchanged in controls and was 7.7-fold higher in the r-BMSC/PLA group (**Figure 2(c)**). In contrast, VEGF production by r-BMSCs on PLA filters was significantly suppressed compared to the control until 3 weeks (**Figure 2(d)**). However, VEGF production by r-BMSC/PLA complexes gradually increased and reached control levels at 4 weeks. VEGF and HGF concentrations in culture medium were <30.0 and <11.0 pg/ml, respectively.

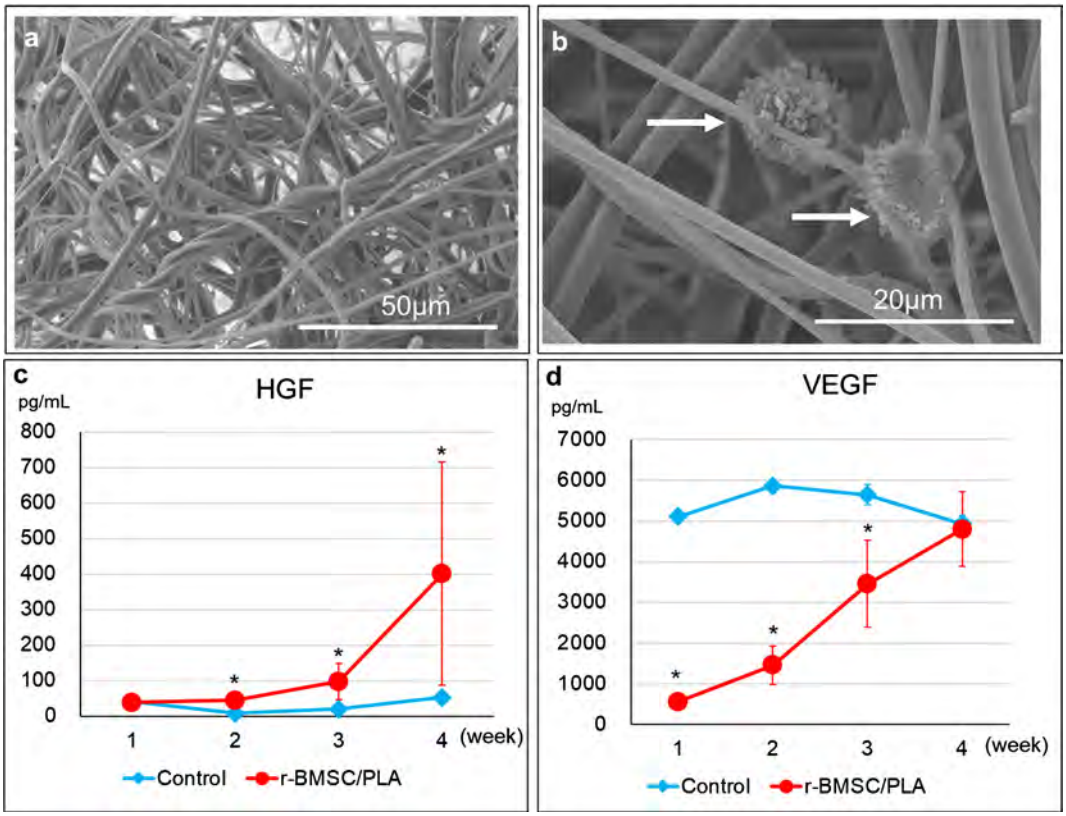


Figure 2. Complex of r-BMSCs on PLA nonwoven filters. (a) Scanning electron photomicrographs of PLA nonwoven filter disks only. (b) Scanning electron photomicrographs of r-BMSCs on PLA filter disks; white arrows indicate r-BMSCs adherent to the fibers. ((c), (d)) Production of growth factors by r-BMSCs on PLA filter disks at 4 weeks of culture. (c) HGF production; (d) VEGF production. Control, conditioned medium from r-BMSCs alone; r-BMSC/PLA, culture medium from r-BMSCs on five PLA filter disks; *$p <$ 0.05 vs. control group; n = 6 per group.

3.2. Effects of r-BMSCs with PLA Nonwoven Filters in CKD Rats

In this study, 5/6-nephrectomized rats were used as models of CKD. Five PLA nonwoven-filter disks (diameter: 25 mm) were used for capturing 3×10^5 r-BMSCs each. The cell/filter complexes, or filters without cells, were then implanted onto the kidney surfaces of 5/6-nephrectomized rats in the r-BMSC/PLA (n = 6) and PLA (n = 4) groups, respectively.

In the group implanted with r-BMSCs alone (r-BMSC, n = 4), suspensions of 3×10^5 r-BMSCs were implanted with type I collagen under the kidney capsule. Untreated 5/6-nephrectomized rats were used as controls (Untreated, n = 10). The urinary protein levels in the r-BMSC/PLA group (32.4 ± 19.6 mg/day) were significantly lower than those in the untreated group (68.1 ± 39.4 mg/day, p = 0.03) at 4 weeks after implantation, indicating that kidney function was improved in CKD rats (**Figure 3(a)**). Moreover, urinary protein levels in the r-BMSC/PLA group remained lower than those in the untreated group for 8 weeks after implantation. However, this difference was not significant (p = 0.25) (**Figure 3(a)**). In contrast to urinary protein levels, serum creatinine levels did not differ significantly between any of the experimental groups (**Figure 3(b)**).

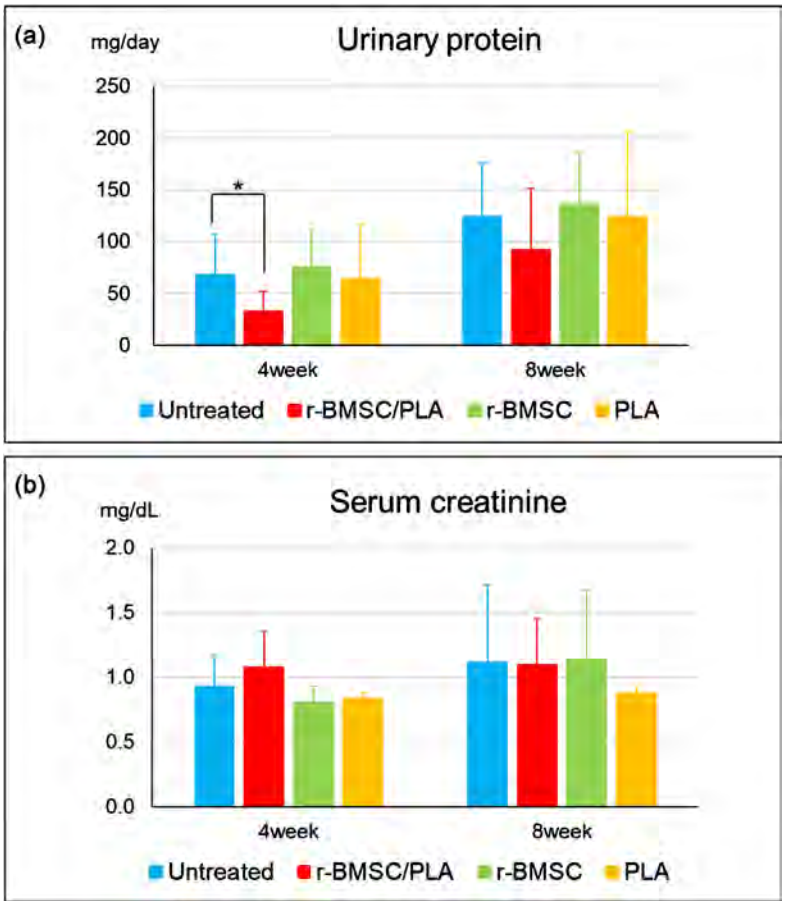


Figure 3. Therapy for 5/6-nephrectomized rats using cells on PLA nonwoven filters. PLA filter disks with or without r-BMSCs were implanted onto the kidneys of 5/6-nephrectomized rats. Control 5/6-nephrectomized rats received renal subcapsular transplants of r-BMSCs or remained untreated. (a) Analyses of urinary protein levels. (b) Analyses of serum creatinine levels. Untreated (n = 10), untreated 5/6-nephrectomized rats; r-BMSC/PLA (n = 6), 5/6-nephrectomized rats treated with r-BMSCs on five PLA filter disks; r-BMSC (n = 4), 5/6-nephrectomized rats treated with r-BMSCs; PLA (n = 4), 5/6-nephrectomized rats treated with five PLA filter disks; *$p < 0.05$ vs untreated group.

3.3. Histopathological Evaluation of Kidneys after Treatment with Nonwoven Filters and Captured Cells

Histopathological analyses of renal cortices at 8 weeks after implantation of PLA nonwoven filters with cells were performed to investigate the morphological improvements related to reduced urinary protein levels in the r-BMSC/PLA group (Figure 4). The right column of Figure 5 (blue rectangle in Figure 4) is an enlarged image of the glomerulus shown in the circle of the figures in the left column. As shown in Figures 5(a)-(d), focal and segmental glomerular sclerosis lesions (black arrows) were observed in all groups. The incidence of glomerulosclerotic lesions was significantly lower in the r-BMSC/PLA group (3.3% ± 3.0%) than in the untreated group (18.4% ± 17.7%; $p = 0.008$) (Figure 5(e)). Moreover, no significant reductions in lesion numbers were observed in the PLA (16.8% ± 13.6%, $p = 0.83$) or r-BMSC (23.3% ± 26.6%, $p = 0.94$) groups compared to the

untreated group (Figure 5(e)).

Next, the contact area of the PLA nonwoven filter and kidney was histopathologically investigated. Figure 6 shows microscopic images of the contact area of the PLA nonwoven filter and the kidney (red rectangle in Figure 4). Black arrows indicate polynuclear foreign body giant cells; these were observed in both the r-BMSC/PLA (Figure 6(a)) and PLA groups (Figure 6(b)), and there were no differences between the groups. The black arrowheads in Figure 6(a) and Figure 6(b) show lymphocytes at the contact area of the PLA nonwoven filters and kidney, as well as those infiltrating the renal cortex. In addition, the fragmented PLA nonwoven filter is indicated by a yellow arrow (Figure 6(a)). The degree of fragmentation of the PLA nonwoven filters was comparable between the two groups.

4. Discussion

The combination of r-BMSCs and PLA nonwoven filters improved or prevented glomerulosclerosis in 5/6-nephrectomy rats with CKD, which is characterized by progressive glomerulosclerosis [15]. Kidney regeneration by MSCs reportedly involves various growth factors and cytokines [16], and several studies have shown that HGF attenuates CKD symptoms. Specifically, injections with HGF attenuated the progression of glomerulosclerosis and proteinuria in 5/6-nephrectomized rats or nephrotic mice [3] [6]. Moreover, implanted MSCs ameliorated glomerular injuries by secreting HGF in a rat model of diabetic nephropathy [17]. The biological effects of HGF include the enhancement of cell growth, anti-apoptotic and angiogenic activity [18], and renoprotective effects [19]. In the present *in vitro* experiments, HGF production was enhanced in MSCs captured on nonwoven PLA filters, and these cells improved glomerulosclerosis after implantation *in vivo*. In contrast, a topical application of nonwoven PLA filters without cells, or of r-BMSCs alone, onto kidney surfaces did not significantly improve or prevent glomerulosclerosis. These data suggest that glomerulosclerosis was ameliorated by enhanced HGF production.

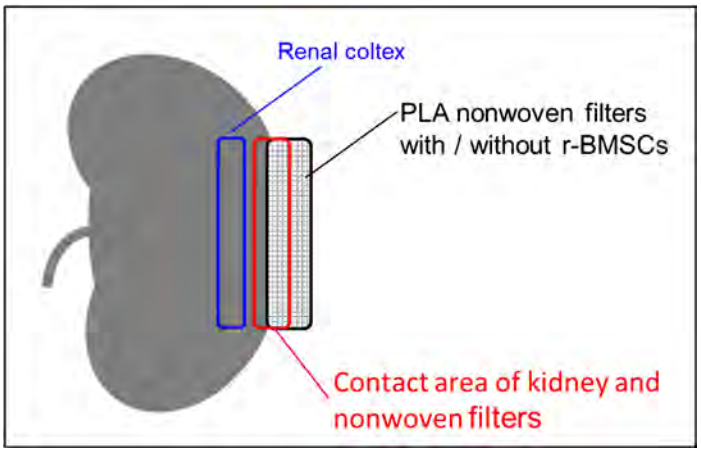
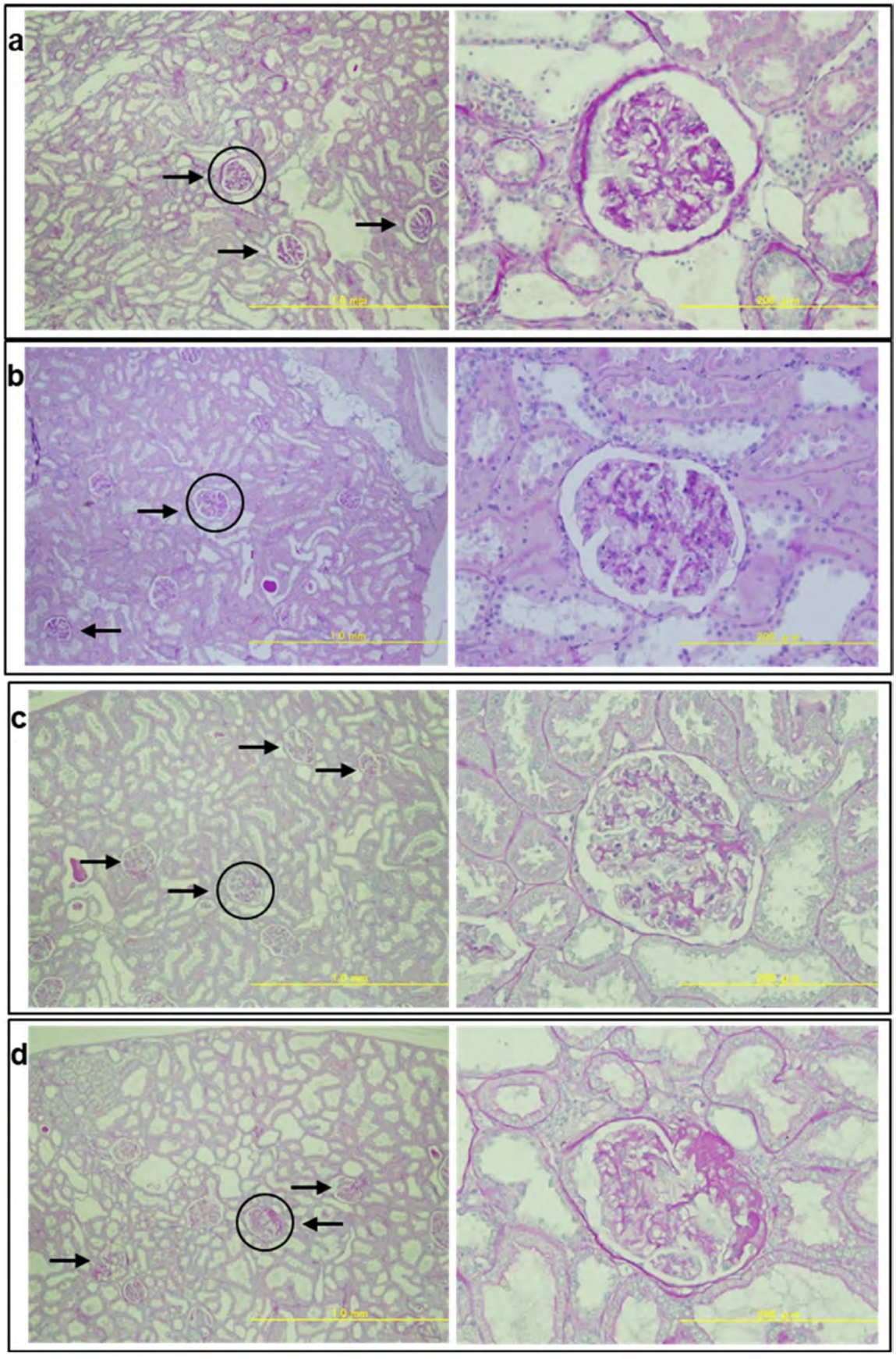


Figure 4. Schema of the contact area of the kidney and nonwoven filter.


a
b
c
d

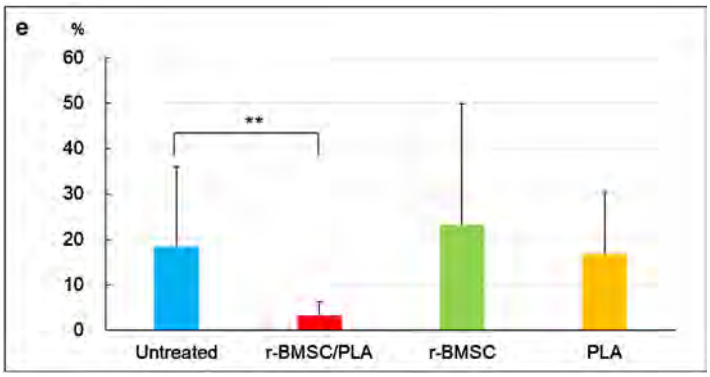


Figure 5. Histopathological evaluations of kidneys after treatment using nonwoven filters with captured cells (PAS). (a) Untreated 5/6-nephrectomized rats. (b) 5/6-nephrectomized rats treated with r-BMSCs on five PLA filter disks. (c) 5/6-nephrectomized rats treated with r-BMSCs. (d) 5/6-nephrectomized rats treated with five PLA filter disks. Black arrows indicate glomerulosclerotic lesions. The right column is an enlarged image of the glomerulus shown in the circle of the left column. Yellow scale bars in bottom right corner of images are 1.0 mm (left column) and 200 μm (right column) insets. (e) Incidence of glomerulosclerotic lesions. Untreated (n = 10), untreated 5/6-nephrectomized rats; r-BMSC/PLA (n = 6), 5/6-nephrectomized rats treated with r-BMSCs on five PLA filter disks; r-BMSC (n = 4), 5/6-nephrectomized rats treated with r-BMSCs; PLA (n = 4), 5/6-nephrectomized rats treated with five PLA filter disks; $^{**}p < 0.01$ vs. untreated group.

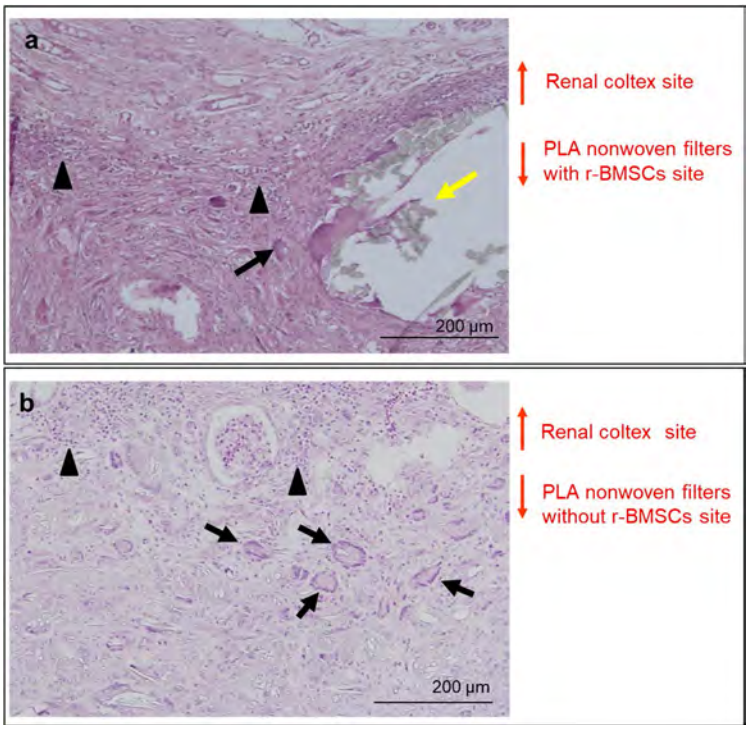


Figure 6. Histopathology of the contact area of kidney and PLA filters 8 weeks after implantation with PLA nonwoven filters with or without cells. (H & E). (a) Pathological photomicrograph of the contact area between the PLA filters with r-BMSCs and the kidney of 5/6-nephrectomized rats. (b) Pathological photomicrograph of the contact area between the PLA filters without r-BMSCs and the kidney of 5/6-nephrectomized rats. Black arrows indicate multinucleated foreign body giant cells; black arrowheads indicate lymphocytes; yellow arrow indicates decomposed matter of PLA filter.

Moreover, PLA filters suppressed VEGF production during the first 3 weeks of culture, whereas VEGF production in the presence of PLA filters with r-BMSCs was similar to that in control groups at 4 weeks. Because the production of VEGF in PLA filters with r-BMSCs gradually increased during the 4-week culture period, the production of VEGF may have been enhanced after culture for longer than 4 weeks. These data further suggest that growth factors such as HGF and VEGF were secreted from r-BMSCs fixed on PLA filters, and that they may contribute to restorative paracrine signaling in wounded glomeruli. When the r-BMSC/PLA complex was implanted after 4 weeks of culture, the subsequent augmented production of both HGF and VEGF was expected to have a major therapeutic effect.

The implantation of r-BMSC/PLA into 5/6-nephrectomized rats attenuated urinary protein excretion after 4 weeks. However, at 8 weeks after implantation, this effect of decreasing urinary protein disappeared. There are two potential reasons behind this development. The PLA nonwoven filter may have been fragmented due to a foreign matter reaction by polynuclear foreign body giant cells; the nonwoven filter indeed appeared to be highly decomposed at 8 weeks after implantation. Moreover, the degree of decomposition did not change depending on the presence or absence of r-BMSCs. Inflammatory reactions are mainly caused by biomaterials embedded in the body, or the degradation of these products [20]. Furthermore, in the cell interaction process during the chronic phase, polynuclear foreign body giant cells are formed, and the formed multinuclear foreign body giant cells degrade biodegradable substances over time [21]. Inflammation is also essential to promote the recruitment of progenitor cells and to initiate healing mechanisms, but at the same time results in tissue damage and fiber inclusion of biocompatible substances [22]. In this study, we did not investigate the presence of r-BMSCs around the implantation site, but polynuclear foreign body giant cells and lymphocytes were observed, suggesting the possibility that fibrosis was initiated in the nonwoven filters. This is a possible reason why urine protein concentrations did not decrease at 8 weeks after implantation. Another possible reason is that the r-BMSCs captured on the PLA nonwoven filter could not adhere to a sufficient area of the damaged kidney surface. Indeed, in some rats it was observed that the r-BMSCs captured on PLA nonwoven filters were partially separated from the kidney surface (data not shown). Therefore, a nonwoven filter affixing method covering a sufficient area of the injured kidney surface, as well as an implantation method allowing the complex of cells and nonwoven filters to exist on the kidney surface for a longer period, requires future study.

5. Conclusion

Here, we report the development of a novel therapeutic device made up of biodegradable PLA filters and r-BMSCs. After implantation into 5/6-nephrectomized rats, these devices reduced urinary protein levels and prevented or ameliorated

glomerulosclerosis. These effects might be related to enhanced HGF production by r-BMSCs that were captured on the PLA filters. This device requires further consideration as a treatment for CKD.

Acknowledgements

The authors thank Drs. Mikitomo Yasutake and Ushio Iwamoto of Asahi Kasei, and Ms. Mikiko Ariga of Asahi Kasei Fibers Corporation for providing the nonwoven filters.

The authors also thank Mses. Miwa Sakata, Kasumi Suzuki, and Shoko Hattori, and Mrs. Koki Kawabata, and Tomohiro Shikata for their technical assistance.

This work was partly supported by JSPS KAKENHI Grant Number 16K05923.

Conflicts of Interest

All authors declare that they have no conflict of interest.

References

[1] Liyanage, T., Ninomiya, T., Jha, V., Neal, B., Patrice, H.M., Okpechi, I., *et al.* (2015) Worldwide Access to Treatment for End-Stage Kidney Disease: A Systematic Review. *The Lancet*, **385**, 1975-1982. https://doi.org/10.1016/S0140-6736(14)61601-9

[2] Just, P.M., Riella, M.C., Tschosik, E.A., Noe, L.L., Bhattacharyya, S.K. and de Charro, F. (2008) Economic Evaluations of Dialysis Treatment Modalities. *Health Policy*, **86**, 163-180. https://doi.org/10.1016/j.healthpol.2007.12.004

[3] Mizuno, S., Kurosawa, T., Matsumoto, K., Mizuno-Horikawa, Y., Okamoto, M. and Nakamura, T. (1998) Hepatocyte Growth Factor Prevents Renal Fibrosis and Dysfunction in a Mouse Model of Chronic Renal Disease. *The Journal of Clinical Investigation*, **101**, 1827-1834. https://doi.org/10.1172/JCI1709

[4] Flaquer, M., Romagnani, P. and Cruzado, J.M. (2010) Growth Factors and Renal Regeneration. *Nefrologia*, **30**, 385-393.

[5] Shimizu, A., Masuda, Y., Mori, T., Kitamura, H., Ishizaki, M., Sugisaki, Y., *et al.* (2004) Vascular Endothelial Growth Factor165 Resolves Glomerular Inflammation and Accelerates Glomerular Capillary Repair in Rat Anti-Glomerular Basement Membrane Glomerulonephritis. *Journal of the American Society of Nephrology*, **15**, 2655-2665. https://doi.org/10.1097/01.ASN.0000141038.28733.F2

[6] Gong, R., Rifai, A., Tolbert, E.M., Biswas, P., Centracchio, J.N. and Dworkin, L.D. (2004) Hepatocyte Growth Factor Ameliorates Renal Interstitial Inflammation in Rat Remnant Kidney by Modulating Tubular Expression of Macrophage Chemoattractant Protein-1 and RANTES. *Journal of the American Society of Nephrology*, **15**, 2868-2881. https://doi.org/10.1097/01.ASN.0000141962.44300.3A

[7] Yang, J., Chen, S., Huang, L., Michalopoulos, G.K. and Liu, Y. (2001) Sustained Expression of Naked Plasmid DNA Encoding Hepatocyte Growth Factor in Mice Promotes Liver and Overall Body Growth. *Hepatology*, **33**, 848-859. https://doi.org/10.1053/jhep.2001.23438

[8] Ezquer, F., Ezquer, M., Simon, V., Pardo, F., Yañez, A., Carpio, D., *et al.* (2009) Endovenous Administration of Bone-Marrow-Derived Multipotent Mesenchymal Stromal Cells Prevents Renal Failure in Diabetic Mice. *Biology of Blood and Marrow Transplantation*, **15**, 1354-1365. https://doi.org/10.1016/j.bbmt.2009.07.022

[9] Zoja, C., Garcia, P.B., Rota, C., Conti, S., Gagliardini, E., Corna, D., *et al.* (2012)

Mesenchymal Stem Cell Therapy Promotes Renal Repair by Limiting Glomerular Podocyte and Progenitor Cell Dysfunction in Adriamycin-Induced Nephropathy. *American Journal of Physiology—Renal Physiology*, **303**, F1370-F1381. https://doi.org/10.1152/ajprenal.00057.2012

[10] Semedo, P., Correa-Costa, M., Cenedeze, M.A., Malheiros, D.M.A.C., dos Reis, M.A. and Shimizu M.H. (2009) Mesenchymal Stem Cells Attenuate Renal Fibrosis through Immune Modulation and Remodeling Properties in a Rat Remnant Kidney Model. *Stem Cells*, **27**, 3063-3073. https://doi.org/10.1002/stem.214

[11] Song, I.H., Jung, K.J., Lee, T.J., Kim, J.Y., Sung, E.G., Bae, Y.C., *et al.* (2018) Mesenchymal Stem Cells Attenuate Adriamycin-Induced Nephropathy by Diminishing Oxidative Stress and Inflammation via Downregulation of the NF-kB. *Nephrology*, **23**, 483-492. https://doi.org/10.1111/nep.13047

[12] Schrepfer, S., Deuse, T., Reichenspurner, H., Fischbein, M.P., Robbins, R.C. and Pelletier, M.P. (2007) Stem Cell Transplantation: The Lung Barrier. *Transplantation Proceedings*, **39**, 573-576. https://doi.org/10.1016/j.transproceed.2006.12.019

[13] Hori, H., Iwamoto, U., Niimi, G., Shinzato, M., Hiki, Y., Tokushima, Y., *et al.* (2015) Appropriate Nonwoven Filters Effectively Capture Human Peripheral Blood Cells and Mesenchymal Stem cells, Which Show Enhanced Production of Growth Factors. *Journal of Artificial Organs*, **18**, 55-63. https://doi.org/10.1007/s10047-014-0794-9

[14] Iwamoto, U., Hori, H., Takami, Y., Tokushima, Y., Shinzato, M., Yasutake, M., *et al.* (2015) A Novel Cell-Containing Device for Regenerative Medicine: Biodegradable Nonwoven Filters with Peripheral Blood Cells Promote Wound Healing. *Journal of Artificial Organs*, **18**, 315-321. https://doi.org/10.1007/s10047-015-0845-x

[15] Shimamura, T. and Morrison, A.B. (1975) A Progressive Glomerulosclerosis Occurring in Partial Five-Sixths Nephrectomized Rats. *The American Journal of Pathology*, **79**, 95-106. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1913032/

[16] Caplan, A.I. and Dennis, J.E. (2006) Mesenchymal Stem Cells as Trophic Mediators. *Journal of Cellular Biochemistry*, **98**, 1076-1084. https://doi.org/10.1002/jcb.20886

[17] Lv, S., Cheng, J., Sun, A., Li, J., Wang, W., Guan, G., *et al.* (2014) Mesenchymal Stem Cells Transplantation Ameliorates Glomerular Injury in Streptozotocin-Induced Diabetic Nephropathy in Rats via Inhibiting Oxidative Stress. *Diabetes Research and Clinical Practice*, **104**, 143-154. https://doi.org/10.1016/j.diabres.2014.01.011

[18] Matsumoto, K. and Nakamura, T. (2001) Hepatocyte Growth Factor: Renotropic Role and Potential Therapeutics for Renal Diseases. *Kidney International*, **59**, 2023-2038. https://doi.org/10.1046/j.1523-1755.2001.00717.x

[19] Cantaluppi, V., Biancone, L., Quercia, A., Deregibus, M.C., Segoloni, G. and Camussi, G. (2013) Rationale of Mesenchymal Stem Cell Therapy in Kidney Injury. *American Journal of Kidney Diseases*, **61**, 300-309. https://doi.org/10.1053/j.ajkd.2012.05.027

[20] Ding, J., Chen, B., Lv, T., Liu, X., Fu, X., Wang, Q., *et al.* (2016) Bone Marrow Mesenchymal Stem Cell-Based Engineered Cartilage Ameliorates Polyglycolic Acid/Polylactic Acid Scaffold-Induced Inflammation through M2 Polarization of Macrophages in a Pig Model. *Stem Cells Translational Medicine*, **5**, 1079-1089. https://doi.org/10.5966/sctm.2015-0263

[21] Ramot, Y., Haim-Zada, M., Domb, A.J. and Nyska, A. (2016) Biocompatibility and Safety of PLA and Its Copolymers. *Advanced Drug Delivery Reviews*, **107**, 153-162. https://doi.org/10.1016/j.addr.2016.03.012

[22] McNally, A.K. and Anderson, J.M. (2003) Foreign Body-Type Multinucleated Giant

Cell Formation Is Potently Induced by Alpha-Tocopherol AND Prevented by the Diacylglycerol Kinase Inhibitor R59022. *The American Journal of Pathology*, **163**, 1147-1156. https://doi.org/10.1016/S0002-9440(10)63474-8

International Journal of Clinical Medicine, 2019, 10, 150-160
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Oscillating Mechanical Stimulation of the Craniocervical Region as Physical Therapy for Chronic Migraine: A Pilot Trial

Makoto Shiraishi[1], Munefumi Hotta[2], Tomohiro Suzuki[3], Noboru Imai[4]

[1]Division of Neurology, Department of Internal Medicine, St Marianna University School of Medicine, Kawasaki, Japan
[2]Hotta Clinic, Shizuoka, Japan
[3]Suzuki Osteopathic Clinic, Shizuoka, Japan
[4]Department of Neurology, Japanese Red Cross Shizuoka Hospital, Shizuoka, Japan
Email: shira@maranna-u.ac.jp

How to cite this paper: Shiraishi, M., Hotta, M., Suzuki, T. and Imai, N. (2019) Oscillating Mechanical Stimulation of the Craniocervical Region as Physical Therapy for Chronic Migraine: A Pilot Trial. *International Journal of Clinical Medicine*, **10**, 150-160.
https://doi.org/10.4236/ijcm.2019.103015

Received: February 16, 2019
Accepted: March 12, 2019
Published: March 15, 2019

Abstract

Objective: We conducted a prospective trial of oscillating mechanical stimulation (OS) of the craniocervical region as treatment for drug-refractory chronic migraine (CM). **Methods:** Ten patients (8 women, 2 men; mean age 47.0 ± 15.1 years) were enrolled. The treatment was administered over an 8-week period to 13, 4, and 9 sites on the face and head, neck, and upper back, respectively, at 5- to 15-pound intensity. The primary outcome measure was the number of days patients suffered a migraine (hereafter "number of migraine days"), and the secondary outcome measures were the six-item Headache Impact Test (HIT-6) and Visual Analog Scale (VAS) scores for migraine pain intensity and the nine-item Patient Health Questionnaire (PHQ-9) and the seven-item Generalized Anxiety Disorder (GAD-7) scale scores. **Results:** Nine patients completed treatment. The number of migraine days remained unchanged, from a mean 21.7 ± 11.6 days/month before treatment to 19.3 ± 7.3 days/month upon completion of treatment. However, the HIT-6 scores improved from 67.0 ± 8.2 to 61.4 ± 7.1 ($p = 0.007$) after 3 weeks, 61.1 ± 11.5 ($p = 0.01$) after 6 weeks, and 59.9 ± 11.6 ($p = 0.035$) upon completion of treatment. Similarly, the VAS scores improved significantly from 7.3 ± 1.7 to 5.7 ± 3.1 ($p = 0.018$) at 6 weeks and 4.8 ± 2.8 ($p = 0.011$) upon completion of treatment. The GAD-7, PHQ-9, and allodynia scale scores remained unchanged. **Conclusion:** Our data suggest that OS is well tolerated and may become a feasible form of treatment for drug-resistant CM.

Keywords

Chronic Migraine, Oscillating Mechanical Stimulation, Drug Resistance,

Nondrug Treatment, Neuromodulatory Effect

1. Introduction

When a patient suffers from headache on more than 15 days a month for 3 months and when the headache manifests as migraine on 8 of those 15 days, the patient is said to be suffering from chronic migraine (CM) [1]. Patients who suffer frequent headache attacks or overuse analgesics are at an increased risk of conversion to chronicity [2]. The European Headache Foundation has proposed that CM be defined as migraine in the absence of drug abuse for which three or more types of migraine prophylaxis have been ineffective [3]. On a global scale, CM is related not only to an increased economic burden, *i.e.*, a drain on medical resources, but also to lost productivity [4]. Peripheral subcutaneous injection of botulinum toxin has been shown to be effective as prophylaxis against CM [5] [6], and randomized, controlled studies of neuromodulation by percutaneous supraorbital stimulation in the absence of drug administration have shown the usefulness of this form of therapy [7]. We have focused on nondrug, percutaneous treatment and developed an oscillating mechanical stimulation therapy (OST), which broadly targets the craniocervical region and has been shown to be effective when administered at weekly intervals [8]. OST was originally attempted in Western countries as a form of physical therapy for medically refractory chronic pain, and a systematic review has been reported of its use in cases of calcific tendinitis of the rotator cuff [9]. There are very few studies from the field of migraine research, however.

2. Material and Methods

The safety of the technique used in the study was verified during preparatory research [8], and the study protocol was approved by the institutional review board of Shizuoka Red Cross Hospital (approval No. 2015-03), and this study was registered by the UMIN (ID: 00017253). The selected patients were given an oral and written explanation of the prospective trial, and they were enrolled between February 2016 and January 2017 after providing written informed consent.

2.1. Patients

The selected patients met the following inclusion criteria: 1) CM had been diagnosed according to the International Classification of Headache Disorders, 3rd edition (beta version) [10]; 2) the CM was refractory, *i.e.*, according to the patient's migraine diary or medical history, the headaches did not improve in severity or frequency, despite acute drug or prophylactic treatment; 3) the patient was ≥20 years of age when providing consent; 4) the patient was deemed capable of outpatient visits; and 5) the patient's migraine drug dosage and method of

administration remained unchanged during the 2-week period before the therapeutic intervention was begun. Refractory CM was defined as CM for which three types of grade A-recommended prophylactic drugs with different mechanisms of action were ineffective, in accordance with the Clinical Practice Guideline for Chronic Headache 2013 [11]. The patients underwent cervical spine radiography before and after OST, and safety was confirmed.

Patients not included in the study were 1) those with chronic headache other than CM; 2) those who were participating in another trial or had participated in another trial up to 1 month prior to the time the intervention would have been started; 3) those with concomitant compression of the spinal cord, resulting, for example, from a facial or craniocervical fracture; and those with severe cervical vertebral disease, intervertebral disc herniation, or ossification of the yellow ligament; 4) those with a history of osteoporosis; 5) those presenting with a severe cutaneous abnormality affecting the face, head, or neck; 6) those whose physical condition had deteriorated subsequent to massage or chiropractic adjustment to the craniocervical region; 7) those who had undergone surgical treatment for a cranial or spinal column disorder within the previous 6 months before this treatment; 8) those scheduled to undergo surgery during what would have been the treatment period; 9) those presenting with dementia; 10) those with malignancy requiring treatment; 11) those with psychological symptoms or a psychological disorder, such as severe confusion, hallucinations, delusions, or abnormal behavior; and 12) those who were pregnant.

2.2. Method of Treatment

OST was delivered by means of an electric percussion hammer (Hammons Impact; Aichi Electronics Industrial Co., Ltd., Aichi, Japan). Treatment was performed once a week over a period of 8 weeks. Each week, percutaneous mechanical OST was performed at several predetermined sites, so that by the end of the treatment period, OST had been performed twice at a total of 26 sites (**Table 1** and **Table 2**): 13 on the face and head, 4 on the neck, and 9 on the upper back (**Figure 1(a)**, **Figure 1(b)**). The device has been approved by the Ministry of Health, Labour and Welfare of Japan (approval no. 23B2X00010), and its use is not limited to specific disorders. It has been used to manage lumbar pain associated with intervertebral disc herniation, neck pain associated with acute sprain and cervical disc herniation, and rehabilitation after surgery for ligament rupture or fracture. In such cases, stimulation of 0.37 N 0.87 N is provided over a period of 2 to 3 months, and although the precise mechanism underlying the therapeutic effects is unknown, the stimulation is presumed to enhance the healing process.

Depending on the stimulation site, the patient is placed in the dorsal or prone position during treatment. The stimulus frequency is set to 6 Hz, and the duration of OS at each site is 6 seconds. An OS intensity of 5 to 15 pounds is selected, depending on the site and the number of treatments. To ensure appropriate transmission of the oscillating mechanical stimulation based on the pressure and

density of the tissue at the stimulation site, the stimulation depth is set to low, medium, or high. A single-prong, double-prong, or wide-prong head, which comes into contact with the patient's skin, is selected on the basis of the site of application.

For the study patients, if the migraine prophylaxis remained unchanged for 3 months before the start of OST and could be provided concomitantly, it was

Table 1. Oscillating craniocervical stimulation sites.

Sites on the face, head, and neck
1. Middle eyebrow: above the pupil
2. Infraorbital foramen: intersection of an imaginary vertical line through the pupil center and imaginary horizontal line through the lower end of the nasal wings
3. Nearby medial ocular angle: 3 mm above the medial ocular angle
4. Upper ear: apex of the head on the pinna
5. Upper lateral forehead: 4 digits from the lower edge of the sphenoidal rostrum
6. Lateral nostril: 1.5 mm from the lateral nostril
7. Medial eyelashes: excavation in medial edge on eyebrow
8. Lateral frontal area: 1 cm behind and 3 cm lateral to the midpoint of the frontal hairline
9. Supraorbital foramen: 1 digit above and 3 cm lateral to the upper center edge of the orbitas
10. Outer edge of the eyelashes: excavation in outer edge of the eyelashes
11. Lower edge of the zygomatic bone: lower zygomatic bone, directly below the outer canthus
12. Mental foramen: lower portion of the second premolars
13. Upper part of the gonial angle: 1 digit below the gonial angle
Sites on the back of the head and upper back
14. Lower portion of the mastoid process: posterior lower excavation of the mastoid process
15. External occipital protuberance: lateral excavation of the external occipital protuberance
16. Neighborhood of the spinous process of the second cervical vertebra: lower edge of the spinous process of the second cervical vertebra
17. Neighborhood of the spinous process of the first thoracic vertebra: lower edge of the spinous process of the first thoracic vertebra, 1.5 digits from the posterior median line
18. Neighborhood of the spinous process of the second thoracic vertebra: lower edge of the spinous process of the second thoracic vertebra, 1.5 digits from posterior median line
19. Neighborhood of the spinous process of the third thoracic vertebra: lower edge of the spinous process of the third thoracic vertebra, 1.5 digits from the posterior median line
20. Neighborhood of the spinous process of the fifth thoracic vertebra: lower edge spinous process of the third thoracic vertebra, 1.5 digits from the posterior median line
21. Scapula: one-third excavation from spina scapulae side on the imaginary line between the midpoint of both the spina scapulae and angulus inferior scapulae
22. Neighborhood of the spinous process of the fifth cervical vertebra: lateral lower cervical portion spinous of the seventh cervical vertebra, 2 digits from the posterior median sulcus
23. Lateral side of the spinous process of the second thoracic vertebra: lateral lower cervical portion spinous of the seventh cervical vertebra, 3 digits from the posterior median sulcus
24. Lateral side of the spinous process of the third thoracic vertebra: 3 digits from the lower edge of the posterior median line
25. Lateral side of the spinous process of the fourth thoracic vertebra: 3 digits from the lower edge of the posterior median sulcus
26. Lateral side of the spinous process of the fifth thoracic vertebra: 3 digits from the lower edge of the posterior median sulcus

Table 2. Treatment schedule.

Weeks	Patient position	Stimulation sites
1, 2	Dorsal	1, 2, 3, 6, 12(S)
	Prone	4, 5(S), 15(W), 16, 17(D)
3, 4	Dorsal	7, 8, 9, 10, 11, 13, 4, 5(S)
	Prone	16(D), 15(W), 18(D), 19(D)
5, 6	Dorsal	4, 5, 7, 8, 9, 10, 11, 13(S)
	Prone	14(S), 22(D), 23, 24, 25, 26(S)
7, 8	Dorsal	4, 5, 7, 8, 9, 10, 11, 13(S)
	Prone	16, 17(D), 21, 23, 24, 25, 26(S)

(S), single probe; (D), double probe; (W), wide probe.

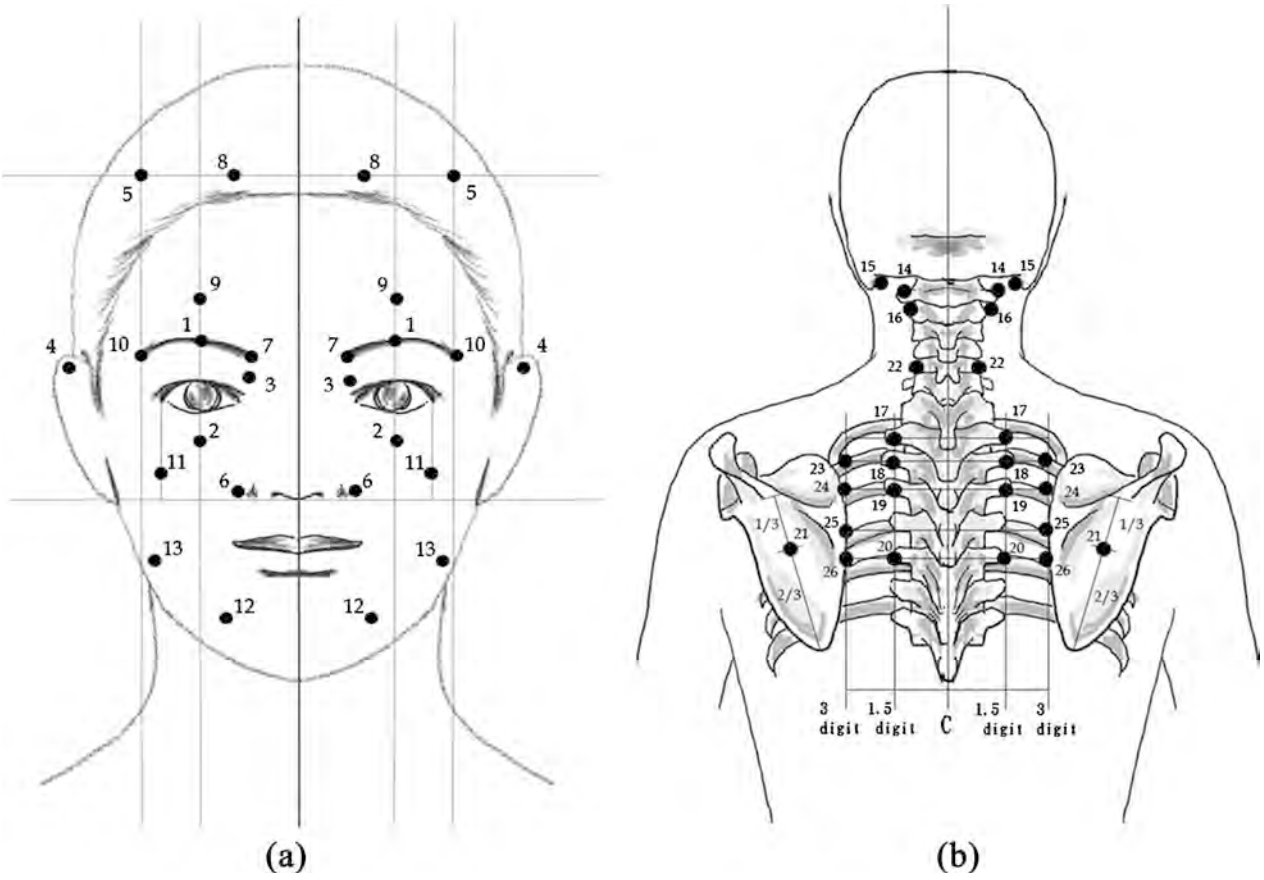


Figure 1. (a) Oscillating craniocervical stimulation sites in the front. 1, Middle eyebrow; 2, infraorbital foramen; 3, nearby medial ocular angle; 4, upper ear; 5, upper lateral forehead; 6, lateral nostril; 7, medial eyelashes; 8, lateral frontal area; 9, supraorbital foramen; 10, outer edge of the eyelashes; 11, lower edge of the zygomatic bone; 12, mental foramen; 13, upper part of the gonial angle. (b) Oscillating craniocervical stimulation sites in the back 14, Lower portion of the mastoid process; 15, external occipital protuberance; 16, neighborhood of the spinous process of the second cervical vertebra; 17, neighborhood of the spinous process of the first thoracic vertebra; 18, neighborhood of the spinous process of the second thoracic vertebra; 19, neighborhood of the spinous process of the third thoracic vertebra; 20, neighborhood of the spinous process of the fifth thoracic vertebra; 21, scapula; 22, neighborhood of the spinous process of the fifth cervical vertebra; 23, lateral side of the spinous process of the second thoracic vertebra; 24, lateral side of the spinous process of the third thoracic vertebra; 25, lateral side of the spinous process of the fourth thoracic vertebra; 26, lateral side of the spinous process of the fifth thoracic vertebra.

continued during the treatment period. Drugs were used for migraine attacks but were not introduced for prophylaxis.

2.3. Data Collection and Statistical Analysis

For evaluation of OST, the following information was obtained: the patient's individual clinical characteristics (age, sex, migraine drug treatment); the number of migraine days, derived from the patient's headache diary; the Headache Impact Test (HIT-6) [12] and Visual Analog Scale (VAS) [13] scores; the frequency of acute therapy; and the allodynia score, derived from the Patient Health Questionnaire (PHQ-9) [14], Generalized Anxiety Disorder (GAD-7) [15], and allodynia scales. These study variables were obtained by neurologists before and after the start of treatment. Values are shown as means ± SD. The HIT-6, VAS, and PHQ-9 scores were evaluated 3 and 6 weeks after the start of treatment. Changes in the HIT-6, VAS, GAD-7, PHQ-9, and allodynia scores were examined by one-way analysis of variance (ANOVA). SPSS version 21 (IBM SPSS Statistics for Windows; IBM Corp, Armonk, NY, USA) was used for statistical analysis, and $p < 0.05$ was considered to indicate a significant difference.

3. Results

The clinical characteristics of the 10 enrollees are shown in **Table 3**. One patient

Table 3. Demographic and baseline characteristics.

Patient No.	Gender	Age	Number of Days Headache Occurred per Month	HIT-6 score	Pain Intensity (VAS) Score	GAD-7	PHQ-9	Allodynia scale	Prophylactic Medicaitons	Acute Medications
1	M	68	23	76	10	0	5	0	Non	Zolmitriptan 2.5 mg
2	F	52	19	64	5	3	3	4	Lomerizine 20 mg, Propranolol 40 mg	Naratriptan 2.5 mg
3	F	40	20	63	4	10	11	7	Lomerizine 20 mg,	Naratriptan 2.5 mg
4	F	70	15	60	7	2	6	0	Non	Sumatriptan 50 mg
5	F	48	28	78	9	22	18	1	Non	Sumatriptan 50 mg, Acetaminophen 800 mg
6	F	51	18	55	8	0	2	3	Amitriptyline 25 mg, Topiramate 200 mg, Valproic acid 200 mg	Aspirin 900 mg, Acetoaminophen 600 mg, Anhydrous caffeine 200 mg, Bromovalerylurea 500 mg
7	F	36	30	71	8	0	11	4	Topiramate 100 mg	Sumatriptan 50 mg
8	F	28	21	74	9	1	9	0	Amitriptyline 50 mg, Topiramate 100 mg,	Loxoprofen 60 mg
9	F	30	27	66	8	6	17	6	Amitriptyline 10 mg	Rizatriptan 10 mg, Acetaminophen 800 mg, Loxoprofen 60 mg
10	M	25	15	66	10	15	12	5	Lomerizine 20 mg	Loxopfofen 60 mg

One patient (patient No.10) withdrew from the study at the request of the family practitioner who wanted the patient to discontinue the treatment. Therefore, 9 patients completed the treatment.

withdrew from the study at the request of the family practitioner who wanted the patient to discontinue the treatment. Nine patients completed the treatment. 9 patients completed the study protocol for 8 weeks. No severe or serious adverse events occurred.

Migraine frequency did not change after treatment; the mean frequency was 21.7 ± 6.4 days per month before treatment and 19.3 ± 7.3 days per month after treatment (Figure 2). However, the HIT-6 scores improved significantly from 67.0 ± 8.2 before treatment to 61.4 ± 7.1 ($p = 0.007$) after 3 weeks, 61.1 ± 11.5 ($p = 0.01$) after 6 weeks, and 59.9 ± 11.6 ($p = 0.035$) when treatment was completed (Figure 3(a)). Similarly, the VAS scores improved from 7.3 ± 1.7 before treatment to 5.9 ± 2.9 ($p = 0.044$) after 3 weeks, 5.7 ± 3.1 ($p = 0.018$) after 6 weeks, and 4.8 ± 2.8 ($p = 0.011$) when treatment was completed. Notably, the VAS scores upon completion of treatment were significantly lower than those after 3 weeks ($p = 0.011$) (Figure 3(b)).

The GAD-7 (Figure 3(c)), PHQ-9 (Figure 3(d)), and allodynia scale scores obtained after 3 and 6 weeks of treatment were not significantly different from the scores obtained before and upon completion of treatment. There were also no significant changes in the patients' recorded use of acute therapy agents during the treatment period or in prophylactic treatment.

4. Discussion

This is the first study to verify the efficacy and safety of percutaneous craniocervical OST applied to multiple sites on the face, head, and neck to treat drug-resistant CM. The overall result of the study was that the patients' migraine symptoms decreased during the 8-week treatment period. Furthermore, OST therapy seems to

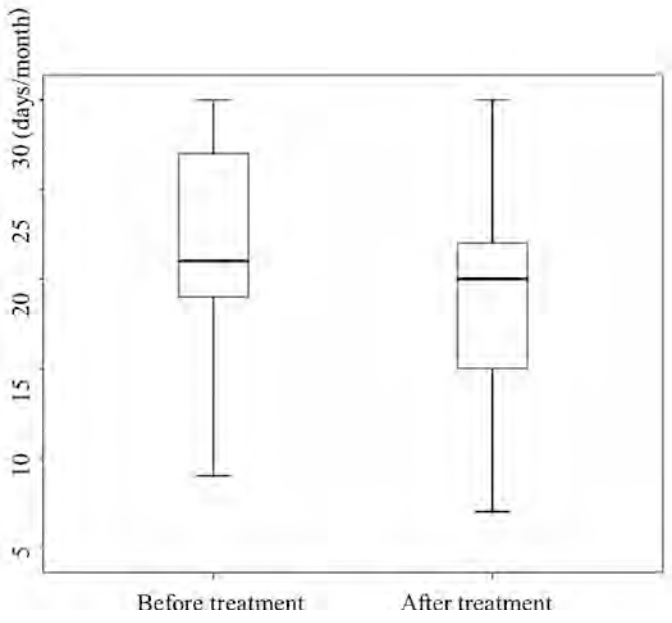


Figure 2. Migraine frequency. Box plots showing distribution of migraine frequency before and after treatment. Whiskers indicate the upper and lower quartiles.

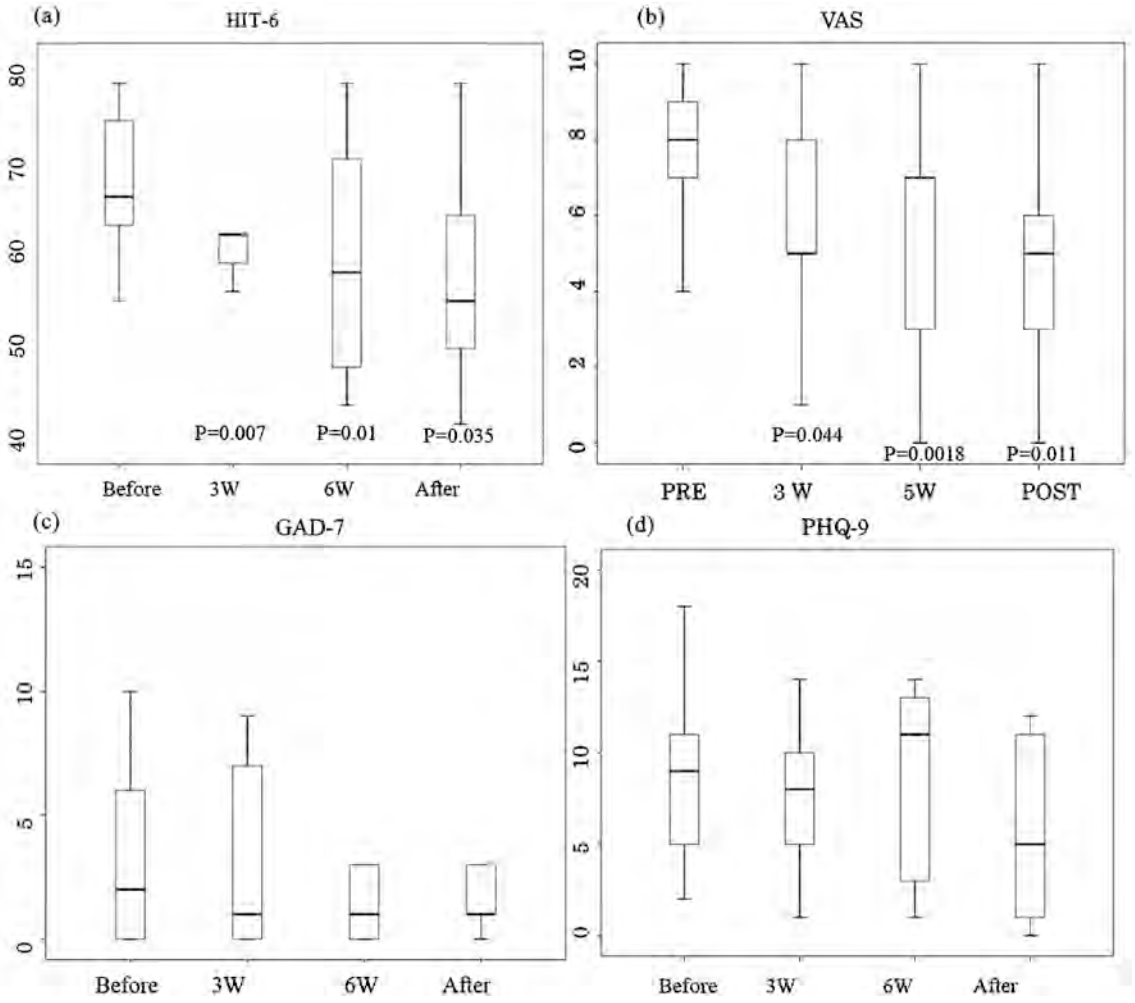


Figure 3. Clinical course of measurements. Box plots showing distribution of (a) HIT-6, (b) VAS, (c) GAD-7, and (d) PHQ-9 scores before treatment, after 3 and 6 weeks (W) of treatment, and after completion of treatment. Whiskers indicate the upper and lower quartiles.

be effective and well tolerated, it can be combined with drug treatments without risking cumulative adverse effects.

The outcome variables that changed after the start of craniocervical OST were the VAS score, which is an indicator of migraine severity, and the HIT-6 score, which is a quality of life (QOL) indicator related to migraines; both scores improved significantly. However, there was no obvious improvement in the number of migraine days and no significant improvement in the GAD-7 or PHQ-9 scores. We believe that the fact that the VAS and HIT-6 scores improved while the number of migraine days remained unchanged suggests that OST controls pain during attacks, despite having no effect on the cause of migraine, and that pain control improves QOL. Therefore, Patients with high VAS or HIT-6 score should be better treated by OST. We also believe that the absence of improvement in GAD-7 and PHQ-9 scores indicates that OST has no effect on psychological symptoms and that the treatment-based improvement in QOL is not due to psychogenic effects. The effects of noninvasive neurostimulation on migraine

were shown in a study in which supraorbital stimulation therapy was used to treat infrequent migraines [7]; the number of migraine days decreased from 6.9 to 4.9. In another reported study, self-administration of noninvasive vagus nerve stimulation by 20 patients with drug-resistant migraine resulted in a decrease in the number of migraine days from 18 to 12 and improvement in depressive symptoms and sleep quality [16]. The OS used in our present study resulted in improved QOL that was attributed to a decrease in migraine severity. We expect, in the future, to see further improvement in the treatment of severe CM if we change the frequency and intensity settings for OS administration.

The mechanism underlying the effects of OST on CM remains unclear. OST is presumed to exert its effects when the stimulus is applied to the peripheral skin, and the main mechanism is assumed to be an effect on nociceptors located within the fascial tissue and intra- and extracranial muscles. Proposed mechanisms for the progression from sporadic migraines to CM include dysfunction of the periaqueductal gray matter that makes up the central descending nociceptive neural network [17] [18] and dysfunction of pain receptors, both due to central sensitization [19] [20]. Animal experiments have shown that the activity of vagal afferents is attenuated by nociceptive neural activity via the spinothalamic and spinoreticular tracts [21]. We presume that the OS applied in our study exerted its pain-modifying effects by passing from the body surface via the vagus nerve, occipital nerve, and trigeminal nerve, following pathways similar to those reported in a study that made concomitant use of percutaneous occipital nerve stimulation and percutaneous orbital stimulation, both of which rely on trigeminal stimulation [22]. Furthermore, we presume that OST also exerts effects on central sites related to chronic conversion of migraines, including the red nucleus, tectum, extrapyramidal system, and pathways descending from the pain matrix [23] [24], which have been verified both by their effects on the stimulation site and by functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies.

Our findings should be interpreted in light of our study limitations. Only a small number of patients were included, and the study was not conducted as a randomized controlled trial. In addition, uniformity of the procedures between centers was not formally verified. Further, OS was applied at numerous sites on the face, head, and neck. The sites at which OS is particularly effective remain to be identified so that the number can be reduced for optimum clinical application and so that the therapeutic mechanism can be elucidated.

5. Conclusion

In conclusion, our data suggest that OS is well tolerated and may become a feasible form of treatment for drug-resistant CM. Further investigations are needed, however, for OST as treatment for refractory CM to become a clinical reality.

Acknowledgements

We also thank Tina Tajima, professor of the Research Institute of Medical Edu-

cation, St. Marianna University School of Medicine for meticulous English editing.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Headache Classification Committee of the International Headache Society (HIS) (2013) The International Classification of Headache Disorders. 3rd Edition (Beta Version). *Cephalalgia*, **33**, 629-808.

[2] Wang, S.J., Fuh, J.L., Lu, S.R., *et al.* (2000) Chronic Daily Headache in Chinese Elderly: Prevalence, Risk Factors, and Biannual Follow-Up. *Neurology*, **54**, 314-319. https://doi.org/10.1212/WNL.54.2.314

[3] Martelletti, P., Katsarava, Z., Lampl, C., *et al.* (2014) Refractory Chronic Migraine: A Consensus Statement on Clinical Definition from the European Headache Federation. *The Journal of Headache and Pain*, **15**, 47. https://doi.org/10.1186/1129-2377-15-47

[4] Lanteri-Minet, M. (2014) Economic Burden and Costs of Chronic Migraine. *Current Pain and Headache Reports*, **18**, 385. https://doi.org/10.1007/s11916-013-0385-0

[5] Aurora, S.K., Dodick, D.W., Turkel, C.C., *et al.*, PREEMPT 1 Chronic Migraine Study Group (2010) Onbotulinumtoxin A for Treatment of Chronic Migraine: Results from the Double-Blind, Randomized, Placebo-Controlled Phase of the PREEMPT 1 Trial. *Cephalalgia*, **30**, 793-803. https://doi.org/10.1177/0333102410364676

[6] Diener, H.C., Dodick, D.W., Aurora, S.K., *et al.* (2014) Onbotulinumtoxin A for Treatment of Chronic Migraine; Results from the Double-Blind, Randomized, Placebo-Controlled Phase of the PREEMPT 2 Trial. *Cephalalgia*, **30**, 804-814. https://doi.org/10.1177/0333102410364677

[7] Schoenen, J., Vandersmissen, B., Jeangette, S., *et al.* (2013) Migraine Prevention with a Supraorbital Transcutaneous Stimulator: A Randomized Controlled Trial. *Neurology*, **80**, 697-704. https://doi.org/10.1212/WNL.0b013e3182825055

[8] Hotta, M., Shiraishi, M., Suzuki, T., Nishiyma, T. and Imai, N. (2016) Preliminary Study on the Effectiveness of Physical Therapy by Craniocervical Oscillating Stimulation for Chronic Headaches. *Japanese Journal of Headache*, **42**, 177-181.

[9] Bannuru, R.R., Falvin, N.E., Vaysbrot, E., Harvey, W. and McAlindon, T. (2014) High-Energy Extracorporeal Shock-Wave Therapy for Treating Chronic Calcific Tendinitis of the Shoulder: A Systematic Review. *Annals of Internal Medicine*, **160**, 542-549. https://doi.org/10.7326/M13-1982

[10] International Headache Society (2014) International Classification of Headache Disorders, 3rd Edition (Beta Version). Igaku-Shoin Ltd., Tokyo, 10-11.

[11] Clinical Practice Guideline for Chronic Headache 2013 (2014) Lists of Members of Chronic Headache Clinical Practice Guideline Development Committee, Members of Evaluation and Coordination Committee, Collaborating Societies. Igaku-Shoin Ltd., Tokyo, 114-117.

[12] Kosinski, M., Bayliss, M.S., Bjorner, J.B., *et al.* (2003) A Six-Item Short-Form Survey for Measuring Headache Impact: The HIT-6. *Quality of Life Research*, **12**, 963-974. https://doi.org/10.1023/A:1026119331193

[13] Katz, J. and Melzack, R. (1999) Measurement of Pain. *Surgical Clinics of North America*, **79**, 231-252. https://doi.org/10.1016/S0039-6109(05)70381-9

[14] Muramatsu, K., Miyaoka, H., Kamijima, K., *et al.* (2007) The Patient Health Questionnaire, Japanese Version: Validity According to the Mini-International Neuropsychiatric Interview-Plus. *Psychological Reports*, **101**, 952-960.

[15] Spitzer, R.L., Kroenke, K., Williams, J.B. and Löwe, B. (2006) A Brief Measure for Assessing Generalized Anxiety Disorder: The GAD-7. *Archives of Internal Medicine*, **22**, 1092-1097. https://doi.org/10.1001/archinte.166.10.1092

[16] Kinfe, T.M., Pintea, B., Muhammad, S., *et al.* (2015) Cervical Non-Invasive Vagus Nerve Stimulation (nVNS) for Preventive and Acute Treatment of Episodic and Chronic Migraine and Migraine-Associated Sleep Disturbance: Preliminary Findings from a Prospective Observational Cohort Study. *The Journal of Headache and Pain*, **16**, 101. https://doi.org/10.1186/s10194-015-0582-9

[17] Smith, G.S., Savery, D., Marden, C., *et al.* (1994) Distribution of Messenger RNAs Encoding Enkephalin, Substance P, Somatostatin, Galanin, Vasoactive Intestinal Polypeptide, Neuropeptide Y, and Calcitonin Gene-Related Peptide in the Midbrain Periaqueductal Grey in the Rat. *Journal of Comparative Neurology*, **350**, 23-40. https://doi.org/10.1002/cne.903500103

[18] Welch, K.M., Nagesh, V., Aurora, S.K. and Gelman, N. (2001) Periaqueductal Gray Matter Dysfunction in Migraine: Cause or the Burden of Illness? *Headache*, **41**, 629-637. https://doi.org/10.1046/j.1526-4610.2001.041007629.x

[19] Burstein, R. and Jakubowski, M. (2004) Analgesic Triptan Action in an Animal Model of Intracranial Pain: A Race against the Development of Central Sensitization. *Annals of Neurology*, **55**, 27-36. https://doi.org/10.1002/ana.10785

[20] Yarnitsky, D., Goor-Aryeh, I., Bajwa, Z.H., *et al.* (2003) 2003 Wolff Award: Possible Parasympathetic Contribution to Peripheral and Central Sensitization during Migraine. *Headache*, **43**, 704-714. https://doi.org/10.1046/j.1526-4610.2003.03127.x

[21] Chandler, M.J., Hobbs, S.F., Bloser, D.C. and Foreman, R.D. (1991) Effects of Vagal Afferent Stimulation on Cervical Spinothalamic Tract Neurons in Monkeys. *Pain*, **44**, 81-87. https://doi.org/10.1016/0304-3959(91)90152-N

[22] Reed, K.L., Black, S.B., Banta, C.J. and Will, K.R. (2010) Combined Occipital and Supraorbital Neurostimulation for the Treatment of Chronic Migraine Headaches: Initial Experience. *Cephalgia*, **30**, 260-271. https://doi.org/10.1111/j.1468-2982.2009.01996.x

[23] Welch, K.M., Cao, Y., Aurora, S.K., Wiggins, G. and Vikingstad, E.M. (1998) MRI of the Occipital Cortex, Red Nucleus and Substantia Nigra during Visual Aura of Migraine. *Neurology*, **51**, 1465-1469. https://doi.org/10.1212/WNL.51.5.1465

[24] Weiller, C., May, A., Limmroth, V., *et al.* (1995) Brain Stem Activation in Spontaneous Human Migraine Attacks. *Nature Medicine*, **1**, 658-660. https://doi.org/10.1038/nm0795-658

International Journal of Clinical Medicine, 2019, 10, 161-173
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Advances in Research on the Pathogenesis of Type 2 Diabetes Complicated with Gallstone

Jianchu Tan[1*], Jiguang Kou[2]

[1]School of Medicine, Wuhan University of Science and Technology, Wuhan, China
[2]Xiaogan Hospital, Wuhan University of Science and Technology, Xiaogan, China
Email: *1425514858@qq.com

How to cite this paper: Tan, J.C. and Kou, J.G. (2019) Advances in Research on the Pathogenesis of Type 2 Diabetes Complicated with Gallstone. *International Journal of Clinical Medicine*, **10**, 161-173.
https://doi.org/10.4236/ijcm.2019.103016

Received: February 24, 2019
Accepted: March 12, 2019
Published: March 15, 2019

Abstract

At present, the incidence of diabetes complicated with gallstones is increasing rapidly, and there are still many problems in the pathogenesis of the disease. Diabetes complicated with gallstones is a chronic complication of diabetes, with diabetes-induced hyperinsulinemia and insulin resistance, gallbladder emptying disorders, Oddis sphincter dysfunction, gastrointestinal hormone disorders, gastrointestinal dyskinesia, fat metabolism disorders, bile Bacterial infection and other factors are related. In recent years, it has been found that in diabetic patients, Telocytes (TC) and Cajal interstitial cells (ICC) are reduced in the biliary system. In addition, the contact between ICC cells and smooth muscle cells and nerve endings is significantly reduced, so it is considered that bile Stone formation has a certain relationship with TC and ICC reduction. This article reviews recent research progress.

Keywords

Type 2 Diabetes, Gallstones, Pathogenesis

1. Diabetes and Gallstones

Gallstones are stones that occur in the gallbladder, mainly cholesterol stones or mixed stones based on cholesterol. They are a common benign disease of the digestive system. The overall incidence of gallstones in Europe and the United States is between 10% and 15% [1]. The incidence of domestic reports is around 10% [2]. Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia caused by multiple causes, caused by defects in insulin secretion and/or action. Acute severe metabolic disorders, such as secondary ketoacidosis, hyperosmolar hyperglycemia, can occur when the condition is severe or stressful. Studies have shown that diabetes is one of the high risk factors

for gallbladder disease, such as gallstones, gallbladder polyps, gallbladder cancer, etc. [3] [4] [5]. The incidence of gallstones is higher in diabetic patients. An autopsy and B-ultrasound survey showed that the incidence of gallstones in diabetic patients was 2 to 3 times higher than that in non-diabetics [6]. Epidemiological surveys have shown that the incidence of gallstones in DM patients is 25% to 30% and the incidence of gallstones in T2DM patients is much higher than that in type 1 diabetes [7]. Aune D *et al.* [8] identified 10 prospective studies that could be included in a meta-analysis that further supports the increased risk of gallbladder disease in diabetic patients. At present, the study believes that the formation of gallstones is a complex process involving multiple factors, but the specific mechanism of formation of gallstones is not completely clear, especially the pathogenesis of gallstones in diabetic patients is not clear. This paper reviews the recent research progress in recent years.

2. The Pathogenesis of Type 2 Diabetes Complicated with Gallstones

2.1. Hyperinsulinemia and Insulin Resistance

Insulin resistance, defined as the reduced sensitivity of the target organ acting on insulin to insulin action. The immediate cause of insulin resistance has not been fully established, but long-term hyperglycemia and hyperinsulinemia are known to cause insulin resistance in human and animal models [9] [10]. In the study of the LIRKO mouse model, the incidence of gallstones in mice was significantly increased after disrupting the intrahepatic insulin receptor in mice [11].

3-Hydroxy-3-methylglutaryl coenzyme (HMG-CoA) reductase is the rate-limiting enzyme for cholesterol synthesis, and the stability of cholesterol in the body can be maintained by regulating the activity of the enzyme. Insulin promotes the synthesis of HMG-CoA reductase, and mouse studies have shown that insulin rapidly increases the expression of hepatic HMGR by increasing the rate of transcription [12]. High insulin accelerates the rate of cholesterol synthesis in the liver and promotes the secretion of cholesterol in the liver, which increases the cholesterol saturation in the bile. Increased serum insulin levels can induce the production of low-density lipoprotein receptor (LDLR), increase its number and up-regulate its activity [13], promote the metabolism of low-density lipoprotein from the blood into the liver and cholesterol synthesis in the liver. The increase in cholesterol synthesized in the liver leads to an increase in the amount of cholesterol secreted by the liver into the bile.

Liver X receptor a(FXR) increases the sensitivity of islet beta receptors and inhibits insulin secretion. Treatment of insulin ob/ob mice with the FXR receptor agonist GW4064 reduced serum insulin levels and increased glucose tolerance [14] [15]. The liver regulates FXRa expression significantly after insulin resistance, leading to inhibition of 7a-hydroxylase activity [16]. 7a-hydroxylase is involved in the bile acid synthesis process as a rate-limiting enzyme in the bile acid conversion process. Its activity is reduced, resulting in a significant reduction in

bile acid and bile salt synthesis in bile, while increasing the relative content of cholesterol and mucopolysaccharide in bile. The imbalance of bile salts, cholesterol and mucopolysaccharide in bile accelerates the formation of cholesterol stones. Insulin resistance leads to decreased adiponectin expression, inhibition of insulin receptor I activation and downstream phosphatidylinositol 3-kinase signaling pathway transduction, decreased expression of fatty acid transporter 1 (FATP1) mRNA, and release of large amounts of free fatty acids by fat cells The synthesis of a large amount of cholesterol lipids leads to an increase in cholesterol in the bile and an increase in the occurrence of gallstones [17].

2.2. Leptin

Leptin (leptin) was discovered in 1994. It is a protein composed of 167 amino acid residues secreted by fat cells. It has functions of regulating neuroendocrine function, energy balance, feeding, growth and development [18]. Numerous studies have shown that leptin can induce cytokine signaling inhibition 3 (SOCS-3), and SOCS-3 can be feedback to regulate the expression of leptin gene [19]. In patients, insulin resistance promotes the release of leptin leading to an increase in leptin levels, and the presence of high concentrations of leptin in the blood further triggers insulin resistance. The experimental results showed that the expression of SOCS-3 was increased in obese individuals, and leptin receptor transduction was inhibited in adipocytes [20]. Decomposition of triglycerides in fat cells and beta oxidation of fatty acids are inhibited, resulting in the release of large amounts of triglycerides and fatty acids into the blood to form hyperlipidemia.

Leptin receptors are distributed in the liver, and leptin can directly act on the liver to regulate lipid metabolism. Animal experiments have found that leptin can activate lipid breakdown and inhibit lipogenesis through JAK-STAT and IRS-PI3K signaling pathways, thereby reducing lipid deposition [21]. Huynh FK *et al.* [22] also found that lack of hepatic leptin signaling leads to liver lipid accumulation and increases of more triglyceride-rich VLDL particles. Himeno *et al.* [23] found that in the absence of significant differences in energy intake and body weight, plasma insulin, triglyceride, and leptin levels were slightly elevated in mice with partial defects in leptin receptors, in the liver. The triglyceride content was significantly higher than that of the wild type, suggesting that some leptin receptor defects can cause abnormalities in liver glycolipid metabolism without affecting the central regulation of feeding activity. In insulin resistance and leptin resistance, glucose is absorbed in the small intestine, while in the liver, it inhibits phosphoenolpyruvate carboxykinase and produces a large amount of ketone bodies, and aerobic oxidation is inhibited and then turned to lipid synthesis.

Leptin can cause oxidative stress to mediate inflammation, induce peripheral blood mononuclear cells to release procoagulant factors, and promote platelet aggregation in microvessels [24], causing insufficient blood supply to the gallbladder smooth muscle to cause ischemic necrosis of gallbladder smooth

muscle, insufficient blood supply Causes vasospasm and inflammation can further lead to inflammation of the gallbladder wall, peripheral nerve cells are also damaged to varying degrees, nerve conduction is blocked, and these factors synergistically reduce the ability of the gallbladder to move. Neuropeptide Y (NPY) and neurotransmitters such as CCK stimulate the contraction of gallbladder and Oddi. When leptin is deficient or leptin resistant, the response of the gallbladder to NPY and CCK is reduced, resulting in reduced gallbladder contractility and increased gallbladder volume, Causing bile emptying disorder [25]. A clinical study found that serum leptin levels are an important factor in the decline of gallbladder motility, because high levels of serum leptin and CCK affect cell membrane receptors, producing receptor resistance, resulting in reduced smooth muscle contractility [26]. Therefore, when leptin is relatively reduced or produces leptin resistance, it will cause gallstones due to the reduction of gallbladder contractility.

2.3. Lipid Metabolism Disorder

Abnormal lipid metabolism caused by diabetes is a more positive risk factor in the formation of cholelithiasis. Abnormal glucose and lipid metabolism in diabetic patients leads to hyperlipidemia [27], serum triglyceride and cholesterol are significantly elevated, and high-density lipoprotein is decreased. Clinical studies have shown that ABCG5 and ABCG8 mRNA expression is significantly increased in cholesterol stones and cholesterol polyps [28]. ABCG5/G8 is mainly expressed in the liver, gallbladder and small intestine. Studies have shown that ABCG5 and ABCG8 proteins with high expression in the liver can increase cholesterol concentration and even reach supersaturation; and the Increased expression of ABCG5 and ABCG8 protein in gallbladder epithelial cells promotes the secretion of more cholesterol from epithelial cells, and cholesterol in bile remains at high levels to form cholesterol crystals and stones [29] [30]. The sharp increase in the expression of ABCG5 and ABCG8 in patients with diabetic stones leads to an increase in cholesterol in bile. In patients with hyperlipidemia, ABCG5/G8 mRNA expression was reduced by 14% after taking atorvastatin [31].

2.4. Bile Bacterial Infection

Diabetic microangiopathy and autonomic neuropathy can reduce gallbladder blood supply, gallbladder emptying and reduce the body's resistance to diabetes, providing conditions for gallbladder and biliary system infections, increasing the risk of stone formation. In recent years, many scholars at home and abroad have verified the existence and role of bacteria in cholesterol gallstones through experiments. Fatemi *et al.* [32] found a link between Helicobacter pylori and acute gallstones. Yang Yulong *et al.* [33] observed 15 cases of cholesterol stones by electron microscopy, and 10 of them found bacteria in the surface structure of stones. In the transmission electron microscopy of 12 cases of cholesterol stones,

it was found that there were 3 cases of cholesterol crystals and 12 cases of stone cores were found to have the presence of bacterial-like structural substances. The results of Tian Zhijie *et al.* [34] showed that Helicobacter DNA could be detected in 38.24% of bile and 79.55% of gallbladder mucosa, and 2 core parts of cholesterol mixed stones. Japanese scholar Kawai *et al.* [35] also detected bacterial DNA from 57% of pure cholesterol stones (100% cholesterol). However, Demir M *et al.* [36] found no significant difference in the prevalence of Helicobacter pylori infection between diabetic patients and non-diabetic controls. It was also found that the incidence of neuropathy in diabetic patients with Helicobacter pylori infection was higher.

The mechanism by which bacteria act may be that B-glucuronidase, phospholipase, and bile acid hydrolase produced by bacteria can catalyze the hydrolysis of bile lipid components and provide a raw material for the formation of calcium precipitates. Moreover, the bacterial metabolite itself is a good substrate for the formation of crystals of cholesterol and accumulation. In addition, bacterial infection can stimulate changes in the body's immune or metabolic state, leading to gallbladder mucosal secretion disorders, gallbladder motor dysfunction, cholestasis. Therefore, some scholars have suggested that bacterial-derived substances may directly participate in the formation of cholesterol stones, and have a cross-effect with non-bacterial stone-forming mechanisms, indirectly affecting the formation of cholesterol stones by affecting or changing human immune metabolites [37]. Zhu Leiming *et al.* [38] found that there was a statistically significant difference in mucosal IgA and IgG between the patients with positive and negative bacterial DNA in the gallbladder mucosa ($P < 0.5$), but no difference in the non-stone group ($P = 0.589, 0.711$). It is speculated that bacteria may cause the secretion of immunoglobulins in the gallbladder mucosa and indirectly participate in the formation of bacterial stones.

2.5. Gallbladder Emptying Obstacles

The gallbladder has the function of storing and enriching bile, and also has the ability to absorb part of the lipid, which depends on the relative concentration of phospholipids and cholesterol. In the case of changes in bile composition, the ability of gallbladder epithelial cells to absorb cholesterol decreases, and the concentration of cholesterol in the bile increases with the absorption of water [39]. Gallbladder contraction under physiological conditions is accomplished by hormones and neuromodulation, and changes in gallbladder dynamics can also affect the formation of gallstones. Patients with long-term fasting and complete parenteral nutrition are prone to gallstones, suggesting an important role for gallbladder contraction in the formation of stones. Ultrasound studies have shown that the fasting volume of the gallbladder and the postprandial residual volume of the gallbladder are positively correlated with obesity and high insulin [40] [41]. Cholecystokinin (CCK) is the main hormone regulating the contraction of gallbladder. CCK receptor exists on the smooth muscle of gallbladder.

The specific binding of the two promotes the hydrolysis of phosphatidylinositol diphosphate to inositol triphosphate and diglyceride, which causes intracellular Ca^{2+}. Induction of gallbladder contraction. Animal experiments have confirmed that the ability of the CCK gene or CCK receptor gene-deficient mice to shrink the gallbladder is significantly weakened, and the incidence of gallstones is significantly higher than that of wild-type mice [42] [43]. Studies of patients with diabetes with gallstones and non-diabetic stones have found that CCK-R and IP3-R are significantly reduced in the former, leading to impaired gallbladder emptying and gallstone formation [44]. Diabetic gallbladder autonomic neuropathy breaks the balance between sympathetic and vagal nerves, and sympathetic excitability is relatively increased. Increased levels of insulin can increase the relative excitability of the sympathetic nerves, causing gallbladder relaxation. The accumulated bile increases in viscosity under the action of gallbladder concentration, and cholesterol is easily precipitated to form crystals.

2.6. Oddis Sphincter Dysfunction

The annular smooth muscle at the end of the common bile duct and the end of the pancreatic duct is combined with the annular smooth muscle around the ampulla of the hepatopancreas. It is called the Oddis sphincter, also known as the hepatic and pancreatic sphincter. It has the function of controlling bile and pancreatic juice discharge. Diabetic patients often have Oddis sphincter dysfunction, which is mainly characterized by an increase in Oddis sphincter tension in the fasting state. Therefore, the increase in pressure in the common bile duct causes the bile that should flow through the Oddis sphincter into the duodenum to flow into the gallbladder, causing the gallbladder to fill too fast, and cholestasis promotes the formation of gallstones. The pathogenesis may be related to the following factors: autonomic neuropathy in diabetic patients leads to sympathetic sympathetic and vagal tone imbalance, resulting in increased Oddis sphincter tone.

2.7. Gastrointestinal Hormone Disorder

According to its regulation of gallbladder movement, gastrointestinal hormones can be divided into hormones that promote gallbladder movement and hormones that inhibit gallbladder movement. The hormones that promote gallbladder movement mainly include cholecystokinin, gastrin, quercetin and gastrin release. Peptides, motilin, substance P, etc.: The hormones that inhibit gallbladder movement mainly include vasoactive intestinal peptide, somatostatin and pancreatic polypeptide family. In diabetes, when the hormones that inhibit gallbladder movement are stronger than the hormones that promote gallbladder movement, gallstones are more likely to form [45] [46].

2.8. Gastrointestinal Dyskinesia

The biliary tract is closely related to gastrointestinal motility. The gallbladder

contraction and the movement of the Oddis sphincter during the digestive phase are consistent with gastrointestinal motility. Changes in intestinal motor function also affect the contractile function of the gallbladder. Studies have shown that gastrointestinal movements are closely related to the emptying movement of the gallbladder. Gastroduodenal emptying during digestive and digestive periods can increase the emptying of the gallbladder. Emptying of the gallbladder promotes emptying of the duodenum, which in turn promotes gastric emptying, while gastrointestinal motility disorders can cause gallbladder emptying disorders. Diabetes is often accompanied by low gastrointestinal motility [47], such as stomach cramps, stomach retention, etc., which can cause gallbladder emptying disorders. Compared with the no-stone control group, the large intestine transit time of patients with gallbladder cholesterol stones, the 7-α dehydroxylation ability of bile acids, the amount of anaerobic bacteria, and the pH value of intestinal lumen were significantly increased in the intestinal flora [48]. These factors lead to a significant increase in deoxycholic acid that is returned to the bile through the enterohepatic circulation, which increases the hydrophobicity of the bile salts, thereby promoting the precipitation of bile cholesterol, increasing the cholesterol saturation index and accelerating the rate of cholesterol crystallization [49].

2.9. Gallbladder Nerve Damage

There are two special cell types in the gallbladder, Telocytes (TC) and Cajal interstitial cells (ICC). The term telocyte (TC) was first introduced in the scientific literature in 2010 [50]. And cells with TC characteristics have been found in almost all mammalian organs [51]. More than a century ago, Santiago Ramon and Cajal [52] described a specific cell type in the gastrointestinal tract (GI) that is located in the interstitial space between nerve endings and smooth muscle cells (SMC) and was eventually named Interstitial cells of Cajal (ICC). However, TC is related to ICC, they have the same embryonic origin (mesenchymal); both form networks that sometimes run the same region in parallel, sometimes some ICCs are embedded in the TC network, and vice versa; this strict relationship indicates TC ICC signals can be transmitted, and TC can represent ICC stem cells [51].

Matyja A *et al.* [53] conducted a controlled study in which bile components may play an important role in the reduction of TC density in the gallbladder. In addition, some scholars believe that TC damage may be associated with blocking c-kit/SCF signaling pathway leading to high cholesterol levels [54] or chronic inflammation of the gallbladder wall [55]. Cholesterol saturation index (CSI) is a well-established parameter associated with bile stone. Pasternak *et al.* [56] compared the lipid content of bile samples from patients with cholelithiasis. The results showed that the CSI of the cholelithiasis group was statistically significant and negatively correlated with the reduction of TC in the gallbladder wall. Cholesterol accumulation in gallbladder smooth muscle cells disrupts protein G-mediated signal transduction by CCK-A (cholecystokinin A) binding to its

receptor. In another study, Pasternak *et al.* [54] further evaluated the relationship between bile lipid composition and TC density in the gallbladder wall of patients with gallstones and found that the concentration of omega-6 polyunsaturated fatty acids (PUFA) was significantly increased. The average concentration of glycocholic acid (GCA) and taurocholic acid (TCA) in bile was significantly reduced. High levels of omega-6 polyunsaturated fatty acids are shown to contribute to the formation of cholelithiasis by enhancing bile cholesterol secretion [57], while GCA and TCA may be based on a significant positive correlation with the mean number of TCs and GCA concentrations on TC Protective effects. Therefore, these observations indicate that higher concentrations of omega-6 polyunsaturated fatty acids and lower amounts of GCA and TCA further induce gallbladder motor dysfunction and thus gallstone formation by increasing the SHI loss index caused by TC loss.

ICC has pacing and signal transduction functions in the gastrointestinal tract, which is essential for the normal operation of gastrointestinal motility. Intestinal irregularities caused by abnormal slow wave activity have been described [58] [59] [60]. ICC defects are characterized by slow wave activity abnormalities [61], and are associated with many dysmotility diseases such as slow transit constipation, hypertrophic pyloric stenosis, and pseudo intestinal obstruction. In recent years, studies have also found that ICC exists in many parts of the biliary system Oddi sphincter, cystic duct, gallbladder, etc., and participates in the regulation of Oddis sphincter autonomous rhythmic movement [62]. In human and animal models, the major cellular defect in diabetic gastroparesis is indeed the loss of ICC, which has been reported [63] [64]. In diabetes, ICC is reduced in the gallbladder and bile ducts, and in addition, ICC cells are significantly less exposed to smooth muscle cells and nerve endings [65].

3. Conclusion and Prospects

In short, diabetes complicated with gallstones is the result of a combination of factors, including lipid metabolism disorders, hypersaturation of cholesterol in the bile, reduction of gallbladder nerves, impaired gallbladder movement, etc., multiple factors affect each other and promote each other, resulting in a common cause Stone formation. Patients with long duration of diabetes, poor glycemic control, and elderly patients with diabetes should be alert to the formation of gallstones. It is recommended to have an abdominal ultrasound every year for early detection and treatment. Diabetes complicated with gallstones is a common chronic complication of diabetes. At present, its pathogenesis is still not fully understood. It is necessary to conduct a more in-depth study on its pathogenesis, and provide a solution for further prevention and treatment of diabetes complicated with gallstones.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this pa-

per.

References

[1] Stinton, L.M. and Shaffer, E.A. (2012) Epidemiology of Gallbladder Disease: Cholelithiasis and Cancer. *Gut Liver*, **6**, 172-187. https://doi.org/10.5009/gnl.2012.6.2.172

[2] Tan, Y.J. and Liu, Y.M. (2009) Current Status and Progress of Research on the Causes of Gallstones. *Chinese Journal of Practical Surgery*, **7**, 602-603.

[3] Toosi, F.S., Ehsanbakhsh, A.R. and Tavakoll, M.R. (2011) Asymptomatic Gallstones and Related Risk Factors in Iran. *Hepatogastroenterology*, **58**, 1123-1126. https://doi.org/10.5754/hge11060

[4] Cojocaru, C. and Pandele, G.I. (2010) Clinical and Paraclinical Features in Diabetic Patients Cholecystectomized for Gallstones. *Revista Medico-Chirurgical a Societatii de Medici si Naturalisti din Iasi*, **114**, 998-1004.

[5] Unisa, S., Jagannath, P., Dhir, V., *et al.* (2011) Population-Based Study to Estimate Prevalence and Determine Risk Factors of Gallbladder Diseases in the Rural Gangetic Basin of North India. *HPB* (*Oxford*), **13**, 117-125.

[6] Fraqudli, M., I'agliarulo, M., Colucci, A., *et al.* (2003) Gallbladder Motility in Obesity, Diabetes Mellitus and Coeliac Disease. *Digestive and Liver Disease*, **35**, S12-S16. https://doi.org/10.1016/S1590-8658(03)00087-2

[7] Wang, C.C., Zhang, W.X. and Cao, Y.M. (2015) Relationship between Gallstones and Diabetes Mellitus. *Journal of Hebei Medical University*, **11**, 1362-1364.

[8] Aune, D. and Vatten, L.J. (2016) Diabetes Mellitus and the Risk of Gallbladder Disease: A Systematic Review and Meta-Analysis of Prospective Studies. *Diabetes Complications*, **30**, 368-373. https://doi.org/10.1016/j.jdiacomp.2015.11.012

[9] Pandey, G., Makhija, E., George, N., *et al.* (2015) Insulin Regulates Nitric Oxide Production in the Kidney Collecting Duct Cells. *Biological Chemistry*, **290**, 5582-5591. https://doi.org/10.1074/jbc.M114.592741

[10] Martínez-Hervás, S., Vinué, A., Núñez, L., *et al.* (2014) Insulin Resistance Aggravates Atherosclerosis by Reducing Vascular Smooth Muscle Cell Survival and Increasing CX3CL1/CX3CR1 Axis. *Cardiovascular Research*, **103**, 324-336. https://doi.org/10.1093/cvr/cvu115

[11] Wang, T.Y., Portincasa, P., Liu, M., *et al.* (2018) Mouse Models of Gallstone Disease. *Current Opinion in Gastroenterology*, **34**, 59-70. https://doi.org/10.1097/MOG.0000000000000417

[12] Ness, G.C., Edelman, J.L. and Brooks, P.A. (2012) Involvement of Tristetraprolin in Transcriptional Activation of Hepatic 3-Hydroxy-3-Methyl Glutaryl Coenzyme A Reductase by Insulin. *Biochemical and Biophysical Research Communications*, **420**, 178-182. https://doi.org/10.1016/j.bbrc.2012.02.138

[13] Ramakrishnan, G., Arjuman, A., Suneja, S., *et al.* (2012) The Association between Insulin and Low-Density Lipoprotein Receptors. *Diabetes and Vascular Disease Research*, **9**, 196-204. https://doi.org/10.1177/1479164111430243

[14] Dufer, M., Horth, K., Krippeit-Drews, P., *et al.* (2012) The Significance of the Nuclear Farnesoid X receptor (FXR) in Beta cell Function. *Islets*, **4**, 333-338. https://doi.org/10.4161/isl.22383

[15] Noel, O.F., Still, C.D., Argyropoulos, G., *et al.* (2016) Bile Acids, FXR, and Metabolic Effects of Bariatric Surgery. *Journal of Obesity*, **2016**, 4390254.

[16] Fang, S., Suh, J.M., Reilly, S.M., *et al.* (2015) Intestinal FXR Agonism Promotes Adipose Tissue Browning and Reduces Obesity and Insulin Resistance. *Nature*

Medicine, **21**, 159-165. https://doi.org/10.1038/nm.3760

[17] lopez-jaramillo, P., Gomez-arbelaez, D., Lopez-lopez, J., *et al.* (2014) The Role of Leptin/Adiponectin Ratio in Metabolic Syndrome and Diabetes. *Hormone Molecular Biology and Clinical Investigation*, **18**, 37-45.

[18] Zhang, Y., Proenca, R., Maffei, M., *et al.* (1994) Positional Cloning of the Mouse Obese Gene and Its Human Homologue. *Nature*, **372**, 425-432. https://doi.org/10.1038/372425a0

[19] Denis, R.G., Bing, C., Brocklehurst, S., *et al.* (2004) Diurnal Changes in Hypothalamic Neuropeptide and SOCS-3 Expression: Effects of Lactation and Relationship with Serum Leptin and Food Intake. *Journal of Endocrinology*, **183**, 173-181. https://doi.org/10.1677/joe.1.05659

[20] Liu, L., Liu, Y.L., Ren, Y.H., *et al.* (2012) Effects of Fat Cells on Leptin Response in Diet-Induced Obese Rats. *Chinese Journal of Public Health*, No. 5, 625-626.

[21] Zhang, L.H., Tan, X.Y., Wu, K., *et al.* (2015) Regulation and Mechanism of Leptin on Lipid Etabolism in Ovarian Follicle Cells from Yellow Catfish *Pelteobagrus fulvidraco*. *General and Comparative Endocrinology*, **222**, 116-123. https://doi.org/10.1016/j.ygcen.2015.06.008

[22] Huynh, F.K., Neumann, U.H., Wang, Y., *et al.* (2013) A Role for Hepatic Leptin Signaling in Lipid Metabolism via Altered Very Low Density Lipoprotein Composition and Liver Lipase Activity in Mice. *Hepatology*, **57**, 543-554. https://doi.org/10.1002/hep.26043

[23] Himeno, K., Seike, M., Fukuchi, S., *et al.* (2009) Heterozygosity for Leptin Receptor (fa) Accelerates Hepatic Triglyceride Accumulation without Hyperphagia in Zucker Rats. *Obesity Research & Clinical Practice*, **3**, 1-52. https://doi.org/10.1016/j.orcp.2008.10.003

[24] Petrini, S., Neri, T., Lombardi, S., *et al.* (2016) Leptin Induces the Generation of Procoagulant, Tissue Factor Bearing Microparticles by Human Peripheral Blood Mononuclear Cells. *Biochimica et Biophysica Acta*, **1860**, 1354-1361. https://doi.org/10.1016/j.bbagen.2016.03.029

[25] Lee, S., Kweon, O.K. and Kim, W.H. (2017) Associations between Serum Leptin Levels, Hyperlipidemia and Cholelithiasis in Dogs. *PLoS ONE*, **12**, e0187315. https://doi.org/10.1371/journal.pone.0187315

[26] Zhou, W., Sun, S., Shen, F., *et al.* (2013) Effects of Serum Leptin and Cholecystokinin Levels on Gallbladder Contraction Function. *Chinese Journal of Experimental Surgery*, **30**, 2275-2277.

[27] Lo, S.F., Chu, S.W., Muo, C.H., *et al.* (2014) Diabetes Mellitus and Accompanying Hyperlipidemia Are Independent Risk Factors for Adhesive Capsulitis: A Nationwide Population-Based Cohort Study (Version 2). *Rheumatology International*, **34**, 67-74. https://doi.org/10.1007/s00296-013-2847-4

[28] Deng, L., Liang, M., Zhang, K., *et al.* (2015) The Role of ABCG5 and ABCG8 Genes in the Formation of Gallstone Cholesterol Gallstones and Cholesterol Polyps. *Chinese Journal of Hepatology Surgery*, No. 2, 125-128.

[29] Wang, F., Li, G., Gu, H.M., *et al.* (2013) Characterization of the Role of a Highly Conserved Sequence in ATP Binding Cassette Transporter G (ABCG) Family in ABCG1 Stability, Oligomerization, and Trafficking. *Biochemistry*, **52**, 9497-9509. https://doi.org/10.1021/bi401285j

[30] Ghanbari-niaki, A., Zare-kookandeh, N. and Zare-kookandeh, A. (2014) ABCG5 Gene Responses to Treadmill Running with or without Administration of *Pistachio atlantica* in Female Rats. *Iranian Journal of Basic Medical Sciences*, **17**, 162-171.

[31] Tremblay, A.J., Lamarche, B., Lemelin, V., *et al.* (2011) Atorvstatin Increases Intestinal Expression of NPC1L1 in Hyperlipidemic Men. *The Journal of Lipid Research*, **52**, 558-565. https://doi.org/10.1194/jlr.M011080

[32] Fatemi, S.M., Doosti, A., Shokri, D., *et al.* (2018) Is There a Correlation between *Helicobacter pylori* and Enterohepatic Helicobacter Species and Gallstone Cholecystitis? *Middle East Journal of Digestive Diseases*, **10**, 24-30. https://doi.org/10.15171/mejdd.2017.86

[33] Yang, Y., Liu, X. and Tan, W. (2005) Bacteria in Cholesterol Stones and Their Role in the Mechanism of Stone Formation. *Journal of Hepatobiliary and Pancreatic Surgery*, **17**, 14-16.

[34] Tian, Z., Han, T., Jiang, Z., *et al.* (2004) Study on Helicobacter DNA of Biliary System of Gallstone Disease. *Chinese Journal of Practical Surgery*, **24**, 84-87.

[35] Kawai, M., Wahashi, M., Uchiyama, K., *et al.* (2002) Gram—Positive Cocci Are Associated with the Formation of Completely Pure Cholesterol Stones. *The American Journal of Gastroenterology*, **97**, 2922-2923.

[36] Demir, M., Gokturk, H.S., Ozturk, N.A., *et al.* (2008) Helicobacter Pylori Prevalence in Diabetes Mellitus Patients with Dyspeptic Symptoms and Its Relationship to Glycemic Control and Late Complications. *Digestive Diseases and Sciences*, **53**, 2646-2649.

[37] Swidsinski, A. and Lee, S.P. (2001) The Role of Bacteria in Gallstone Pathogenesis. *Frontiers in Bioscience*, **6**, E93-E103.

[38] Zhu, L., Cai, D. and Lu, Y. (2003) A Comparative Study of Biliary Bacterial Infection Status and Immunoglobulin Correlation between Cholesterol Gallstone Patients and Non-Cholelithiasis Patients. *Chinese Journal of Hepatobiliary Surgery*, **9**, 419-422.

[39] Chen, W. and Liang, L. (2015) Cholecystectomy Recognition of Gallbladder Function. *Chinese Journal of Practical Surgery*, **35**, 926-928.

[40] Lu, L., Yan, X. and Ma, S. (2011) 40 Cases of Gallbladder Emptying Dysfunction in Diabetic Patients. *World Chinese Journal of Digestology*, No. 3, 301-304.

[41] Jiang, Z., Han, T., Yi, F., *et al.* (2013) Improved B-Ultrasound Three-Dimensional Gallbladder Function Test and Judgment Criteria. *Journal of Hepatobiliary and Pancreatic Surgery*, No. 3, 229-231.

[42] Sato, N., Miyasaka, K., Suzuki, S., *et al.* (2003) Lack of Cholecys-Tokinin-A Receptor Enhanced Gallstone Formation: A Study in CCK-A Receptor Gene Knockout Mice. *Digestive Diseases and Sciences*, **48**, 1944-1947. https://doi.org/10.1023/A:1026110002713

[43] Wang, H.H., Portincasa, P., Liu, M., *et al.* (2010) Effect of Gallbladder Hypomotility on Cholesterol Crystallization and Growth in CCK-Deficient Mice. *Biochimica et Biophysica Acta*, **1801**, 138-146. https://doi.org/10.1016/j.bbalip.2009.10.003

[44] Zhang, Z., Tian, J., Liao, Q., *et al.* (2014) The Analysis of Expression of CCK and IP3 Receptors in Gallstones Patients with Type 2 Diabetes Mellitus. *Hepatogastroenterology*, **61**, 2173-2176.

[45] Pendleton, H., Ekman, R., Olsson, R., *et al.* (2009) Motilin Concentrations in Relation to Gastro Intestinal Dysmotility in Diabetes Mellitus. *European Journal of Internal Medicine*, **20**, 654-659. https://doi.org/10.1016/j.ejim.2009.05.015

[46] Chen, L., Zhang, X.F., Ku, B.Q., *et al.* (2012) Effects of Acupoint Injection of Autologous Blood on Symptoms and Plasma Motilin and Gastrin Levels of Diabetic Gastroparesis Patients. *Acupuncture Research*, **37**, 229-232, 246.

[47] Fan, J.G., Zhu, J., Li, X.J., *et al.* (2005) Prevalence of and Risk Factors for Fatty Liver in a General Population of Shanghai, China. *Hepatology*, **43**, 508-514. https://doi.org/10.1016/j.jhep.2005.02.042

[48] Thomas, L.A., Veysey, M.J., Bathgate, T., *et al.* (2000) Mechanism for the Transit-Induced Increase in Colonic Deoxycholic Acid Formation in Cholesterol Cholelithiasis. *Gastroenterology*, **119**, 806-815.

[49] van Erpecum, K.J. and van Berge Henegouwen, G.P. (2003) Intestinal Aspects of Cholesterol Gallstone Formation. *Digestive and Liver Disease*, **35**, S8-S11. https://doi.org/10.1016/S1590-8658(03)00086-0

[50] Popescu, L.M. and Faussone-Pellegrini, M.S. (2010) Telocytes—A Case of Serendipity: The Winding Way from Interstitial Cells of Cajal (ICC), via Interstitial Cajal-Like Cells (ICLC) to Telocytes. *Journal of Cellular and Molecular Medicine*, **14**, 729-740. https://doi.org/10.1111/j.1582-4934.2010.01059.x

[51] Vannucchi, M.G. and Traini, C. (2016) Interstitial Cells of Cajal and Telocytesin the Gut: Twins, Related or Simply Neighbor Cells? *Biomolecular Concepts*, **7**, 93-102. https://doi.org/10.1515/bmc-2015-0034

[52] Ramon, Y. and Cajal, S. (1911) Histologie du Systeme Nerveux de L'Homme et des Vertebres. Volume 2, A. Maloine, Paris.

[53] Matyja, A., Gil, K., Pasternak, A., *et al.* (2013) Telocytes: New Insight into the Pathogenesis of Gallstone Disease. *Journal of Cellular and Molecular Medicine*, **17**, 734-742. https://doi.org/10.1111/jcmm.12057

[54] Pasternak, A., Gugajska, J., Szura, M., *et al.* (2017) Biliary Polyunsaturated Fatty Acids and Telocytes in Gallstone Disease. *Cell Transplant*, **26**, 125-133. https://doi.org/10.3727/096368916X692717

[55] Fan, Y., Wu, S., Fu, B., *et al.* (2015) The Role of Interstitial Cajal-Like Cells in the Formation of Cholesterol Stones in Guinea Pig Gallbladder. *Hepatology International*, **9**, 612-620. https://doi.org/10.1007/s12072-015-9623-3

[56] Pasternak, A., Matyja, A., Gil, K., *et al.* (2013) Interstitial Cajal-Like Cells and Bile Lithogenicity in the Pathogenesis of Gall-Stone Disease. *Polski Przeglad Chirurgiczny*, **85**, 311-316. https://doi.org/10.2478/pjs-2013-0046

[57] LaMorte, W.W., O'Leary, D.P., Booker, M.L., *et al.* (1993) Increased Dietary Fat Content Accelerates Cholesterol Gallstone Formation in the Cholesterol-Fed Prairie Dog. *Hepatology*, **18**, 1498-1503.

[58] Angeli, T.R., O'Grady, G., Du, P., *et al.* (2013) Circumferential and Functional Re-Entry of *in Vivo* Slow-Wave Activity in the Porcine Small Intestine. *Neurogastroenterology & Motility*, **25**, e304-e314. https://doi.org/10.1111/nmo.12085

[59] O'Grady, G., Du, P., Paskaranandavadivel, N., *et al.* (2012) Rapid High-Amplitude Circumferential Slow Wave Propagation during Normal Gastric Pacemaking and Dysrhythmias. *Neurogastroenterology & Motility*, **24**, e299-e312. https://doi.org/10.1111/j.1365-2982.2012.01932.x

[60] Lammers, W.J. (2013) Arrhythmias in the Gut. *Neurogastroenterology & Motility*, **25**, 353-357. https://doi.org/10.1111/nmo.12116

[61] Farrugia, G. (2008) Interstitial Cells of Cajal in Health and Disease. *Neurogastroenterology & Motility*, **20**, 54-63. https://doi.org/10.1111/j.1365-2982.2008.01109.x

[62] Ahmadi, O., Nicholson Mde, L., Gould, M.L., *et al.* (2010) Interstitial Cells of Cajal Are Present in Human Extrahepatic Bile Ducts. *Gastroenterology & Hepatology*, **25**, 277-285.

[63] Grover, M., Farrugia, G., Lurken, M.S., *et al.* (2011) Cellular Changes in Diabetic

and Idiopathic Gastroparesis. *Gastroenterology*, **140**, 1575-1585e8.

[64] Choi, K.M., Gibbons, S.J., Nguyen, T.V., *et al*. (2008) Heme Oxygenase-1 Protects Interstitial Cells of Cajal from Oxidative Stress and Reverses Diabetic Gastroparesis. *Gastroenterology*, **135**, 2055-2064.

[65] Faussone-Pellegrini, M.S., Grover, M., Pasricha, P.J., *et al*. (2012) Ultrastructural Differences between Diabetic and Idiopathic Gastroparesis. *Journal of Cellular and Molecular Medicine*, **16**, 1573-1581. https://doi.org/10.1111/j.1582-4934.2011.01451.x

International Journal of Clinical Medicine, 2019, 10, 174-182
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Solasodine Glycosides from the Eggplant in a Topical Cream Psorend^{BEC} Are Effective against Psoriasis

Tania R. Chase, Kai E. Cham, Bill E. Cham

Australasian Medical Research, Port Vila, Republic of Vanuatu
Email: bill.cham@gmail.com

How to cite this paper: Chase, T.R., Cham, K.E. and Cham, B.E. (2019) Solasodine Glycosides from the Eggplant in a Topical Cream Psorend^{BEC} Are Effective against Psoriasis. *International Journal of Clinical Medicine*, **10**, 174-182.
https://doi.org/10.4236/ijcm.2019.103017

Received: February 11, 2019
Accepted: March 15, 2019
Published: March 18, 2019

Abstract

Background: Psoriasis is a chronic disease that can have significant effects on quality of life. **Aim:** To test whether the antineoplastic, antipathogenic plant derived secondary metabolites, solasodine glycosides, can treat psoriasis. **Case Presentation:** We report a case of a 54-year-old French-Vietnamese male who presented with diagnosed erythematous scaly annular recalcitrant psoriasis scattered throughout his body. **Method:** After failing conventional treatment regimens for over 10 years the patient received a trial with a topical cream formulation Psorend^{BEC}, containing solasodine glycosides, for his psoriasis. **Result:** Topical applications of Psorend^{BEC} twice daily resulted in complete resolution of cutaneous lesions after 4 weeks of treatment with no recurrence post 1 year after therapy. **Conclusion:** Topical Psorend^{BEC} therapy rapidly removes recalcitrant psoriasis with no apparent side effects.

Keywords

Psoriasis, BEC, Solasodine Glycosides, Psorend^{BEC}, Eggplant, Devil's Apple, Black Nightshade

1. Introduction

The chronic disease psoriasis is a common skin condition that speeds up the life cycle of skin cells and causes cells to build up rapidly on the surface of the skin. The well-demarcated plaques consist of extra skin cells that form scales and red patches, which are itchy and sometimes painful.

Psoriasis affects people of all ages, and in all countries. The reported prevalence of psoriasis in adults ranges from 0.5% - 11.4% and 0 - 1.4% in children, making psoriasis a serious global problem [1]. According to the International

Federation of Psoriasis Associations (IFPA), around 125 million people have some form of psoriasis.

The exact cause isn't fully understood but it is thought to be the result of several factors, including genetic and environmental conditions [2]. Overactive immune system (T-lymphocytes) that creates inflammation inside the body leading to the symptoms of psoriasis on the skin has been implicated [3]. There is no cure for psoriasis and there is no known way to prevent psoriasis.

People who have psoriasis tend to have a higher risk of developing certain other types of health conditions, called comorbidities [4].

Psoriasis has a profound emotional and social, as well as physical, impact on the affected patients.

A wide variety of psychological effects of psoriasis have been described in a large number of patients ranging from poor esteem, sexual dysfunction, depression and suicidal ideation [5].

There are several ways that lead to some relief from the symptoms of psoriasis. Psoriasis treatments generally reduce inflammation to clear the skin. Treatments can be divided into three main types: topical treatments, light therapy, and systemic medications including biologics. The side effects of these medications range from mild to major. Psoriasis is currently not curable, but it can go in remission.

Topical treatments can effectively treat mild to moderate psoriasis. For severe psoriasis, creams are combined with oral medications, light therapy and/or biologics [6].

There is an increase in prevalence of psoriasis [7]. Therefore, there is a need for more effective therapies with fewer side effects for the treatment of psoriasis in general, and in particular, for moderate to severe psoriasis.

In 1987, it was first reported that BEC and its individual components elicit antineoplastic activities with high therapeutic indices [8]. BEC is a plant extract obtainable from various Solanum species such as *S. linnaeanum* (Devil's Apple), *S. nigrum* (Black nightshade), *S. melongena* (Eggplant) and is composed of solasodine glycosides. BEC consists of 33% solamargine, 33% solasonine and 34% mono-and diglycosides of solasodine [9].

Since then, hundreds of independently published articles have confirmed and elaborated on the original findings, to the extent, that these compounds are the ongoing hope for treating terminal tumours [10]. In addition, currently, there is an established effective topical cream formulation Curaderm for the treatment of skin cancers [10] [11].

BEC glycoalkaloids are also effective against infections of bacteria [12], fungi [13], viruses [14], malaria [15], parasites [16] [17], leishmaniasis [16] [17], and show promise as trypanocidal agents [16] [17].

The antimicrobial mechanisms of action of BEC are similar to the antineoplastic mechanisms of action of BEC with cancer [17].

Pathogenic microbial infections, cancer and psoriasis share an important commonality, in that, there are increases in unchecked cell growths, although

the aetiologies may be different.

Therefore, it not surprising that BEC was tested against psoriasis. Proof of concept studies with an aqueous cream formulation containing BEC with a twice-daily application over the psoriatic lesions resulted in amelioration of symptoms [18].

Subsequently, Phases I/Ila clinical studies of a topical cream containing BEC was carried out in healthy subjects and subjects with mild to moderate psoriasis. The results of these studies determined that the primary endpoints of safety and tolerability were achieved. Unfortunately, the secondary endpoint of efficacy was not achieved. Reportedly, the lack of efficacy was caused by the inappropriate cream formulation used in the clinical studies [18].

2. Psorend^{BEC}, the Natural Novel Topical Treatment Formulation

The importance of cream formulations when using BEC was previously highlighted, and it was shown that at low concentrations of BEC in topical creams, it was essential to choose the appropriate excipients for a specific application to obtain high efficacy.

Excipients were at one time considered to be "inactive" ingredients but presently they are considered to be able to serve as "key determinants of dosage form performance".

In the context of cream compositions, an excipient is a natural or synthetic substance formulated alongside the active ingredient of the medication to confer a therapeutic enhancement on the active ingredient in the final dosage form, such as, facilitating drug interaction with targeted diseased state, solubility and stability of the medication over the expected shelf life [19] [20].

This concept was investigated when BEC was added to various cream formulations, specifically to obtain a possible treatment for psoriasis.

After investigating a wide variety of topical formulations, it was concluded to add BEC to an existing cream formulation that reduces dryness, itching, redness, soreness, and scaling in patients with eczema.

The eczema cream is an oil in water formulation consisting of coal tar as the "active" ingredient.

3. Case Report

A 54-year-old French-Vietnamese male builder was referred to our research facility with well-demarcated erythematous scaly annular plaques, scattered on his torso, legs, arms, face, and scalp, which had not responded to a wide variety of conventional psoriasis treatments. The patient gave his consent for his Case Report to be published.

3.1. History

The initial lesions first appeared on the knees ten years ago and new lesions

gradually appeared over two to three years thereafter. There was no joint pain and/or a history of infections prior to lesions development. His past medical history was significant only for depression. There was no personal or family history of psoriasis or other dermatologic diseases. Prior to the presentation in our research facility, he had skin biopsies and the diagnosis was considered as psoriasis vulgaris (common plaque psoriasis). He was under the care of a general practitioner and dermatologist for 10 years who had treated his psoriasis with oral and topical medications. Topical therapies included Corticosteroids creams, Donovex ointment, Anthranil ointment, Salicylic acid ointment, Coal tar cream, and UV therapy.

However, despite years of follow-ups and treatment adjustments, including Methotrexate 25 mg per week, he not only remained poorly controlled, but his condition worsened and the patient experienced various side effects such as thinning of the skin, erythema, skin irritation, tinnitus, itching of the skin, tiredness, nausea and easily bruising from his medications.

Consequently, therefore, the patient did not persist with any particular treatment. The patient is a non-smoker with no alcohol or illicit drug use.

3.2. Appearance on Admission

Scattered erythematous medium-to-large plaques on the chest, back, arms, lower limbs, thighs, scalp, and face with silvery scales. There were flexural involvements and erythema, scale and induration, which were severe throughout a large body surface area. Some affected areas were quite sore. As the patient was not experiencing efficacy with appropriately administered conventional psoriasis treatments, he decided to stop all treatments and on admission to our research facility, he was off all previous treatments for at least six months.

3.3. Treatment

Psorend^{BEC} cream contains coal tar, zinc oxide, allantoin, vitamin E acetate, vitamin A palmitate and BEC plant extract in an aqueous cream formulation. Psorend^{BEC} was applied twice daily and was gently rubbed onto the psoriatic lesions.

The objective of this communication was to establish whether BEC in a topical cream formulation, Psorend^{BEC}, is effective against a serious case of psoriasis that had not responded to a wide variety of therapies.

4. Results

Before treatment with the Psorend^{BEC} cream, the psoriasis lesions were widespread across his body, some of which were bleeding, caused by scratching the itchy affected areas (**Figures 1-5**).

It was found that the eczema cream formulation without BEC only had a marginal antipruritic effect on psoriasis when applied to the lesions over 4 weeks with twice daily applications. However, when BEC was added to the now modified

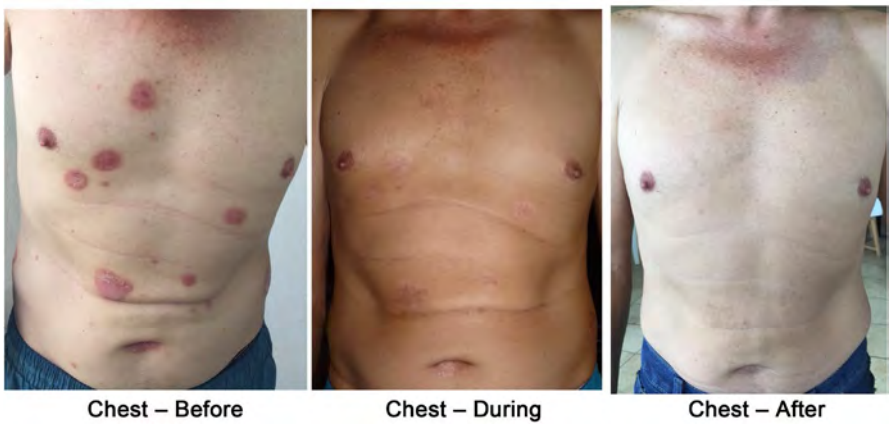


Figure 1. Psoriasis on the chest before, during (2 weeks) and after (4 weeks) of Psorend-[BEC] therapy.

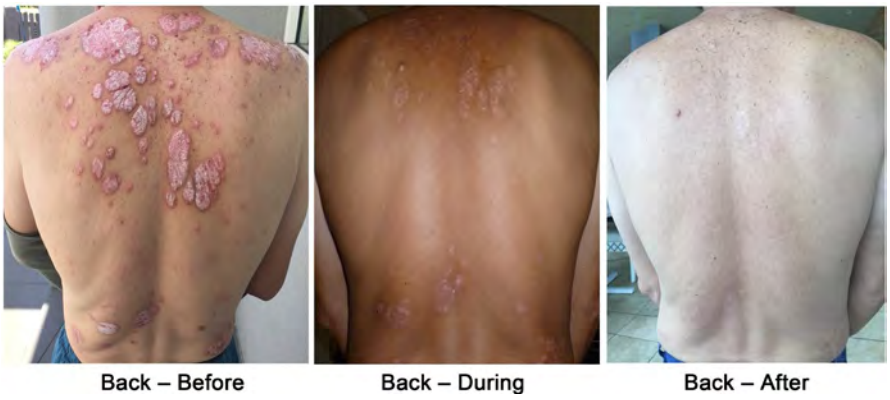


Figure 2. Psoriasis on the back before, during (2 weeks) and after (4 weeks) of Psorend[BEC] therapy.

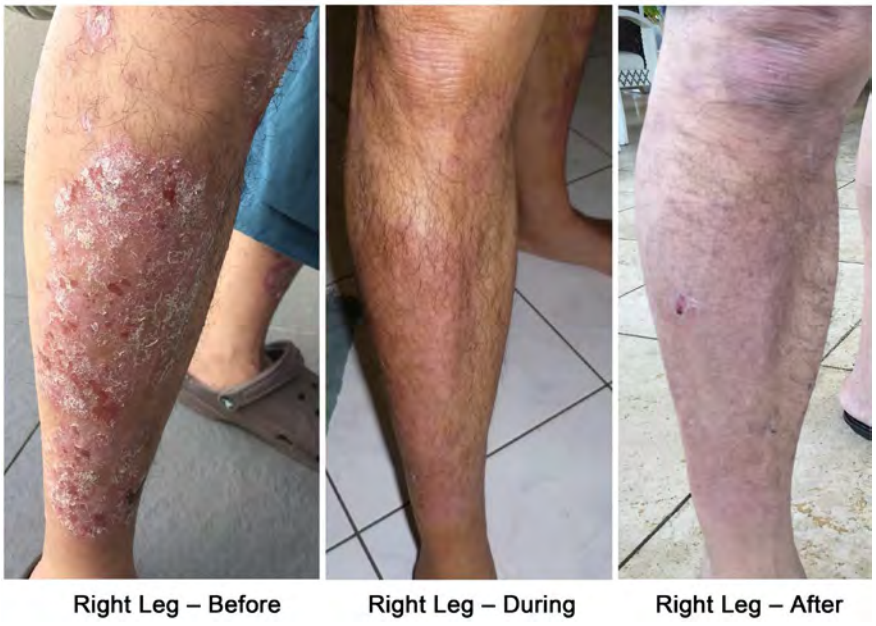


Figure 3. Psoriasis on the right leg before, during (2 weeks) and after (4 weeks) of Psorend[BEC] therapy.

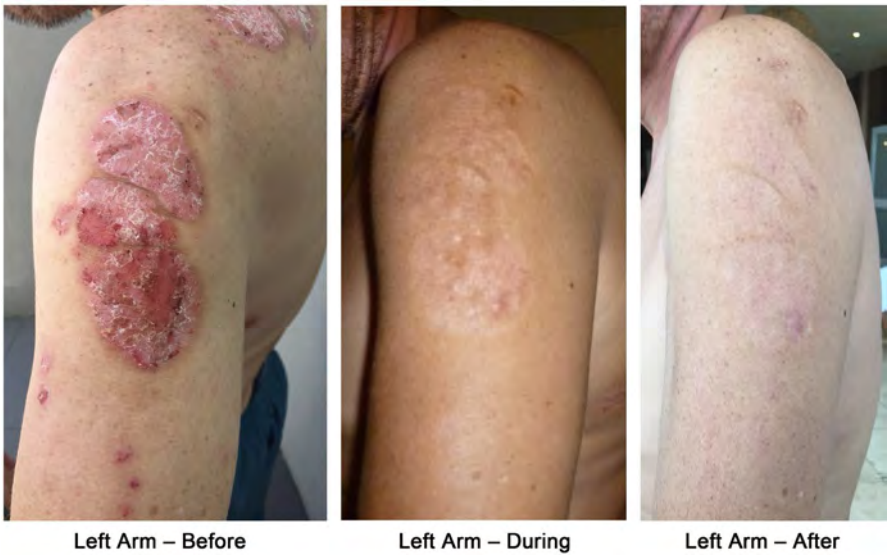


Figure 4. Psoriasis on the left arm before, during (2 weeks) and after (4 weeks) of Psorend[BEC] therapy.

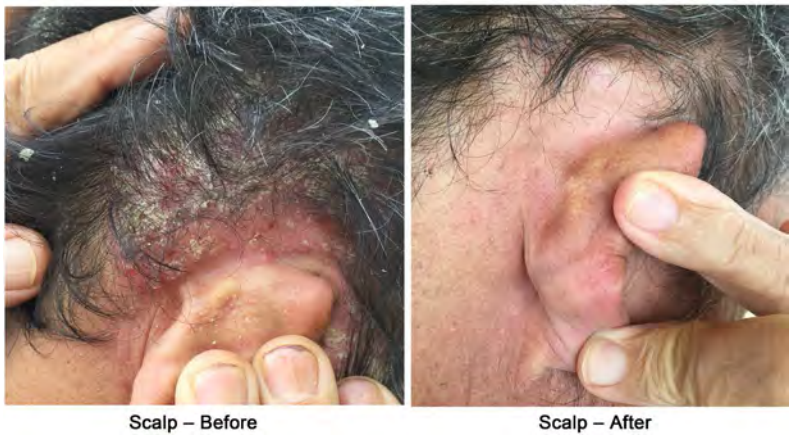


Figure 5. Psoriasis on the scalp before and after (4 weeks) of Psorend[BEC] therapy.

eczema cream (Psorend[BEC]) and then applied twice daily to psoriatic lesions, a rapid positive effect was apparent.

The use of Psorend[BEC] for two days resulted in considerable reduction in itching all over the body where the cream was applied. Continuation of Psorend[BEC] therapy for two weeks resulted in much improvement of the lesions (**Figures 1-4**).

After 4 weeks of Psorend[BEC] treatment, the scaly patches and wounds disappeared and the skin obtained its normal appearance without any blemishes (**Figures 1-5**). Treatment was stopped after the disappearance of the symptoms (4 weeks of treatment).

There have been no recurrences of any lesions to date, which is one year after cessation of Psorend[BEC] therapy. No further treatment has been required to date. This observation is important because retention rates or persistence rates in a given treatment protocol are very useful in assessing the "added value" of ther-

apy in daily clinical practice.

The treatment was well tolerated and no local or systemic side effects were observed.

5. Discussion

Before 2003, dermatologists had limited options to treat psoriasis. Many of those treatments caused side effects and did not always result in satisfactory results.

Since then, biologics have become available that affect the immune system, resulting in the reduction of inflammation and consequently slow down the growth of skin cells. In that context, it is interesting that naturally occurring BEC exerts a positive outcome on the immune system [21] and also affects the proliferation of unwanted cells [10].

A biologic is a pharmaceutical drug product manufactured in, extracted from, or semi-synthesized from biological sources resulting in high treatment costs. The side effects of biologics can be very serious, ranging from suicidal ideation, susceptibility of infection and may even cause cancer [22].

Thus, there is a need for new highly efficacious, safe, low cost treatments for psoriasis.

In 1987 it was first reported that BEC consisted of a mixture of solasodine glycosides, of which solamargine and solasonine are the major components. BEC has high antineoplastic efficacy and low toxicity when treating a wide variety of cancers [10].

Subsequently, it was reported that the BEC glycoalkaloids were effective against bacteria, fungi, viruses, parasites and malaria. These observations supported the accepted understanding that in plants, BEC glycoalkaloids are secondary metabolites, which are regarded as defensive agents against pathogens and predators including fungi, bacteria, viruses, insects and worms.

Here, it is reported for the first time that Psorend^{BEC} may prove to be a good candidate for the treatment of severe psoriasis.

The severity of psoriasis is determined by how much of the body's surface is covered and how much it affects a person's quality of life.

In this case report, a large portion of the patient's body surface was covered with psoriasis at multiple sites.

The quality of life was very poor and the patient had to endure his condition for a decade. This was despite the use of various prescribed medications.

When using Psorend^{BEC} the relief was virtually immediate, the itching had stopped shortly after the application of Psorend^{BEC}. Reduced symptoms of the psoriasis lesions were observed within one week of Psorend^{BEC} treatment. After 2 weeks treatment, striking remission was observed and after 4 weeks treatment the lesions had completely regressed.

Importantly, there was no recurrence after one-year follow-up post treatment and there were no observable side effects.

It is interesting that the solasodine glycosides present in the formulation

Psorend[BEC] was able to eliminate recalcitrant psoriasis. The same solasodine glycosides, but in a different topical cream formulation, Curaderm[BEC5], is effective against a wide variety of non-melanoma skin cancers [10], including recalcitrant basal cell carcinomas [23].

We now show for the first time that BEC in combination with other commonly used substances is highly effective against a serious case of psoriasis.

At this stage, it is not known whether the other components in the cream formulation work synergistically with BEC. Importantly, a cream formulation containing all the other components at identical concentrations, but without BEC, had no effect on psoriasis.

Evidence is now presented that BEC elicits efficacy against another medical condition, psoriasis.

6. Conclusions

Although this is only a case study with unknown modes of action of the treatment medication, the results are nevertheless very rapid, long lasting, and impressive.

Many further studies are required to establish the possible value of Psorend[BEC] as a potential treatment for psoriasis. Such clinical studies are underway.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Michalek, I.M., Loring, B. and John, S.M. (2017) A Systematic Review of Worldwide Epidemiology of Psoriasis. *Journal of the European Academy of Dermatology and Venereology*, **31**, 205-212. https://doi.org/10.1111/jdv.13854

[2] Menter, A., Gottlieb, A., Feldman, S.R., Van Voorkees, A.S., Leonardi, C.L., Gordon, K.B., Lebwohl, M., Koo, J.Y., Elmets, C.A., Korman, N.J., Beutner, K.R. and Bhushan, R. (2008) Overview of Psoriasis and Guidelines of Care for the Treatment of Psoriasis with Biologics. *Journal of the American Academy of Dermatology*, **58**, 826-850. https://doi.org/10.1016/j.jaad.2008.02.039

[3] Boehncke, W.H. and Schön, M.P. (2015) Psoriasis. *Lancet*, **386**, 983-994. https://doi.org/10.1016/S0140-6736(14)61909-7

[4] Christophers, E. (2007) Comorbidities in Psoriasis. *Clinics in Dermatology*, **25**, 529-534. https://doi.org/10.1016/j.clindermatol.2007.08.006

[5] Ramsay, B. and O'Reagan, M. (1988) A Survey of the Social and Psychological Effects of Psoriasis. *British Journal of Dermatology*, **118**, 195-201. https://doi.org/10.1111/j.1365-2133.1988.tb01774.x

[6] Feldman, S.R. (2019) Treatment of Psoriasis in Adults-UpToDate. https://www.uptodate.com/contents/treatment-of-psoriasis-in-adults

[7] Icen, M., Crowson, C.S., McEvoy, M.T., Dann, F.J., Gabriel, S.E. and Maradit Kramers, H. (2009) Trends in Incidence of Adult-Onset Psoriasis over Three Decades: A Population-Based Study. *Journal of the American Academy of Dermatol-*

ogy, **60**, 394-401. https://doi.org/10.1016/j.jaad.2008.10.062

[8] Cham, B.E., Gilliver, M. and Wilson, L. (1987) Antitumour Effects of Glycoalkaloids Isolated from *Solanum sodomaeum* L. *Planta Medica*, **53**, 34-36. https://doi.org/10.1055/s-2006-962612

[9] Cham, B.E. and Wilson, L. (1987) HPLC of Glycoalkaloids from *Solanum sodomaeum*. *Planta Medica*, **53**, 59-62. https://doi.org/10.1055/s-2006-962621

[10] Cham, B.E. (2017) Solasodine, Solamargine and Mixtures of Solasodine Rhamnosides: Pathway to Expansive Clinical Anticancer Therapies. *International Journal Clinical Medicine*, **8**, 692-713. https://doi.org/10.4236/ijcm.2017.812064

[11] Cham, B.E. and Meares, M.M. (1987) Glycoalkaloids from *Solanum sodomaeum* L. Are Effective in the Treatment of Skin Cancers in Man. *Cancer Letters*, **36**, 111-118. https://doi.org/10.1016/0304-3835(87)90081-4

[12] Gubarev, M.I., Enioutina, E.Y., Taylor, J.L., Visic, D.M. and Daynes, I.A. (1998) Plant-Derived Alkaloid Protects Mice against Lethal Infection with *Salmonella typhinurium*. *Physiological Research*, **12**, 79-88. https://doi.org/10.1002/(SICI)1099-1573(199803)12:2<79::AID-PTR192>3.0.CO;2-N

[13] Giron, L.M., Aguilar, G.A., Aceres, A.G. and Arroyo, G.L. (1998) Anticandidal Activity of Plant Used for the Treatment of Vaginitis in Guatemala and Clinical Trial of *Solanum nigrescences* Preparation. *Journal of Ethnopharmacology*, **22**, 307-313. https://doi.org/10.1016/0378-8741(88)90241-3

[14] Chataing, B., Christancho, N.B. and Usubillaga, A. (1998) Topical Treatment of Herpes Simplex, Herpes Zoster and Genital Herpes with a Mixture of Solanaceous Glycoalkaloids. MedULA. *Universidad de Los Andes*, **7**, 30-34.

[15] Chen, Y., Li, S., Sun, F., Han, H., Zang, X., Fan, Y., Tai, G. and Zhou, Y. (2010) *In Vivo* Antimalarial Activities of Glycoalkaloids Isolated from Solanaceous Plants. *Pharmaceutical Biology*, **48**, 1018-1024. https://doi.org/10.3109/13880200903440211

[16] Kumar, P., Sharma, B. and Bakshi, N. (2009) Biological Activity of Alkaloids from *Solanum dulcamara* L. *Natural Product Research*, **23**, 719-723. https://doi.org/10.1080/14786410802267692

[17] Cham, B.E. (2013) Inspired by Nature Proven by Science. The New Generation Cancer Treatment That Causes Cancer Cells to Commit Suicide. Colorite Graphics Printers Book, 260 p.

[18] Coramsine and Psoriasis. archive.li/rZOqR.

[19] Patent EP1181022A1.

[20] Patent WO2017147659A1.

[21] Cham, B.E. and Chase, T.R. (2012) Solasodine Rhamnosyl Glycosides Cause Apoptosis in Cancer Cells, Do They Also Prime the Immune System Resulting in Long Term Protection against Cancer? *Planta Medica*, **78**, 349-353. https://doi.org/10.1055/s-0031-1298149

[22] Dinarello, C.A. (2010) Anti-Inflammatory Agents: Present and Future. *Cell*, **140**, 935-950. https://doi.org/10.1016/j.cell.2010.02.043

[23] Batsev, A.F., Dobrokhotova, V.Z. and Cham, B.E. (2016) Topical Cream Curaderm^{BEC5} Treats a Recalcitrant Basal Cell Carcinoma. *Clinical Medical Review and Case Reports*, **3**, Article No. 098.

International Journal of Clinical Medicine, 2019, 10, 183-249
http://www.scirp.org/journal/ijcm
ISSN Online: 2158-2882
ISSN Print: 2158-284X

Manifestation of Pathological States of Numerous Diseases in the Largest Organ of the Human Body: (I) Basics and the Diseases of Tendon

Peter Chin Wan Fung[1*], Regina Kit Chee Kong[2]

[1]Division of Medical Physics, Department of Medicine, University of Hong Kong, Hong Kong, China
[2]School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China
Email: *peterallegro333@gmail.com, *hrspfcw@hku.hk

How to cite this paper: Fung, P.C.W. and Kong, R.K.C. (2019) Manifestation of Pathological States of Numerous Diseases in the Largest Organ of the Human Body: (I) Basics and the Diseases of Tendon. *International Journal of Clinical Medicine*, **10**, 183-249.
https://doi.org/10.4236/ijcm.2019.103018

Received: January 21, 2019
Accepted: March 15, 2019
Published: March 18, 2019

Abstract

We analyze the crucial biochemical and biophysical properties of the basic constituents—connective tissues (CT), and interstitial fluid (IF) constituting the non-cellular part of the fascia. We provide ample evidence that the resident cells and cells in transit in the fascia are continuously interacting with the non-cellular constituents to form an active organ with well-defined functions. We show evidence that pathological states of diseases of internal organs, as well as that of the constituents of the fascia itself, manifest in certain CTIF domains of the fascia. Numerous diseases originate from imbalance of the digestion and synthesis of the native collagen triple helices. Review on the scanning electron microscopy examination of cross-section of tendons indicates that micro-fibrils of collagen I form regular geometrical structures, supporting the hypothesis that the collagen fibrils assemble like liquid crystals. Information on the age of Achilles tendons has been reported, based on dating of the ^{14}C atoms generated from the nuclear bomb tests in 1955-1963. The causes of spontaneous tendon rupture and tendinopathy are analyzed. Plausible clinical measures to treat tendinopathy are briefly discussed, including the application of synthetic mechano-growth factor, glyceryl trinitrate patch (to supply nitric oxide), platelet rich plasma, proteomic profile analysis and microRNA 29a therapy.

Keywords

Fascia, Mechanotransduction, Collagen Degradation and Synthesis, Durotaxis of Cells, Self-Assembly of Tropocollagen, Thermal Stability of Collagen, Fibroblasts, Stem Cells, Proteoglycans, Glycoproteins, Interstitial Fluid,

Achilles Tendon, Tendinopathy

1. Introduction

1.1. Mechanotransduction through Organisms on Earth

All living organisms on earth are inevitably subjected to (a) the gravitational force that continuously acts as a mechanical pull on all parts of the body, and (b) frequently occurring external mechanical stimuli/forces of various degrees from other objects. In the past twenty years, research on intra- and inter-cellular signaling with reference to cellular structure variation indicates that (i) external stimuli of mechanical or electrical nature control or trigger many fundamental biochemical interactions; (ii) tissues are composed of a network of proteins joining organs; (iii) the nucleus and other functional components inside individual cells are not floating in a fluid; rather many protein/peptides readily join up to communicate between the extracellular matrix and the nucleus; (iv) certain intact cells are migrating in the connective tissues to carry out important physiological functions.

Putting fourth these observations and recalling the two "disturbances" (a) and (b) on organisms on earth, one is inclined to consider that a special architecture system must be at work in each living entity particularly suitable for the transmission of mechanical signals, if the organism is to remain as that entity. Such an architectural system is one important basis for allowing physical and biochemical signals to be transmitted inter- and intra-cellularly to certain parts of the body. Mechanical signal, as one type of physical signal, is inferred to be transmitted through mechanotransduction to various parts of the body based on the hierarchy nature of the body [1] [2] [3].

1.2. The Basic Components of the Largest Organ of the Human Body—The Fascia

In a multi-cellular organism, after a (fermented) cell has completed its first mitosis into two and then more cells, a system of communication must have existed to cater for the coordination of the function of a group of cells in a controlled environment and morphogenic development, for the survival and growth of the organism. Such a communication system must allow the exchange of various forms of energy and materials (such as the exchange of biomolecules via osmosis, diffusion) for both biophysical and biochemical interactions. A system that consists of both solid and liquid components could serve these purposes. The connective-tissue system, which is developed from the mesoderm, is a natural solid platform for communication between organs; the embryonic water is a natural fluid to develop into a fluid for the transportation of both soluble and insoluble (via vehicles such as chylomicrons) [3]. The connective tissues include 28 types of (a) fibrous (including collagen fibers,

elastic fibers and reticular fibers) and (b) non-fibrous collagen structures, plus (c) non-collagenous proteins to support integrity of various visceral organs and the body as a whole [3] [4] [5].

Part (c) contains two main classes of non-collagenous protein complexes (A) and (B). Classes (A) *i.e.* Proteoglycans (PG) has subclasses aggrecan, fibromodulin, heparan sulfate, decorin, perlecan, glycosaminoglycan (GAG, such as hyaluronan, keratan, heparin) and others. Note that hyaluronan, which is the most abundant and ubiquitous member of the GAGs, forms negatively charged long chains. The hydronium ions together with layers of water (originated from the blood stream) are then bound to the hyaluronan chains, forming a thick interstitial fluid (IF) in the interstitium; IF, also named the ground substance, is just the fluid for communication mentioned above. There is about 3 liters of interstitial fluid processed through the human body per day. The other non-collagenous protein complex (B), called Glycoproteins, have subclasses such as fibronectin, tenuscin, thrombospondins-1 and -4 (as adhesive proteins), laminin. Members of (B), together with members of (A), provide a potential bridge between parts (a) and (b) and the cells adjacent/within the interstitium. The connective tissue proper (CT) includes parts (a), (b) and (c). Since blood vessels do not "connect" cells of organs in the body, all communications are transmitted through the connective tissue-interstitial fluid (CTIF) system. The fascia is meant to be the grand anatomical structure joining all the CTIF systems associated with different parts of the body.

This fascia frame, including the solid parts and special fluid mentioned above, has various types of linked, connective tissue layers which embed the neurovascular tracts, extending to form tunicae around the visceral organs. The frame also wraps around muscles (striations/spindles); the connective tissues of the frame grow deep to form the periosteum which supplies vascular structure to the bone [6] [7]. Since the bone is also built of collagen, it is not unreasonable even to define the skeletal bone as the very "hard part" of the CTIF system.

There are intact cells migrating in the fascia. These cells are called the resident cells. They are also called indigenous cells [8] and have a stable population; they are mainly responsible for the synthesis and maintenance of the heterogeneous extracellular matrices of CTIF associated with different organs; these cells are also serving some other physiological functions. There are also cells in transit when in need, to perform immunity duties. The "hard part" and "soft part", which are both non-cellular, together with the cells mentioned, constitute an integrated network to maintain the integrity of body shape against gravity, as well as to serve as a grand active communication network. Research in the past decade recognizes the fascia to be highly dynamic and versatile (see force transmission aspect in [9]). Since this grand communication platform with solid and fluid constituents, has anatomical structure and has well defined functions, it is rightful to call it an organ—in fact, the largest organ of the human body. **Figure 1** summarizes the basic compositions of this organ.

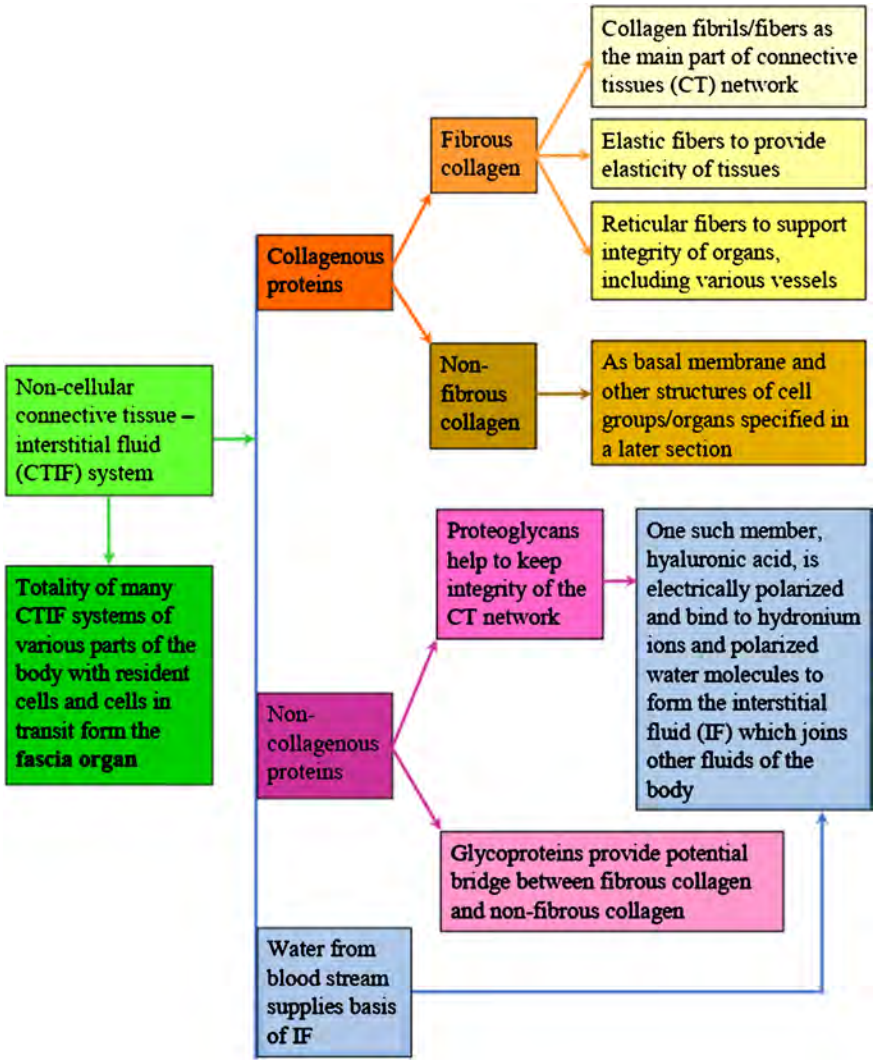


Figure 1. Basic structure of the largest organ of the body.

1.3. Functions of the Circulation of Fluids in the Largest Organ of the Body

We emphasize that as blood vessels do not "connect" cells of organs in the body, all communications between two organs pass through the connective tissue-interstitial fluid (CTIF) system. The stated communications include the transportation of molecules and various forms of energy signals (such as mechanical, sound, electrical signals). In particular, the IF is responsible for the following main processes: 1) Exchange of O_2 and CO_2 in muscles and organ cells; 2) Provision and re-absorption of nutrients to organ cells; 3) Transportation of metabolic debris and harmful cells to the peripheral lymphatic system via the flow of the IF to the lymphatic collecting ducts; 4) Supplying shear forces (acting as signals) during durotaxis/chemotaxis [10] of fibroblasts, chondrocytes, osteocytes, osteoblasts, mesenchymal stem cells and stem cells in the CT, including those in the hair-follicles, for tissue homeostasis as well as repairs/turnover of the CTIF system, cartilages and bones; 5) Provide a medium for the migration of

leucocytes from the blood vessels to the CTIF during inflammation; 6) It has been discovered only in the recent few years that a lymphatic system in the brain exists—called glymphatic system and the fluid is named the glymphatic fluid (see review of [11]). This fluid is a mixture of the classical cerebrospinal fluid flowing in the subarachnoid space and the brain interstitial fluid in the brain parenchyma. One of the important functions of the IF flow is to provide a route for the glymphatic fluid to join the peripheral IF which flows into the peripheral lymphatic fluid system. 7) The IF, carrying debris and fragments of damaged cells in the CTIF system, joins the subclavian vein vessels. These vessels have branches flowing through the renal system; wastes are then discharged by urination [3] [11].

1.4. Intracellular Mechanotransduction

Integrins are cell membrane receptors, being composed of α- and β-subunits with various combinations. They can bind to proteins in the extra-cellular matrix (ECM) with specificity [12]. When the integrins are stimulated mechanically, they connect intracellular proteins such as focal adhesion kinase (FAK), src-family kinases. Activation of these kinases triggers off a series of pathways associated with cytoskeletal remodeling. In particular, *in vitro* experimental results show that the stress-induced mechanical strain stimulated conformational activation of α- and β-integrins of NIH3T3 cells [13].

Such activation is followed by new integrin ligand binding to ECM proteins; this process then mediates c-Jun NH2-terminal kinase (JNK), initiating a series of cellular responses without upsetting the existing integrin-ECM binding. Responding to mechanical stimulation, "focal adhesion" is said to be formed, leading to a stress-dependent increase in cytoskeletal (CSK) stiffness. The CSK stiffness changes because there is rearrangement of the microfilaments (MF), microtubules (MT) and intermediate filaments (IF), which are the three important elements to participate in mechanotransduction, intracellularly. These connecting structures, together with other components, eventually join the nucleus surface after such CSK remodeling [14].

Then expressions of different genes are affected to produce the relevant proteins to maintain the cell integrity and to initiate the necessary physiological processes [15]. There are different integrins for different cell types to trigger different functions in response to mechanotransduction. For example, the integrins of the endothelial cells of blood vessel are born to react sensitively to mechanical stimulus arising from fluid flow, in addition to "pull" from collagen fibers. Thus, mechanical signals at the cell membrane can cascade a series of signals reaching the cell nucleus with the integrin proteins, as mechanical sensors, starting the story.

1.5. Intercellular Mechanotransduction

Gap junction proteins, which are built by various connexin proteins, are "connection proteins" between adjacent cells [16]. It has already been known, based

on analysis of synchronous contraction of myocardial cells, that the gap junction proteins participate in the transfer of mechanical stimulation, indicating that mechanotransduction can be "passed on" between adjacent cells of a tissue/organ [17]. It has also been shown that electrical synaptic transmission between nerve cells depends again on the function of gap junctions [17]. The connexin protein Cx43 has been found to bind directly or indirectly to some intracellular proteins like caveolin, 20-β-catenin, tubulin, src, which have various functions, providing evidence that gap junctions take part also in mechanotransduction [18].

1.6. Objectives and Layout of This Paper

As analyzed in Sections (1.2) and (1.3), all "ingredients" and signals for visceral organ cells pass through part of the fascia. It is thus important to learn the physiological conditions of various parts of this large organ. Now collagen is the main protein constituting the solid part of the fascia, and collagen forms loose connective fibers/fibrils in the skin and supporting tissues, integument layers of all organs, including even small blood vessels, nerve fibers, and lymphatic ducts. We review the key features of synthesis of the basic collagen molecules and their formation of fibrous morphology of different sizes, as well as some important structural characteristics in Sections (2.1) to (2.4). Relevant features of the residence cells are briefly outlined in Section (2.5). The hierarchy nature of the fibrous collagen constituents, together with roles played by resident cells and cells in transit will be briefly analyzed in Section (2.6). The crimp structure of collagen fibrils is important in absorbing force during movement of different parts of the body, and two examples are presented to illustrate the morphology of the crimps in Section (2.7). A living organism is a dynamical complex system. Homeostasis includes turnover procedures and repairing mechanisms of collagen. Therefore, we review the classification and functions of the degrading enzymes and the endogenously generated inhibitors of those enzymes in Sections (3.1) - (3.4). Based on the brief review presented in Sections (1) - (3), we can then analyze the first class of disease of our series—rupture and tendinopathy of the tendons which are parts of the fascia proper. Section (4) is devoted to the discussion of the interesting structural arrangement of the collagen fibrils that allows tendons to withhold tensile strength based on *in vitro* investigation of tendon specimens obtained from animal models. Thermal stability of collagen triple helices is a debatable key issue in maintaining the mechanical strength of tendons. We therefore present two types of contrasting experimental results in Section (5). Every triple helix is built of three peptide chains, and wrong combinations (due to genetic mutation or incomplete folding correction by heat shock proteins in the RER) lead to diseases of the fascia proper, which could trigger off pathological states of other organs. This issue is discussed in Section (6). It is difficult to predict tendon rupture, and tendinopathy is difficult to treat clinically. In Section (7), we present some updated information of the causes and introduce

plausible treatments. Section (8) emphasizes the evidence of viewing the fascia to be the largest organ of the body and highlight the notion that pathological states of many diseases manifest in the fascia, paving the way for the next related paper. Section (9) gives concluding remarks. This paper is lengthy as a rather large amount of basic materials need to be introduced for meaningful discussion of any disease involved.

2. Synthesis and Special Properties of Collagen Molecules in the Fascia

2.1. Collagen as the Most Abundant Protein in the Body

Collagen fibrils have been supporting life of numerous species for over 600,000,000 years, and collagen is now known to be the most prominent protein component of the hierarchy connective tissue systems of nearly all members of the animal kingdom. It is indeed amazing to find that the basic architecture of collagen fibrils is conserved across different animal species during evolution [19] [20] [21] [22].

In the human body, collagen constitutes about one third of the body mass [23]. The basic unit of collagen is composed of three helical structures of amino acids, with details specified later. According to different compositions of these helices, collagen can be classified, up to the present, into at least 28 types.

Types I, II, and III account for quantitatively over 70% by weight of the total connective tissues [24] which compose the biggest tissue system of the body. In particular, type I collagen makes up 80% - 90% by weight of the collagenous proteins.

2.2. Types of Collagen Molecules, Fibrous and Non-Fibrous

Since both fibrous and non-fibrous collagen structures are involved in the pathogenesis of many diseases, we show **Table 1** to summarize the classes of collagen so far found, their key characteristics and main locations in the human body. There can be more than one isoform for one type of collagen. For example, in the second column, $[\alpha_1(I)]\cdot^2\alpha_2(I)]$ means that a type I collagen triple helix can be composed of two α_1 chains and one α_2 chain of collagen I; the second isoform is simply built of three α_1 chains of collagen I. The super-script 3 of $[\alpha_1(I)]^3$ means that there are three α_1 (I) chains in a type I tropocollagen molecule. The subscript 1 means that the chain is of class α_1 (rather than the α_2 chain). The former structure is called a heterotrimer and the latter structure is called a homotrimer. These two classes of structure respond differently to enzyme degradation, as will be explained later in Section (6). The other symbols for other types of collagen follow the same way. There are totally 28 types of collagen found up to now. We show only 14 types out of 28 here as they are relevant to our discussion on the physiological/pathological aspects involved in this paper. When we come to some other diseases involving other types of collagen, they will be specified in other papers to follow.

Table 1. Types of collagen [25].

Type of collagen	Chains	Basic structural characteristics	Distribution in body
I	$[\alpha_1(I)]\cdot^2\alpha_2(I)$; $[\alpha_1(I)]^3$ (not native)	fibrous	skin, tendon, ligament, bone, cornea, general connective tissues
II	$[\alpha_1(II)]^3$	fibrous	cartilage, vitreous humour, inner core of vertebral disc—nucleus pulposus, less organized-meshwork
III	$[\alpha_1(III)]^3$	fibrous	co-distributes with type I in smaller amount, endotenon
IV	$[\alpha_1(IV)]\cdot^2\alpha_2(IV)$; $\alpha_3(IV)\cdot\alpha_4(IV)\cdot\alpha_5(IV)$; $[\alpha_5(IV)]^2\cdot\alpha_6(IV)$	forming network	basement membrane of organs
V	$[\alpha_1(V)]\cdot^2\alpha_2(V)$; $\alpha_1(V)\cdot\alpha_2(V)\cdot\alpha_3(V)$; $[\alpha_1(V)]^3$	fibrous	co-distributes with type I in smaller amount
VI	$\alpha_1(VI).\alpha_2(VI).\alpha_3(VI)$	with beaded-filament structure	around muscles, in "cross-structure between fibrils"
VII	$[\alpha_1(VII)]^3$	forming anchoring fibrils	epidermal-dermal boundary
VIII	$[\alpha_1(VIII)]^2\cdot\alpha_2(VIII)$	forming network	basement membrane between corneal proper and endothelial-Descement's membrane
IX	$[\alpha_1(IX)]\cdot\alpha_2(IX)\cdot\alpha_3(IX)$	fibril associated collagens with interrupted triple helices (FACIT)	co-distributes with type II
X	$[\alpha_1(X)]^3$	forming network	hypertrophic structure of cartilage
XI	$\alpha_1(XI)\cdot\alpha_2(XI)\cdot\alpha_3(XI).$	fibrous	co-distributes with type II
XII	$[\alpha_1(XII)]^3$	FACIT	co-distributes with type I in small amount
XIII	$[\alpha_1(XIII)]^3$	transmembrane protein	cell-matrix and cell—cell adhesion, neuromuscular junctions
XIV	$[\alpha_1(XIV)]^3$	FACIT	co-distributes with type I in small amount

2.3. Procollagen Molecules Are Synthesized, with Folding Checked by Heat Shock Proteins and Exit to the Extra-Cellular Domain via Exocytosis

Interstitial collagens consist of three α chains of approximately 1000 residues with repeating Gly-X-Y triplets, where X and Y are often proline and hydroxyproline, respectively. Because of the high imino-acid content and the tripeptide unit repeats, the α-chain adopts a left-handed poly-Pro II-like helix, and three left-handed α-chains intertwine with each other to form a right-handed superhelix [25]. It is the occurrence of amino acid glycine as every third residue throughout 95% of the α chains as well as the presence of large amounts of proline and hydroxyproline (which together amount to approximately 22% of the amino acid residues) that would allow each (alpha) polypeptide chain to be arranged in a stretched polyproline helix. In other words, collagen triple helices are said to be packed into a "quarter-staggered" pattern so that the nearest neighbor molecules are staggered longitudinally by about 22% of their molecular

lengths with a space gap between the N-terminal of one molecule and the C-terminal of the next [25].

Each peptide chain of collagen is encoded by one gene in general. However, many of the collagen types have two, or even three peptide chains of the same class. In Figure 2, suppose three different collagen genes, α_1, α_2, α_3 are triggered to synthesize a collagen molecule. In the nucleus, the double helical gene structure is broken to form RNAs. Each RNA works as an mRNA and one associated peptide is synthesized in the usual way in the ribosome; and the peptide is fed into the (rough) endoplasmic reticulum (ER or RER) and then the Golgi apparatus, as shown schematically in Figure 2. The three raw peptides assemble, and the resulting structure is quality checked for proper folding by heat shock proteins in the RER and Golgi apparatus (see [26]), with nucleation taking place at the C terminal [27]. A number of sugars are added to the peptides in the RER-Gorgi apparatus "factory" too. The "tentative collagen molecule", called "procollagen" of ~325 nm in length, is then excreted from the cell by the usual exocytosis process, with the help of the Golgi apparatus. The dotted arrow represents the fact that the last synthesis processes are carried out in the RER and Golgi apparatus, not in the cytosol.

It was already known that HSP 47 is an ER-resident stress inducible glycoprotein that specifically and transiently binds to newly synthesized procollagens [28].

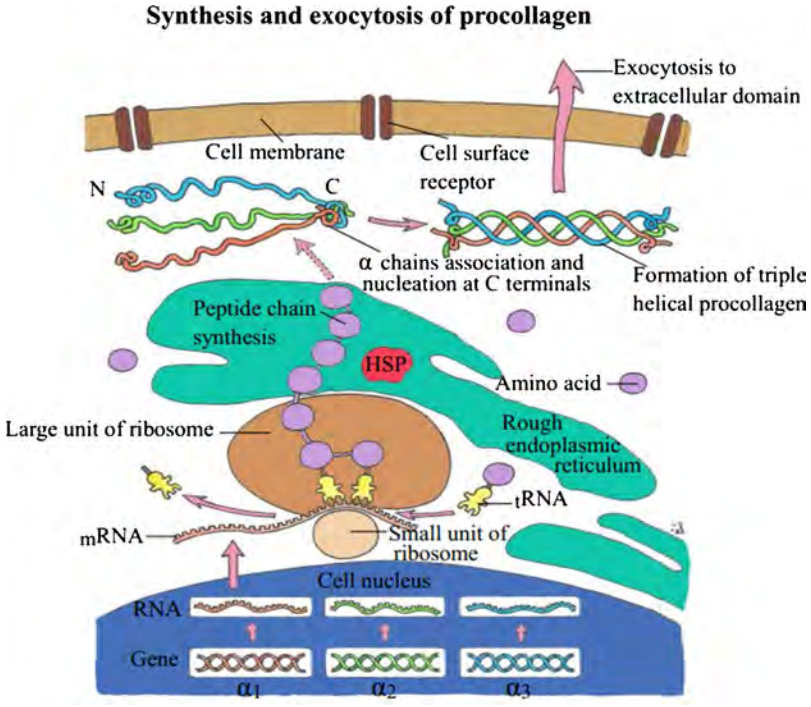


Figure 2. Schematic representation of the synthesis of collagen peptide chains, which may be called pre-procollagen chains, in the Ribosomes close to the Rough Endoplasmic Reticulum (RER). These peptide chains are secreted into the lumen of the RER where they are coiled to form a relatively loose triple helix, starting with nucleation [25] [26] [28]. A procollagen molecule is secreted from the cell. This figure was hand-painted by author PCWF.

2.4. Exocytosis of the Procollagen Molecules to Form Tropocollagen Molecules and the Formation of Micro-Fibrils and Fibrils in the Extra-Cellular Domain

A procollagen has a typical length of 325 nm, including a rod-like, tightly-packed triple helix of 300 nm, plus loosely-structured N terminal (15 nm) and C terminal (10 nm) (**Figure 3**). When a procollagen molecule is secreted via the secretory vacuole originating from the Golgi apparatus into the extra-cellular space, the N and C terminals are respectively cleaved by procollagen amino-protease and procollagen carboxy-protease, leaving a regular helical structure called tropocollagen molecule [25] [29]. That five triple helices build up a micro-fibril is generally accepted as the highly likely model based on the observed values of the cross-section of a triple helix and the length of a helix, but there are other alternatives; for example, it was long ago that another 5-strand model, with four helices in the cross-section, forming tetragonal stacking, plus another one helix attached to any side of the tetragonal (see Figure 2 of [30]) was proposed. For our analysis, the specific geometrical arrangement is not important at this stage. Five tropocollagen molecules can self-assemble to build up a micro-fibril, whose cross-section shows five helical structure in the over-lapped region, but the cross-section shows only four helical structure in the cross-section cutting across a gap. These two cross-sections are shown on the upper left part of the above figure. Micro-fibrils build up a fibril with different configurations. The one shown on the upper right of **Figure 3** is just one possibility with radial symmetry in the cross-section. This tight-pack model shows a quasi-hexagonal boundary. The four-strand model of micro-fibril, built by four tropocollagen molecules is another model structure of a micro-fibril, which is not shown here because the structure is very simple—with four molecules arranged to form a quasi-rectangular shape. A three-strand model is also proposed based on fractal nature of the hierarchy [31]. These micro-fibrils, with effective diameter around several nm, build up fibrils with effective diameter in the range around a few tens of nm to around 1 μ-meter.

2.5. Resident Cells of the Fascia

Fibroblasts, reticular cells and stem cells

Fibroblasts are derived from primitive mesenchymal cells which develop from the mesoderm of the embryo; they are called fibrocytes in general. In the dormant state, a fibroblast appears as an ellipsoid or said to have a spindle shape. An active fibroblast has branches, crawling on collagen fibers so that the fibers attain a certain degree of mechanical tension. The fibroblasts can be connected among themselves, via inter-cellular gap-junction and desmosome proteins, lining up along collagen fibers. The main function of fibroblasts is to secrete precursors of collagen, elastic, fibers, proteoglycans (PG), glycosminoglycans (GAG), together with glycoproteins [3]. Reticular fibers are synthesized by reticular cells [5].

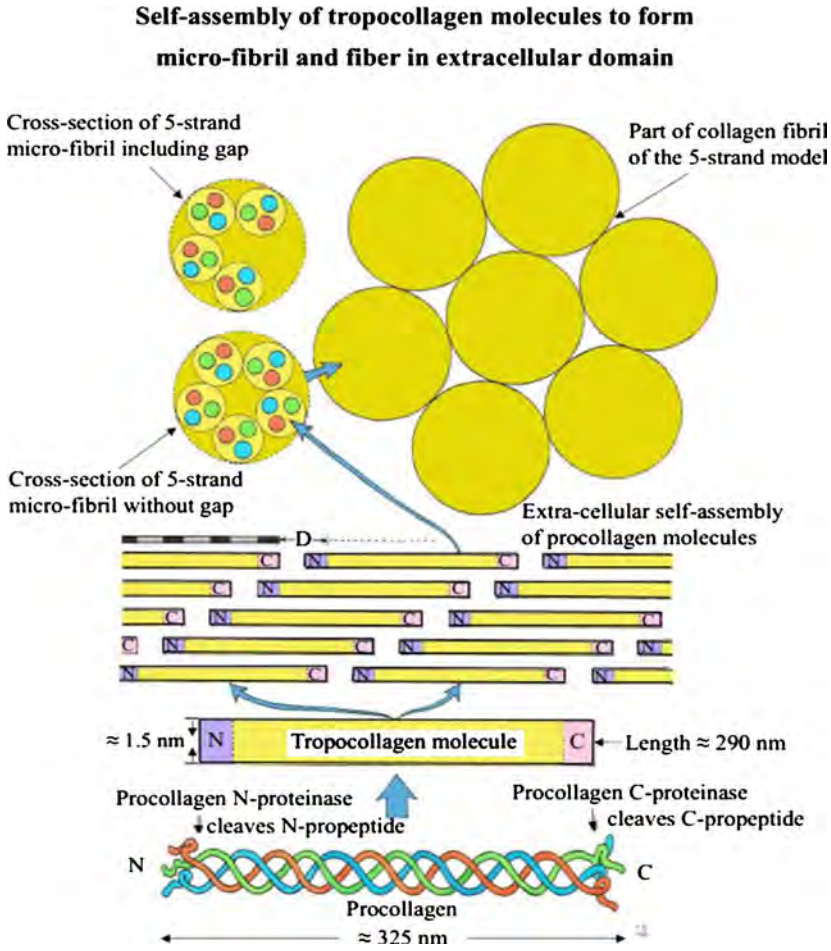


Figure 3. When a procollagen molecule is exocytosed from the fibroblast, the N and C terminals are respectively cleaved by procollagen amino-protease and procollagen carboxy-protease, leaving a regular helical structure called tropocollagen molecule, of length about 300 nm [25]. Micro-fibrils build up a fibril/sub-fibril with different configurations [29]. The one shown on the upper right is just one possibility. This figure was painted by author PCWF.

GAG with binding water molecules builds a jelly-like IF as the ground substance of the fascia, and the IF, together with other fluids forms an integrative five-fluid system [11]. The fibroblasts are the most important, and most abundant cells in the largest organ of the body. Fibroblasts migrate to many parts of the body, and they can change their phenotyping to become other cells. There is evidence that gradient of the mechanical tension of collagen fibers controls the direction along which cells would crawl—a process called durotaxis, which is the key factor for the stated change in phenotyping. In that respect, fibroblasts function like stem cells, in response to mechanical clue. Stimulated by cytokines, fibroblasts can also migrate (chemotaxis) to participate in wound healing [32]; they become myo-fibroblasts which may be involved in carcinogenesis if not properly regulated, as explained in the next related paper.

Mesenchymal stem cells (MSCs) also originate from the mesoderm of the embryo and are bone-marrow-derived. There is evidence that MSCs could pro-

mote synthesis of collagen type I and collagen type III in tissue-engineered ligaments [33]. It is not surprising that a certain number of stem cells, which are reside in many locations of the fascia, would differentiate into fibroblasts and other fibroblast-like cells, participating in repair and growth when in need [33]. In addition to MSCs, there are stems cells found in the hair follicles [34] in the dermis and the fasica [3] [35]. These stem cells are interacting actively with the collagen molecules of the fascia.

Chondroblasts, chondrocytes and cartilages

A chondroblast or cartilage cell, like a fibroblast, originates from a mesenchymal stem cell. The extracellular matrix of cartilage is secreted by these chondroblasts, which reside in the outer covering layer of the cartilage. As the chondroblasts secrete matrix and fibers, they become trapped inside it, and mature into cells called chondrocytes. The active chondrocytes are large secretory cells with basophilic cytoplasm, containing many rough endoplasmic reticula. Older chondrocytes contain fat droplets [36].

The cartilage also contains a mixture of collagen and elastic fibers plus other non-fibrous protein components, of which there is 10% aggrecan (formed by aggregating chondroitin sulfate) and hyaluronic acid. Cartilage is avascular and can only be nourished (oxygen and nutrients) by long range diffusion from nearby capillaries in the perichondrium. Therefore, cartilage is a layer which does not grow to become thick. The ECM of cartilage entraps the PG (aggrecan) molecules to form a sponge-like structure to soak up water, producing high water content ~75%. The water combines with the hyaluronic acid in the matrix to form a gelatinous ground substance [37] [38].

The cartilage matrix, together with the thick fluid, is flexible, yet very durable, has strong resistance to compression forces. The disorders of this intriguing structure lead to the known diseases of the knee and other joints [38].

Osteoblasts and osteoclasts

Being mesenchymal in origin, osteoblasts are mononucleate, specialized fibroblasts which express bone sialoprotein and osteocalcin as well as other fibroblasts products. Osteoblasts are responsible for bone formation [37] [39]. First, osteoblasts produce a matrix of osteoid, composed mainly of Type I collagen, completely extra-cellular. To form a bone, osteoblasts are also responsible for the mineralization of this matrix, with Zinc, Copper, Sodium plus other minerals in this process. Osteoblasts separate bone from the IF by tight junctions [40]. Note that bone is a dynamic tissue: it is broken down by another specialized fibroblasts—osteoclasts, and is constantly being reshaped by osteoblasts [41]. The number of osteoblasts decrease with age, leading to osteoporosis [39].

Mast cells

It is known that mast cells (MC) develop, like other leukocytes, from haematopoietic stem cells but do not mature before exiting the bone marrow and circulate as committed progenitors [42]. It has been demonstrated that these undifferentiated but committed progenitors do not develop into mature MC but

traverse the vascular space and complete their maturation after migrating into diverse peripheral tissues of the CTIF regions, such as skin, submucosa of stomach and intestine, breast parenchyma, myocardium, lymph nodes, conjunctiva and synovium and other CTIF domains of the fascia [43]. The sizes of mast cells in CT range from 20 - 30 μm; these cells are filled with deeply basophilic granules [44]. Human MC are conventionally divided mainly into two types, depending on the expression of different proteases in their granules. Mast cells are not only responsible for allergy reactions, but are also responsible for numerous physiological functions [3] [44], of which the relevant one in our study is that mast cells are sources and inducers of fibroblasts synthesis, at least in the skin [45].

Macrophages

Macrophages, very well known, also called histiocytes, are phagocytic cells derived from monocytes in the blood stream. They migrate in most loose CT, as one of the major lines of defense against infection.

Adipocytes

These cells, distributed throughout the subcutaneous connective tissue layer of the fascia, serve to aggregate a reserve store of energy and as a shock cushion, as well as heat insulator for internal organs. They are long-lived, and do not divide. Apart from the basic function mentioned above, they are involved in other important pathological states. We will discuss their special properties associated with different diseases in later papers.

Melanocytes

These cells are pigment cells found in the skin and choroid of the eye. Melanocytes produce melanin, a pigment in the skin, eye, and hair via a process called melanogenesis, protecting the hypodermis from the ultra violet B light that causes photodamage to the DNA.

During inflammations or injuries of organs, leukocytes extravasate from the blood stream and enter the CTIF system to carry out their immunity duties. They are well-known, and we will only discuss their interaction with the constituents of the fascia when we analyze pathogenesis of various diseases. In our nomenclature, we define leukocytes as cells in transit of the largest organ of the body.

2.6. The Hierarchy Structure of the Fibrous Collagen

The micro-fibril has a very stable structure. In **Figure 3**, we have shown the 5-strand micro-fibril model. Based on the fractal and spectral dimension analysis of 26 biopsy samples from fibrotic livers of patients, regular crystalline structure of the collagen has been reported and confirmed [46]. Whatever geometric model is used to describe the cross-section of collagen fibril, there is evidence that the boundary of a fibril is a polygon. In **Figure 4**, at the lower right corner, four tropocollagen molecules may form a tetragon, which can also be squashed to become a squashed-4-strand model, with one tropocollagen molecule as one

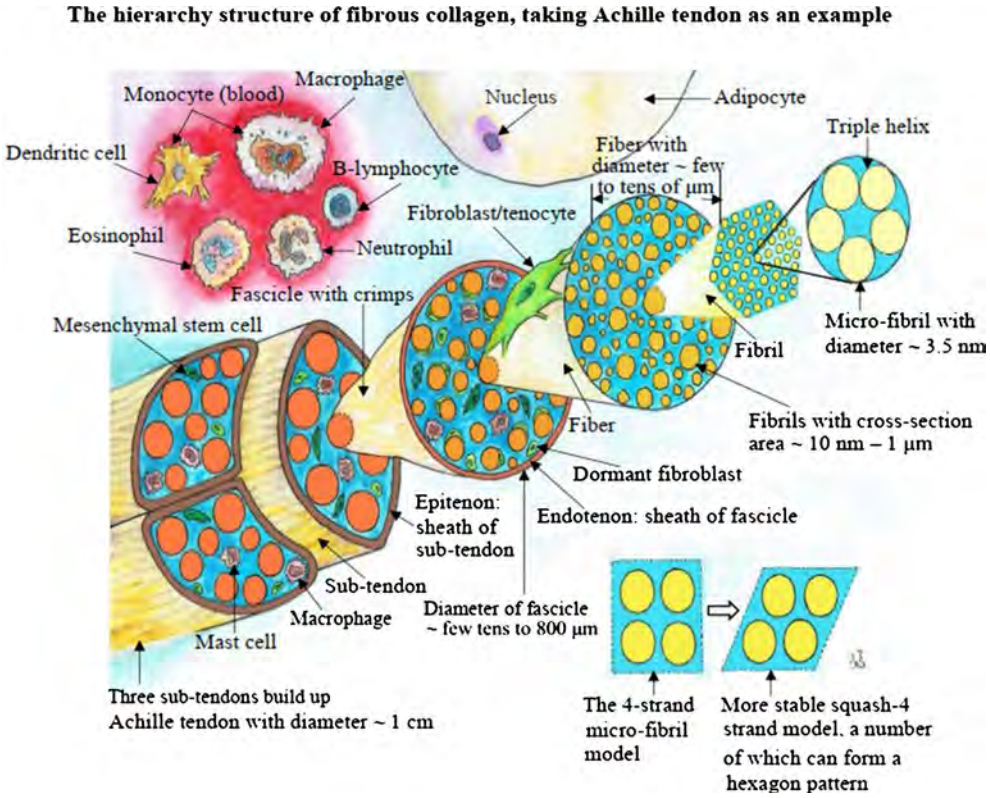


Figure 4. The right-hand corner at the bottom indicates that the quasi-square strand of four micro-fibrils would become the "squashed 4-strand model", which is more stable. We propose that many of these units form the hexagon or hexagonal like polygons observed in the cross-sections of animal fibrils, as confirmed in [46]. The resident cells—fibroblast/tenocytes, (mesenchymal) stem cells, mast cells, macrophages are painted in the ground substance of the CTIF system. The red patch are immunity cells from the blood stream during inflammation. We present three sub-tendons for an Achille tendon in human, and the tendon is closed by a layer called a paratenon (not painted here). The bright green tenocyte crawls on a fiber, giving the fiber a tensile force. A dormant or inactive fibroblast/tenocyte becomes ellipsoid like in shape and is not attached to a collagen fiber; an inactive fibroblast/tenocyte is marked in the figure. Note that the endotenon, epitenon, paratenon form extrinsic compartments consisting synovium-like tissues where some cells and signals (such as cytokines) of the immune, vascular, nervous systems can migrate/pass through [54] [55] [56]. This diagram is hand-painted by author PCWF.

basic unit. In view of the fractal nature mentioned, the basic unit can also be a microfibril (instead of a tropocollagen). At this point, we do not specify which strand model is the correct one; we take the squashed-4-strand model to show that with 64 tropocollagen molecules, the cross-section can become a hexagon, as shown in the figure. In Section (4.2), scanning electron microscopy (SEM) examination of human Achille tendon shows that the cross-sections of many fibrils in a tendon are polygons—in fact, quite a number are quasi-hexagon within the resolution of revelation (see e.g. **Figure 1** of [47]). When the number of sides of a polygon is large, the cross-section becomes like a circle. The effective cross-section diameters (diameter of a circle having an area equal to that of the polygon) of fibrils vary from about ~10 nm to 1 μ-m in human. Each fibril is composed of parallel tropocollagens shown in **Figure 4**, bound by hydrogen and

other chemical bonds, depending on the class of collagen involved. However, due to the very specific chemical composition of a tropocollagen (Section (2.3)), there are always gaps and overlapping regions as shown in **Figure 4**. Thus the "white and black" bands with D values within a narrow range of (64 - 67 nm) appear in the optical/SEM micrographs of the fibrils. Many fibrils form a fiber; the blue background of the "second circumference" from the right of **Figure 4** represent some proteins (proteoglycans and glycoproteins) plus the viscous interstitial fluid (IF); it is basically the inter-tropocollagen bonds that keep the integrity of a fibril. Collagen fibrils would self-assemble in physiological fluid [48], and there is evidence that the collagen fibrils are in fact liquid crystals—that is where the regular structure comes from [49] [50]. Many fibrils constitute a collagen fiber, having diameters in the range of ~few to tens of micro-m. This size scale is important because the cells in the human body have diameters/length size starting from ~7 μ-m to 35 μ-m. Therefore cells, the living units, are only interacting with fibers (or fibrils) around the same order of magnitude. Collagen fibers must have the benefit of the service of repair by the cells—the fibroblasts—which synthesize them. When some parts of the fiber or fibrils are damaged, enzymes called matrix metalloproteinases (MMPs) are secreted by fibroblasts to degrade the fragments/damaged portions, starting from very specific sites inside a fibril. When a fibroblast is activated, it stretches out with pseudopodia, crawling along a fiber with force. A string of fibroblasts line-up by cell-cell-interaction (see Fig.6 of [51]), subjecting the fibers to a certain amount of mechanical tension. The active sites for MMP degradation are then hidden, protecting the good fibers. A fibroblast (bright green) was painted to crawl on a fiber in **Figure 4**. The whole solid body structure of the connective tissues between two organs (even the small blood/lymphatic vessels, nerve fibers) is embedded in the viscous interstitial fluid as mentioned in Section (1). Since the connective tissue is developed from the mesoderm, evolution leaves some number of stem cells (deep green) around in the CTIF system [52], including some in the hair follicles [53]. They can change the phenotyping and become cells performing specific functions, such as wound healing. There are also some macrophages (one is labeled in the **Figure 4**) residing always in the CTIF system for surveillance. There are also mast cells which carry "bags of biochemical treasures"; a mast cell can secret one or more such bags by exocytosis to carry out physiological functions, including response to allergy [3] [44]. We have taken the environment of an Achille tendon as an example. In many parts of the body, there are adipocytes in the sub-cutaneous layer of the CTIF system. As the fat cell also plays important roles in physiology (details to be discussed in a future paper on obesity), a part of fat cell has been painted in the upper part of the figure. The fascicle (with diameter ~few tens to ~ 800 μ-m, even up to 1 mm [51]) is formed by fibers. These fascicles are bounded by a sheath/layer also of collagen in origin, called endotenon. The fascicles have crimp structure to absorb tensile force, as required during locomotion, because they form the (macroscopic) force enduring units of a sub-tendon. The little curvy lines on the fascicle in

Figure 4 represent the crimp structure. A crimp structure automatically develops in a fibrous collagen structure which endures tensile force during daily life. In fact, one can find fibers (of various sizes) having crimps in different parts of animal models. Several fascicles build up the sub-tendon (with size ~ several mm in human), bounded by a sheath called epitenon. Several (three are shown in the figure) sub-tendons form an Achille tendon, having diameter ~1 cm.

During inflammation, the white blood cells extravasate to the CTIF domain. The basophils become the mast cells which cannot reverse their identity and remain as resident cells there. In order to enforce immunity power, monocytes develop into dendritic cells (as antigen presenting cells) and macrophages. The B-lymphocytes, the neutrophils, and eosinophils can also extravasate to the tissues. These cells, called cells in transit in our nomenclature, are schematically painted within the "red patch", indicating that they originate from the blood circulation, and enter the CTIF during inflammation. We would emphasize that in different parts of the body, other cells are actively interacting with the CTIF to perform their physiological functions. For example, the chondrocyte, chondroblasts interact with the CTIF in the knee region; we would discuss activities of these cells elsewhere. Note that the endotenon, epitenon, paratenon form extrinsic compartments consisting of synovium-like tissues where some cells and biomedical signals (such as cytokines) of the immune, vascular, nervous systems can migrate/pass through [54] [55] [56].

2.7. Crimp Structure of Collagen Structure Observed in Animal Models and Humans—Presenting Two Examples

Crimps observed in the Achille tendon of rat

Polarized light micrograph of a relaxed Achille tendon of the rat model after dehydration is obtained in Fig.3 of [57]. The crimps look like flat sheets bent into triangular-like structures, (instead of a wavy pattern) as shown in **Figure 5(A)**, a simplified representation by a hand-painted picture of the discovery in [57]. From **Figure 4**, we have learned that the cross-sections of fibrils are polygons in general, and many appear circular when the orders of the polygons are high. Due to inter-fibrillar chemical binding force, the fibrils can form flat structures. In a later study, highly regular planar crimps of Achille, flexor digitorum profundus tendons, collateral ligament of knee (of animal models) were also revealed [58].

When similar Achille tendon specimens were stretched, the bending became flattened, showing some "wrinkles" which are the small parts that were not stretched, as shown by the polarized light micrograph in Fig. 3 of [57]. Based on such micrograph, another water color painting was done and presented as **Figure 5(B)** here. Below we shall present an example of wavy crimps, rather planar ones.

Crimps revealed in the chordae tendineae of the mitral valve, as a miniature tendon, of humans

The chordae tendineae of the human mitral valve shows orderly, wavy crimps in the scanning electron microscopy (SEM) micrograph of [59]. The effective

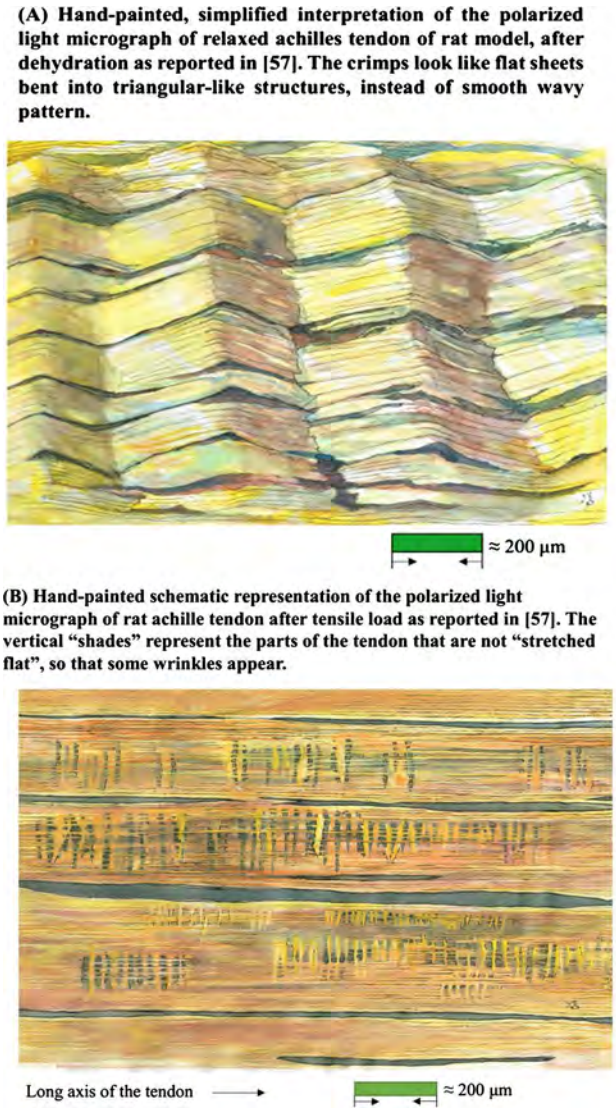


Figure 5. (A) Polarized light micrograph of a relaxed Achille tendon of the rat model after dehydration is obtained in [57]. The crimps look like flat sheets bent into triangular-like structures. This is a simplified, hand-painted representation of their micrograph in the above reference by author PCWF; (B) When the rat Achille tendon specimens of [57] were pulled by tensile force, the "triangular structures" disappeared, leaving some wrinkles, indicating certain small parts were not stretched. This is a hand-painting adaptation, simplified, following the work of [57].

diameters are around ten to several tens of nm, being equal to the smallest fibrils in the Achille tendon of humans to be discussed in Section (4.2). Degenerative disease of the mitral valve is known to be always accompanied by lengthening and/or rupture of chordae tendineae, as shown in Figure 8(b) to (d) of [59]. In **Figure 6(A)**, we present a hand-painted interpretation of the SEM micrograph of Fig. 8(a) of [59], showing clearly crimps of normal human chordae tendineae. The magnification is about 2400 times. Three green spindle-shaped cells are fibroblasts. Therefore, crimps can be found in fibrils and fibers in the collagen hierarchy; we have not indicated crimps of fibers and fibrils in **Figure 4** for the

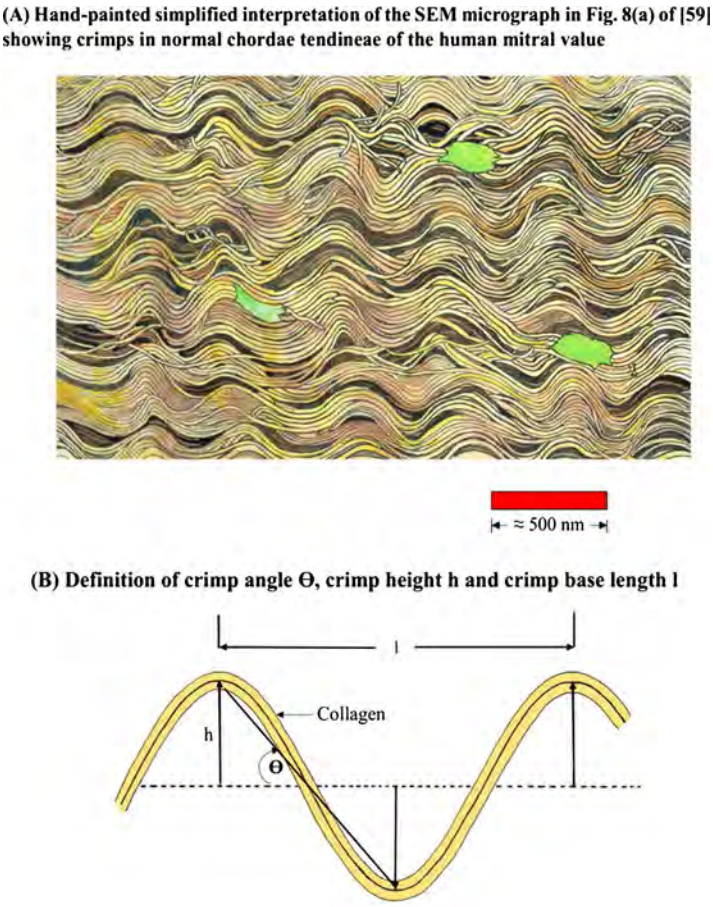


Figure 6. (A) In a healthy human mitral valve of the heart, the wavy crimps are rather ordered and very few ruptures are found. During the diseased state, the crimps disappear, and the fibrils are ruptured. The organization of the fibrils becomes disordered. We present only a simplified hand-painted interpretation (by PCWF) of the discovery of the healthy chordae tendineae of the mitral valve in [59]. The green spindle-shaped cells are fibroblasts, already "pressed" during the preparation of the SEM sample. The reader is referred to Figure 8(b)-(d) of [59] for the morphology of the diseased chordate; (B) Definitions of the crimp angle θ, crimp hight h, and crimp length l.

Achille tendon. In fact, the existence of crimp in fibrous collagen structure is well established. It is intuitively true that any heavy-duty tendon or ligament would have developed such a structure to absorb force. Only after the tendon is straightened, the collagen fiber/fascicles would experience force to unwind the helices.

Collagen crimps are developed to absorb tensile force and protect the host organism. To estimate the degree of such "protection", normally the parameters of crimp angle θ, crimp height h and crimp base length l are used in literature. The definitions of these terms are depicted in **Figure 6(B)** for convenience of discussion.

3. Degradation of Collagen by Metalloproteinases (MMPs) and Inhibition of Such Degradation by Tissue Inhibitors Metalloproteinases (TIMPs) in the CTIF Regions

We already know that collagens are the major structural proteins of connective

tissues such as skin, tendon, bone, cartilage, blood vessels and basement membranes of all organs, irrespective to their size. The scaffoldings of various types of collagen provide the most important communication network and integrity of these organs. The degradation of these collagen macromolecules, with various fibrous and non-fibrous structures, is naturally an integral part of many physiopathological processes such as embryogenesis, organ morphogenesis, tissue remodelling in tissue homeostasis, wound healing, angiogenesis, arthritis, atherosclerosis, aneurysm, fibrosis and carcinogenesis. A group of degrading enzymes, the matrix metalloproteinases (MMPs) feature to digest collagen molecules in various very specific ways in the above processes; on the other hand, inhibitors, called TIMPs regulate the degradation until a physiological balance is reached. Upsetting the balance either way could lead to important consequences—various kinds of diseases manifest themselves in certain CTIF domains of the fascia. We shall summarize below some characteristics of MMPs and TIMPs; much research on the balance of these classes of enzymes is going on.

3.1. Types of Digestive Enzymes for Degradation of Collagen in Humans

The triple-helical conformation makes interstitial collagens resistant to most proteinases because the MMPs, produced by many cell types such as epithelial cells, macrophages and other leukocytes, fibroblasts, marrow stromal cells have to bind to specific sites of the collagen macromolecules (for interaction) and these sites are "hidden" inside the triple helix structure (see evidence of collagen IV, as an example in [60]). Likewise, specific sites for MMP degradation are also hidden in the triple helix in general, an aspect mentioned in many papers on collagen degradation. In **Table 2**, we list some characteristics of the MMPs discussed in this paper. Briefly, MMP-1, 3, 8 are the endogenous digestive enzymes for the ECM collagen fibrils, and MMP-2 and -9 are more relevant to degrade basal membranes in the ECM of cell-groups/organs, whereas MMP-10 digests some important proteoglycans and some other collagen molecules, the types of which are yet to be confirmed. MMP-14, 15, 16 are anchored on the external side of the cell membrane in their dormant states. When activated by ECM proteinases, they can digest some collagen and non-collagenous molecules and regulate the activity of MMP-2, which is a powerful digestive enzyme to break the basal membrane of organ; for this reason, if these MT-MMPs are not regulated, they are involved in cancer invasion, as will be discussed in another paper.

3.2. Atomic Force Microscopy Investigation of the Action during Collagen II (Obtained from Cartilage, Rather than Tendon) Degradation by MMP-9, as an Example

Technically, by now, there is ample evidence that other members of the MMP family, cooperatively degrade a variety of extracellular proteins, including collagens which are resistant to other proteases due to their tightly packed structure

[71] [72]. As an example, native human collagen type-II can be cleaved by the collagenases MMP-1, MMP-8, and MMP-13. These MMPs will cleave all three α-chains of interstitial collagens by a single scission at a specific site, located 3/4 from the N terminal and 1/4 from the C terminal, leaving two fragments of the collagen molecule [73]. In fact, a more recent study [74] provides a direct visualization of MMP-9 action on the triple helical structure of intact collagen II molecule, using AFM observation. It is interesting to note that their single molecule imaging techniques provide direct evidence that during the initial state of the degradation process, the protease diffused laterally along the collagen type II fragment (to find and) to bind the relatively loose tail. During this process, the MMP-9 molecule changed its elongated conformation in the free state to a more globular conformation upon binding to the tail of the collagen fragment.

As the reaction progressed, and before collagen degradation, gelatin-like morphology arising from the denaturation of the triple helical collagen was observed [74]. Following the first cleavage, other MMPs, like MT1-MMP [75], MMP-1, MMP-8, MMP-13 (mainly gelatinases and stromelysins) can collectively further degrade the collagen fragments. Thus, the MMPs work collectively to cleave some collagen molecules, meaning that to inhibit "over-digestion" (such

Table 2. Characteristics of some relevant metalloproteinases (MMPs) and tissue inhibitors of MMPs discussed in this paper.

MMP member	Collagen types of the proteins being substrates	Remarks
MMP-1	I, II, III	when gene is mutated, member is associated with obstructive pulmonary disease (COPD) [61]
MMP-2	IV, V, elastin	when gene is mutated, member is associated with Winchester syndrome, Nodulosis-Arthropathy-Osteolysis (NAO) syndrome [62]
MMP-3	III, IV, IX, X	involved in wound healing, progression of atherosclerosis, tumor initiation [63]
MMP-8	I, II, III	involved in embryonic development, reproduction, tissue remodelling, arthritis, metastasis [64]
MMP-9	IV, V	animal studies suggest member is involved in tumor associated tissue remodelling [65]
MMP-10	fibronectin, elastin, proteoglycan core protein, laminin and some collagen types to be confirmed	involved in tissue remodelling, arthritis, metastasis [66]
MMP-13	I, II, III	involved in articular cartilage turnover and its pathology [67]
MMP-14	I, II, III, fibronectin, vitronectin	also known as membrane type MMP (MT-MMP); expressed at the cell membrane and is activated when cleaved by ECM proteinase. This member also activates MMP-2 and is suggested to be involved in tumor invasion [68] [69]
MMP-15	ProMMP-2, fibronectin, laminin, very small amount of I	this member is also an MT-MMP; suggested to be involved in tissue remodelling, development, reproduction, arthritis and metastasis [69] [70]
MMP-16	III, ProMMP-2, gelatin, fibronectin, laminin	this member is also an MT-MMP; suggested to be also involved in tissue remodelling, development, reproduction, arthritis and metastasis [69]

as during cancer invasion), we might need to break off one of the processes in the progressive cleavage phenomenon.

Normally, the proteolytic activities of MMPs are regulated precisely by endogenous tissue inhibitors of metalloproteinases (TIMPs). Recent research in the past few years have shed light on the complexity of the functions and activities of these MMP and MT-MMP as they can be "re-absorbed" back into the cells that synthesize them, and the processes are not fully understood up to now.

3.3. Another Example: Two Domains in MMP-1 Work Together to Cleave Collagen II in Three Steps

MMP-1 consists of an N-terminal catalytic (Cat) domain containing an active-site zinc ion and a C-terminal hemopexin (Hpx, the plasma protein with the highest binding affinity to heme and is mainly expressed in liver) domain comprised of a four-bladed β-propeller, which are connected by a "linker region" [76]. Although the Cat domain can cleave some non-collagenous proteins plus heat-denatured collagen (gelatin), its activity on native triple helical collagen is negligible. However, experimentally, the combination of the Cat and Hpx domains is required for MMP-1 to degrade native collagen. The statement holds true also for MMP-2, MMP-8, MMP-13, and MMP-14 [77]. It has been demonstrated that MMP-1 first unwinds triple-helical collagen locally before peptide bond hydrolysis. Employing combined biochemical experiments and crystallographic structure analysis (*i.e.* screening of a triple-helical peptide library of collagen II with MMP-1, (see Fig.2 of [78]) the authors of [78] discovered that extensive interaction of all three chains (of a collagen II triple helix) with the two stated domains of MMP-1 was required for collagenolysis in three steps: (i) positioning of the scissile bond (*i.e.* a covalent chemical bond that could be broken by the MMP enzyme) near the active site; (ii) locally unwinding the triple helix; (iii) chemical interaction of the enzymatic action; (iv) the Cat and Hpx domains worked cooperatively in a temperature-dependent manner, up to 37˚C for degrading collagen II, and the interaction (of degradation process) decreased sharply, indicating that MMP-1 prefered a looser triple helix but not denatured collagen. This result is very interesting, as it implies, at least for collagen II, that degradation is insignificant above core body temperature. Above core body temperature, there are supposedly more triple helices being denatured (gelatin-like). We interpret such a result as: at least for collagen II, MMP-1 degrades the injured triple helices below and up to body temperature. Since the fibroblasts would synthesize collagen molecules as explained in previous sections, a natural healing process is at work if the activities of MMPs are being regulated. The very subtle problem on folding and refolding of triple helices will be followed up in Sections (5) and (8.5).

3.4. There Are Only Four Types of Endogenous Inhibitors of MMPs Found So Far to Regulate the Digestion of Collagen in Humans

On the other hand, the proteolysis of the collagen molecules, whatever their

forms, must be regulated. So far, four members of the tissue inhibitors of metalloproteinases (TIMPs) have been identified. The N-terminal domain of a TIMP interacts with the active site of the associated MMP, resulting in inhibition of catalytic activity. Table 3 presents some characteristics of these TIMPs only, because such inhibitors participate in many other functions, and are candidates of rather intensive research, and they have been applied to treat various cancers. Both Table 2 and Table 3 are by no means comprehensive but show special features of those versatile proteins.

4. Probing the Reasons for Tendons Having the Ability to Sustain Lengthy Periods of Mechanical Tensile Loading

Tendons in many parts of the body are prone to sustain heavy mechanical loading duties, though we have learned that the crimp structure can absorb some tensile loading in Section (2.7). Clinically, there are many cases of spontaneous tendons ruptures, without alarming pre-rupture symptoms [94]. To prevent tendon rupture and to design treatments when rupture does occur, it is important to understand the physiological process of tendons' response to tensile loading. The following sub-sections serve this purpose.

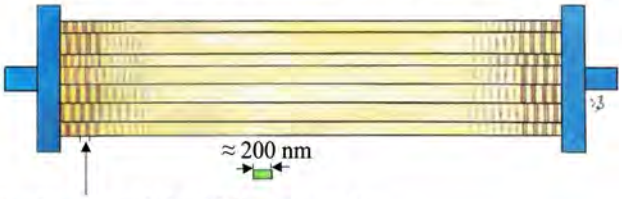
4.1. Distribution of Kinks in Overload Bovine Tail Tendon

Specimens of tendons from the tails of steers were employed to analyze the response of the collagen fibrils to tensile force in [95]. The bundle of collagen fibrils was anchored in the manner specified in Figure 2 of [95]. We show schematically in Figure 7(A) such a bundle following their SEM micrograph—without showing crimps in the length scale of the experiment; it is difficult to distinguish the difference between fibrils and fibers. Collagen tubes having diameters within a range of a few hundreds of nm are called fibrils by many authors—e.g. authors of [95]. We follow such nomenclature here. Tensile force

Table 3. Some characteristics of tissue inhibitors of metalloproteinases (TIMP).

TIMP member	Source of TIMP member	Types of MMPs inhibited	Remarks
TIMP-1	human skin [79] fibroblast; tendons [80]; many other cells	most of the known MMPs	in addition to tissue remodelling, it promotes proliferation of a wide range of cells; it has anti-apoptotic function; involved in pancreatic cancer, melanoma, and glioblastoma [81]
TIMP-2	endothelial and other cells [82]	forms a complex with collagen IV, but inhibits also other MMPs in tissues	suppresses proliferation of endothelial cells; involved in cardiac fibroblast ECM remodelling; osteoarthritis in Chinese Han population [83]
TIMP-3	gingival fibroblasts [84]; intestinal epithelial cells [85]; liver cells plus other cells	most of the dominant MMPs in tissue	mutation of this gene is associated with Sorsby's fundus dystrophy; after myocardiac infarction, over expression of this member promotes angiogenesis; on the other hand, it inhibits vascular endothelial factor (VEGF)—mediated angiogenesis [86] [87]; prevent fatty liver and carcinoma [88]
TIMP-4	brain cells, fat cells [89]	anticipated to inhibit a number of MMPs but results are not yet confirmed	regulates carcinogenesis through enriching tumor progenitor cervical cancer cells [90]; triggers apoptosis in cervical cancer cells [91]; related to focal epilepsy in Malaysian Chinese [92]; involved in human gliomas [93]

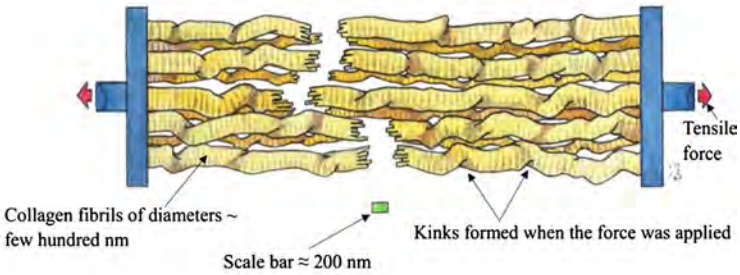
(A) Before tensile overload was applied, the fibrils of bovine tail tendon under SEM examination showed clear D bands in ref. [95]



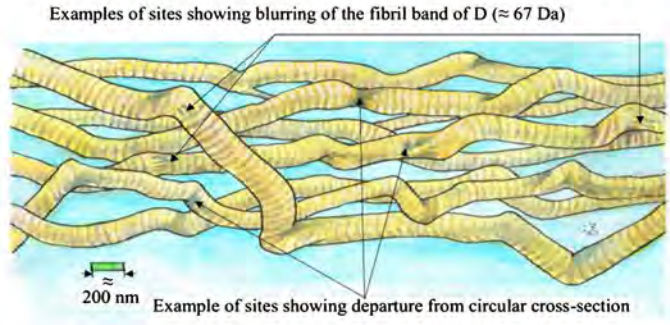
D bands are revealed in SEM micrographs of the tendon fibrils published in ref. [95]

(B) Interpretation of the experiment of reference [95]

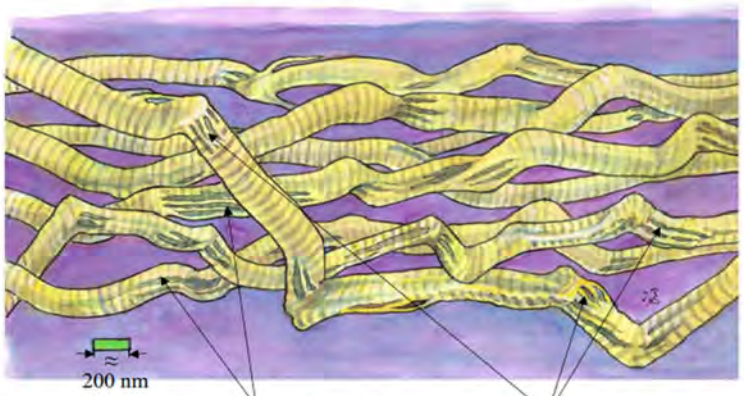
rupture of the tendon specimen by tensile force



(C) After the application of tensile force which caused rupture eventually there were changes in structure of the collagen fibrils according to the micrograph of [95]



(D) Interpretation of the structure of the ruptured fibrils after digestion of the denatured collagen molecules according to the micrograph of [95]



Example sites showing damage of the circular cross-section of fibrils, due to denaturing of tropocollagen molecules directly by mechanical force

Sites showing sub-fibrils with diameters ~ a few tens of nm

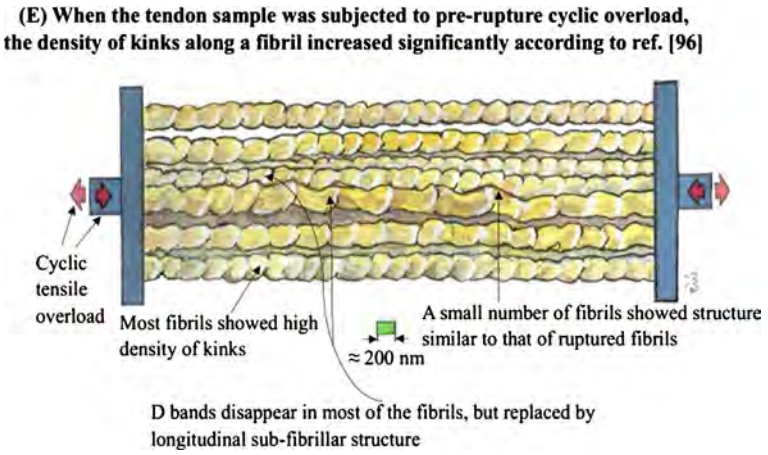


Figure 7. (A) Schematic representation of the bovine tail tendon specimen of [95] before overload tensile force was applied. The D bands showed up clearly in the collagen fibrils. The rough scale is for the effective diameters (ranging around one to three nm roughly) of the fibrils only, as the length of the sample is 1.2 mm, much longer than that shown in the painting. (B) Overload tensile force was applied at both ends of the tendon specimen of (A), causing it to rupture, and kinks were found along the collagen fibrils as shown in the SEM micrograph in Figure 3(A) of [95]. (B) was a hand-painted interpretation of such kinks in the gauge length of the fibrils. In their experiment, about half of the whole cross-section of the tendon was ruptured. (C) SEM micrographs of [95] indicate that at the kinks of the ruptured tendon fibrils, the cross-sections became irregular in shape, and the D bands became a little blurred. Instead, longitudinal small tubes appeared; these tubes are obviously sub-fibrils. However, other parts of the fibrils still showed clear D bands. This hand-painted picture is interpreted based on Figure 3(C) of [95]. (D) Hand-painted interpretation of the SEM micrograph of Figure 3(D) in [95], showing the sub-fibrils with effective diameters around a few tens of nm after the specimen was treated with digestive enzyme which dissolved the uncoiled/damaged triple helices. Many portions of the fibrils showed irregular cross-sectional shape. (E) Now refer to the work of reference [96]. After 5 cyclic tensile overloaded with strength below rupture as described in the text, a hand-painted, simplified interpretation of Figure (1B) of [96] is presented here. Special features of the damaged tendon are described in the text. The pictures were painted by author PCWF.

was then applied to the tendon specimen until rupture occurred. The SEM mircrographs indicate that many kinks occurred along the ruptured fibrils. The distance between two consecutive kinks along a fibril was reported to vary between about 300 to 800 nm, though some samples showed values up to 1100 nm according to their **Figure 5**. Interpretation (of Figure 2 of [95]) of the present authors on the rupturing process is shown schematically in **Figure 7(B)** by a painting. The two red arrows indicated that tensile forces were applied at both ends of the sample. Kinks formed along fibrils before rupture, after tensile force was applied. The green bar scale is about 200 nm on the vertical scale, as the length of the specimen is out of the scale here. **Figure 7(C)** is the simplified interpretation of the present authors with a painting, based on their SEM micrograph (*i.e.* Figure 2 of [95]), highlighting the few main features only. In **Figure 7(C)**, while kinks were formed, there were sites indicative of "blurring of the D-band of 67 Dalton"; a few are marked in this figure. At these sites, tubes with

diameters around a few tens of nm appeared vaguely, indicative of sub-fibrillar structure. There are also many regions along the fibrils suggesting that the "close to circular" cross-sections of the fibrils were altered. In their original experiment, the digestive enzyme trysin (an enzyme stronger than the human MMPs) was applied to the ruptured fibrils specimens, dissolving the denatured tropocollagen molecules. We should remark that denature is defined here to be uncoiling of the triple helical structure of a tropocollagen molecule. Note that the fibrils have a range of sizes, depending on the anatomical sites of the body. In order just to point out only the main features, we present a picture in **Figure 7(D)** to represent the structure of the ruptured fibrils after the denatured triple helices have been digested by the degrading enzyme based on the SEM micrographs published in [95]. We highlight the clear appearance of the sub-fibrils with diameters around a few tens of nm after the specimen is treated with digestive enzyme. Many portions of the fibrils showed irregular cross-sectional shape, because quite a number of sub-fibrils were uncoiled during the rupturing process. We would like to emphasize that despite the fibrils being ruptured, the appearance of the D bands between kinks of the fragments implies that the ordered molecular packing of micro-fibrils in the fragments of the fibrils was still retained. This property is important. Following up, the researchers applied a cyclic overload tensile force to similar bovine tail tendon specimens [96]. Before proceeding, the displacement (effectively the extension) versus load graph was plotted, in order to find out at what load the sample would rupture. When the data points reached a plateau region, further increase of load would not cause any extension, and the sample would rupture. The rupture force was found to be 25 Newton. In the "cyclic experiment", a similar sample was stretched using a strain rate of 0.5% per second. Before rupture occurred, the force was decreased at the same strain rate of −0.5% per second, until the tendon resumed its original length. After 5 sub-rupture overload cycles (pertaining to the SEM micrograph discussed; 5 - 15 cycles of overloading were carried out in other parts of their experiment), the specimen was examined by SEM as before. Based on the SEM micrographs published in [96], the authors present here a painting of the structure of the tendon after overloading below the rupture threshold in **Figure 7(E)**. Note that (a) Only a small number of fibrils show a structure similar to that of the ruptured fibrils (*i.e.* **Figure 7(C)**), with smaller kinks. (b) The D bands disappeared in most of the fibrils, and longitudinal sub-fibrillar structure appearred instead. (c) Most fibrils demonstrated high density of kinks (with distance between consecutive kinks around 200-400 nm). (d) In some parts of overloaded fibrils, D bands still appeared in the regions between kinks, again implying that ordered molecular packing of micro-fibrils in these regions was retained. Interpretations (a)-(d) of the present authors are marked in **Figure 7(E)**. After digestive action of trysin, the micrographs of [96] show that the outer layers of the fibrils were dissolved, leaving fibrils with smaller diameters, with some sub-fibrillar "fragments" attached to the host fibril. These smaller fibrils showed the D bands again, as expected, because they were triple helices with definite order of ar-

rangement. While in real life, the fibrils would remain stable, many of the damaged fibrils should be dissolved and repaired by new tropocollagen molecules, if the tendon is to keep its physiological state. As emphasized in [95], the damaged sites were spread out so that no crowding of big kinks occurred in a small spatial region. The failure to sustain the strength of the fibril group, *i.e.* the tendon, occurred in many parts which were spaced apart. This is an important aspect, enabling the tendon to endure tensile forces within a rather wide range. In fact, the rupture process is like the rupture of a rope [97] in our daily life during a tug-of-war game. We would emphasize that the tendons of the tail of a bovine is not normally subjected to strong tensile force. Tendons in some other parts of the body, e.g. the Achille tendons of the ankle, would have to endure strong tensile force in exercise. Nature has allowed this to happen by synthesizing fibrils with crimp structure in many of our tendons. This important aspect has already been mentioned in Section (2.7). Note that the loaded Collagen I fibrils as elements of the ECM would promote cell adhesion, accompanied with reorganization of the actin cytoskeleton inside the fibroblasts. The fibroblasts, therefore, are in their active states, stretching out to form a network-like structure among themselves; repair work can be carried out readily [3] [98].

4.2. Evidence of Ruptured Human Achille Tendons Have Fewer Number of Larger Fibrils, Smaller Crimp Angle, and the Occurrence of Disrupted Crimp Continuity

Achille tendon tissues were obtained at the central core/central plus the posterior peripheral/distal superficial as well as the proximally intact (proximal superficial) part of the of the Achille tendons of subjects who suffered from complete rupture core [47]. These specimens of Achilles tendons were taken during routine forensic autopsy. The cross-sectional area density and diameter distribution of fibrils were analyzed using stereological techniques of digitized SEM biopsy cross-sections. It is interesting to note that the "fibrils" are polygons, and some displayed "quasi-hexagonal" shape or even rectangular shape. We notice that sizes of these fibrils cover a wide range. Notice also that the fibrils are separated—the space is occupied by the interstitial fluid and some non-collagenous proteins.

We present the finding of the SEM micrograph in Fig.1(a) of [47] by a schematic painting in **Figure 8**. As the collagen fibrils are liquid crystals, it is not difficult to understand that such orderly geometrical shapes appear, as briefly discussed in Section (2.7) with **Figure 4**.

Clearly, there is variation of the cross-sectional areas of the fibrils. The "diameter", strictly speaking, can only be defined as the "effective circular diameter" of the fibril/fiber. The magnitude of the effective diameter is in the range of 250 nm down to 10 - 20 nm. Revelation of such patterns of the cross-sections of fibers/fibrils is not surprising, as there are different possible ways of forming the micro-fibril and bigger basic regular collagen units (Section (2.6)). There is clear evidence, using optical interferometry measurement, that liquid crystalline

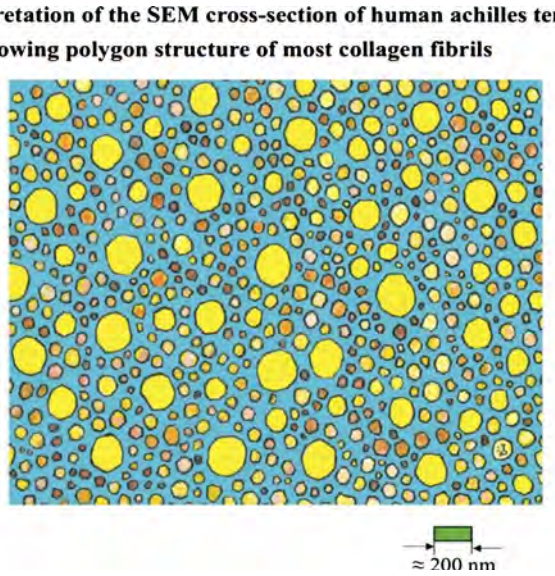


Figure 8. Schematic representation of the scanning electron microscopy result of the cross-section of a typical Achille tendon of ten subjects under study reported in Fig.1 of [47]. We observe that most of the cross-sections of the collagen fibrils have polygonal shapes of various types. See also **Figure 4** of the present paper. The scale bar is painted in green. The painting was done by PCWF.

mesophases exist in living organisms [49]. Later, other studies also indicate that collagen molecules (and other polypeptides of living organisms) behave like liquid crystals with self-assembly ability [50] [99] [100] [101]; they can assemble into regular geometrical shapes. The longitudinal, tube-like structure is maintained by the special tropocollagen molecules with "quarter-staggered" pattern (Section (2.3)), a well-established aspect in collagen studies. By analyzing the distribution of the effective diameters of these fibers/fibrils, we observe from Figure 2(a) of [47], that there were clearly fewer comparative larger fibers (effective diameter in the range from 70 to 190 nm) in the ruptured tendon. We show the schematic representation of their discovery in **Figure 9**, where the first graph (histogram) of Fig.1(a) of [47] is approximated by two continuous functions (solid line for the intact tendon, and dotted line for the ruptured tendon); the vertical axis gives the number of fibrils per micrometer squared and the horizontal axis indicates the effective averaged diameter as defined above. It is sufficient to show qualitatively that the ruptured Achille tendons of the 10 subjects had fewer relatively large fibrils (represented by the deep red region), and had a higher number of smaller fibrils (represented by the pink region) as compared to the intact tendon.

Since the variation of the crimp angle plays a role on the absorption of force, it was also measured in [47]. In short, the periodic banding pattern of collagen fibers was revealed to be changed when it was rotated between crossed optical polaroids. When the transmission direction of a fiber coincided with one of the polarizers, a dark extinction band became visible. When the sample was rotated with respect to the polaroids, the extinction band moved continuously along the

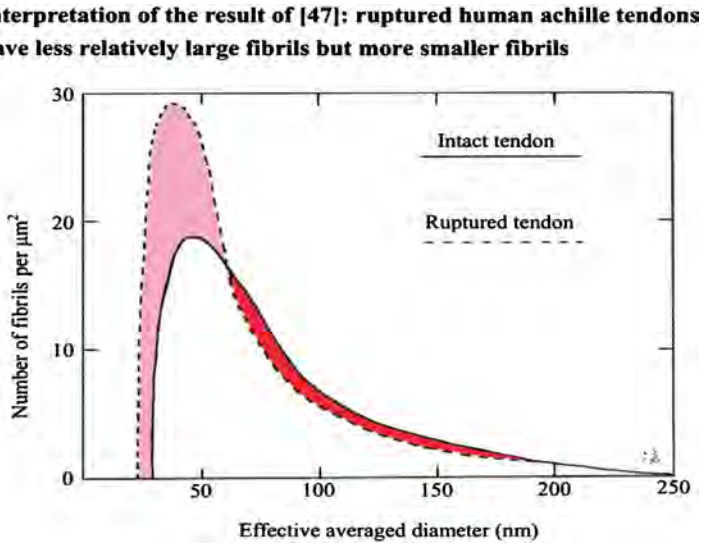


Figure 9. A rough estimation of the variation of the number of fibrils per micro-meter squared with respect to variation of effective averaged diameters in the distal core region of the tendon samples, where the histograms are approximated by two continuous functions here—solid line for the intact Achille tendon and dotted line for the ruptured tendon, according to the data of [47]. The ruptured tendon has fewer relative large fibrils (read portion) and more smaller fibrils (pink portion). The graph was painted by PCWF.

tendon; from the data on the variation of the extinction band, the crimp angle could be calculated according to a method established long ago [102]. The averaged crimp angle was found to be smaller in the ruptured tendons [47].

In another study, 66 spontaneously human ruptured tendons, including those in Achilles, quadriceps, biceps brachii and extensor pollicis longus, were studied. Even though the size distribution of the crimp angle and effective averaged diameters varied a lot in these samples, the measured effective averaged diameter (using SEM) and crimp angle were found to be smaller in the localized raptured sites, showing also disrupted crimp continuity [103].

4.3. Evidence of Tendons under a Certain Degree of Mechanical Tension Resist Degradation by MMPs

Types I, II, III, V and XI form fibrils, fibers which are the predominant tensile load-bearing proteins in the connective tissue of various parts of the body such as skin, blood vessels, corneal stroma, tendon, ligaments, cartilage, intervertebral disc and bone. The load-bearing fibrils persist to adapt to applied strain during daily life, including the early growth period. During adaptive matrix remodelling under load, mechanically-activated fibroblasts synthesize both procollagen and MMPs; the detailed mechanism of removal of (damaged) collagen and repairing them by the new collagen molecules is still under research, but we know that such repair must have happened. Also, there is evidence to suggest that the collagen molecules are stabilized by mechanical strain against both thermal denaturation [104] and enzymatic degradation [105] [106] [107]. We will review briefly below the work of [108], relevant to proceeding with our analysis.

A "differential interference contrast (DIC)/edge detector" has been built to take optical image of the kinetics and pattern of the samples of collagen micro-networks during enzymatic degradation (see Figure 1 of [108]). A pepsin-extracted, commercial bovine sclera type I sample of atelo-collagen monomers (the outer collagen layer that protects the eye) at a concentration of 3.0 mg ml^{-1} was obtained commercially for the experiment. The collagen specimen was strained gently between micro-pipettes; the intensity of the CID recording indicated the strained sample was significantly persistent to degradation by the established bacterial enzyme Clostridium histolyticum as compared to the unstrained one.

Following up, the same CID technique was applied to measure the resistance of reconstituted bovine type I collagen (at Ph 7.4) to digestion by MMP8, which is rather specific to degradation of collagen I [109]. In more detail, there is evidence that MMP-8 cleaves the native type I collagen triple helix preferentially at a site located between Gly775 and Ile776 [110]. This experiment is one step ahead (of those using bacterial degradation enzymes) because MMP-8 is physiological encountered.

Intuition tells us that MMPs cannot cleave all three alpha chains simultaneously due to size restrictions at the catalytic cleft. With the above background, we proceed to review briefly the experiment of [111], leading to hysteresis loops discussed in many papers. A single fibril mechanochemical erosion assay with nN force resolution was developed to detect the loss of a few layers of monomer from the surface of single native bovine sclera collagen specimen. The specimens were subjected to zero or finite tensile loads. When the unstrained specimens were subjected to digestion by Clostridium histolyticum collagenase A, they were degraded rapidly in 20 min. When a tensile force of 1.8 pN/monomer was applied, the fibrils were degraded in a longer time of 35 - 55 min. Moreover, if the load was increased to 23.9 pN/monomer, the fibrils were not degraded at all. The enzymatic degradation of collagen follows two steps; (i) binding; (ii) cleavage. This process as a whole is called the "Michaelis-Menten reaction process", discovered long ago [112]. The amount of monomer being cleaved is estimated by the binding and cleavage rates. For insoluble monomers, as in this case, only the cleavage rate k_c is nonzero. Analysing the decrease of diameter of the monomer in time, the strain-stress relation, the k_c values, the authors concluded that the result strongly supports the hypothesis of "mechanical tension stabilizes collagen monomers against enzymatic degradation" [111] [113]. The action of such degradation was called a mechanochemical switch. Therefore, there is evidence that the collagen monomer has an intrinsic protection against enzymatic digestion. Using the data of [111], incorporating mathematical model analysis (which is omitted here) the effective diameter (of the collagen monomer) versus time graphs for the zero load, a load of 46 nN, and a large load of 1054 nN was plotted with discrete points in [114]. To show the idea of their discoveries, in **Figure 10** we show three lines of best fit (roughly) based on the data presented in Fig. 2 of [114].

Such data suggest that tension may generally pull the collagen alpha chains into a tighter apposition, limiting enzyme access all along the molecule.

In view of the experimental investigations analysed above, we consider that the healthy tendons, ligaments, skin collagen fibrils/fibers, cartilages, bones must be under certain, but various degrees of tension to sustain homeostasis, avoiding over degradation. It will be fruitful if there is a way in future to quantify the tensions needed for each collagen structure of a subject.

4.4. While Repair Work of Degradation and Neo-Synthesis of Tropocollagen Is Going on Through Life, Cores of Tendons Are Not Turned over Since the Age of 17, Based on Radioactive Examination of Specimens of Humans during the Period of Atomic Bob Tests

From the rupture and pre-rupture overload experiments on bovine tail tendon discussed in Section (4.1), we have learned that when the tail tendon was overused, the damaged sites spread over a vast domain all over the tendon. If the tensile force is below the threshold of rupture, after degradation by a strong bacterial degrading enzyme to digest the damaged fragments, the tendon is still intact (see Figures 7(A)-(D)). In a human body, the digestive enzymes (MMP-1, 3, 8) employed to degrade collagen I are not so strong as the bacterial enzyme. Moreover, there are stringent conditions under which collagen fibrils can be degraded— the enzyme has to interact to a full length to detect the site of interaction; the enzyme has to unfold the deformed portion (*i.e.* the kinks) of the fibrils, and then chemical reaction takes place to degrade. There is evidence, as presented in Section (4.3) with Figure 10 that when a tendon is under stretched,

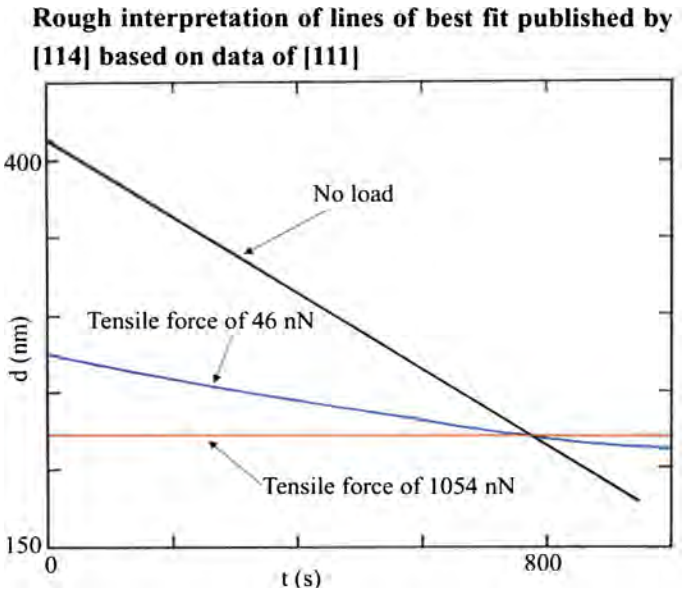


Figure 10. Effective diameter d (nm) of collagen monomer versus time (s). Three lines of best fit are shown, based on the data presented in Fig.2 in [114] with reference to the data obtained in [111]. The red line with a longer time scale substantiates the previous result presented in Fig.4 of [111].

the activation site of degradation is "hidden" inside so that tension on the tendon actually protects it from degradation. Moreover, in the experiments reported in [95] and [96], the repair process of synthesis of new collagen was excluded. In real life, when a tendon is stretched, the fibroblasts are activated, with their pseudopodia stretched out. Procollagen molecules are synthezised and their exocytosis would bring them to an extra-cellular domain where they are trimmed at both ends by enzymes as described in **Figure 3**. The resulting tropocollagen molecules can self-assemble and repair the damaged regions. During wound healing, collagen III molecules are produced first and are replaced by collagen I molecules on completion of healing [115]. The tendons are very heavy-duty structures of the body. It might be expected, at first thought, that tendon turnover should be fast to enable the tendons to carry out their heavy duties. However, measurement/estimates of the lives of tendons of the past few decades give rather controversial results: a wide range from 2 months to 200 years have been reported [116]. In order to give a precise answer to the turnover time accurately, it was noted that there were nuclear bomb tests in the time period 1955-1963, releasing the isotope C^{14} which can be distinguished from the common isotope of $C^{12,}$ according to accelerator mass spectrometry (AMS) analysis. Levels of C^{14} were measured using AMS in 28 forensic samples of Achilles tendon core and 4 skeletal muscle samples (donors were born in the period 1945-1983). Such investigation indicates that the tendon core is formed at about 17 years of age (during height growth) and is essentially not renewed thereafter. The accurate experimental result explains why Achille tendon tear is so common and difficult to treat. For similar results, the diseases of other tendons, due to overuse in many cases, are also difficult to treat clinically, an issue to be followed up in this paper.

5. On the Controversial Issue of Thermal Stability of Collagen Triple Helices below and above Body Temperature

There have been controversial results on the thermal stability of collagen triple helices for decades. It started off in the work [117] in which collagen samples from human lungs were heated and differential scanning calorimetry as well as circular dichroism experiments were conducted on the samples. Their analysis led to the conclusion that collagen fibrils are unstable even at body temperature. Note that the magnitude of the heating rate is one decisive factor, and debates went on for over one decade. We will not review the pros and cons but describe two sets of controversial results below and draw our conclusion based on common experience in daily life, together with the ability of self-assembly of collagen molecules in the discussion section.

5.1. Newly Synthesized Peptide Chains of Collagen I in Chick Tendon Fibrobasts Fold Faster to Form Triple Helices as Temperature Was Increased, Up to 40˚C

Chick embryo metatarsal tendon cells were cultured and collagen I peptides were synthesized as a natural process [118]. These cells were pulse-labelled for 4

- 10 min. with ^{14}C proline which is the amino acid that would preferentially label collagen, so that the synthesized collagen peptides would contain radioactive ^{14}C. As the synthesis of the peptide begins with the N-terminal, and the labelling occurred in time series, the young peptides were labelled near the N-terminals, whereas the oldest peptides were labelled near the C-terminals. The peptides with intermediate ages were labelled throughout sites of the chain. The experiment was carried out at different temperature steps. Once the peptides were synthesized, they began to fold. Note that the chromotography technique was used in order that the constituents (folded helices and peptides/loose coils) could be separated due to difference of their mobilities. In that way, the relative amounts of folded helices and procollagen peptides were determined at specific temperature points.

Based on the briefly reviewed experimental steps, the authors demonstrated that the rate of triple-helix formation increased with increasing temperature. Roughly, at 33˚C, it took 14 min. for the newly synthesized peptides to form triple helices; it took only 6 min. for such folding at 40˚C. The fast folding rate was explained by the occurrence of a series of cis-tran isomerization of peptide bonds; in short, such a process means a rotation of the interactive part of the peptide (from a "cis" orientation to a "tran" direction) favorable to the elongation of the folding, governed by chemical force. It was also found that the triple helices resisted the action of collagen digestive enzyme within the temperature range studied.

It was noted that the temperature-dependence of rates of proteins synthesis, being conserved during evolution, are similar in all organisms known so far; such rates are characterized by an approximate activation energy of 125 kJ/mol. Taking that value to estimate the rate of elongation/production of procollagen molecules at 37˚C, a general equation expressing the time of elongation as a function of temperature was derived. The derived results on kinetics was found to be consistent to the time of folding found experimentally.

In a follow-up study along the line of [118], it was noted that (cyclosporin A)-bind protein (cyclophilin) is chemically identical to peptidyl-prolyl cis-trans-isomerase. Using chick leg tenocytes as specimens, it was demonstrated that cyclosporin A would slow down collagen triple helix formation, as an indirect support for the cis-trans-isomerization process for folding mentioned above [119].

5.2. Investigation on Folding of Procollagen Derived from Human Skin, with the N-Terminal Cleaved, to Form Fibrils in the Temperature Range of 29˚C - 41˚C

^{14}C-labelled type I procollagen was purified from cultured fibroblasts of human skin in [120]. We have noted in **Figure 2** that the procollagen includes loosely-structured N terminal (about 15 nm) and C terminal (about 10 nm). The typical sample was digested by N-proteinase, leaving a specimen called pC-collagen with the C-terminal still attached. In our terminology, it is the intermediate form from the propagation from procollagen to tropocollagen (**Figure 2** and **Figure**

3) with one "surplus C-terminal". The pC-collagen specimen and the C-proteinase solution was heated in the temperature range of 29˚C-41˚C. When the C-terminal was cleaved, the pC-collagen became a tropocollagen (**Figure 3**). Tropocollagen molecules, once formed, would fold and form triple helices, and the solution would become turbid. The amount of turbidity, representing the amount of folded collagen, was measured by recording the absorbance of light at a wave length of 313 nm by a spectrophotometer fitted with a temperature recorder. Fluorography (or photofluorography) is the technique of photographing an image produced by light emitted from a fluorescent screen on collagen solution. Light is produced by the excitation of a fluorescent material by ionizing radiation which is produced when charged particles (electrons from the ^{14}C-labelled collagen) can interact with the fluorescent material. In the experiment, the amount of soluble collagen ("unfolded collagen") was assayed by the stated technique of "fluorography". In fact, the fluorograms revealed the α_1(I) band, confirming the identity of the soluble collagen I in the buffer. The (supposed) collagen fibrils were centrifuged out and the effective diameters of the fibrils were measured using specimens from SEM examination (to justify that the folded protein molecules were triple helices) and the authors reported (without showing micrographs) that the effective diameter was inversely proportional to temperature. Based on the turbidity measurement as a function of temperature, Fig.3 of [120] shows effectively that the folding of tropocollagen molecules to form triple helices increased with temperature until 37˚C. This result contrasts that using collagen III as reviewed briefly in Section (5.3) below. At a higher temperature range of 37˚C - 41˚C, the assembly rate decreased. The authors interpreted that result is due to the formation of micro-unfolded monomer states, *i.e.*, a collagen monomer has some portions of unfolded α chains along the monomer. It was also noted that fibronectin binded readily to denatured collagen, and that the resulting unfolded collagen chains would have a better chance of self-assembling to form fibrils according to the relevant authors in their later paper, in line with an earlier study of [121]. We interpret that the rate of triple helices assembled is proportional to the degree of turbidity in the experiment of [120], though the relationship may not be linear. To show the basic idea qualitatively, we present the schematic representation of the rate of tropocollagen folding versus temperature in **Figure 11**, showing positive slope up to body temperature, and negative slope when T was higher than 37˚C.

This result is supporting the experiment described in Section (5.1), contrasting to another typical *in vitro* experimental result reviewed below.

5.3. Heating Bovine Fetal Skin beyond Body Temperature Will Cause Denaturing of Triple Helical Collagen Molecules, but the Resulting Molecules Will Refold during Cooling to Resume Their Intrinsic Helical Structure—An Example of Results Contrasting that Presented in 5.1 and 5.2

The skin is built up of molecules of collagen I and III, and collagen I was studied

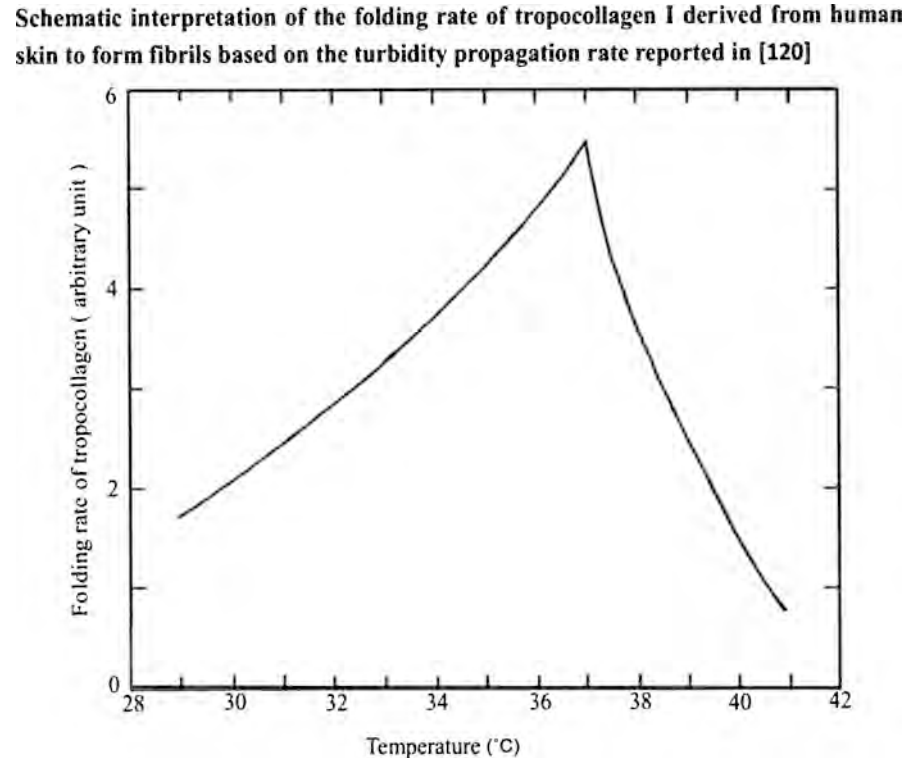


Figure 11. ^{14}C-labelled type I procollagen was purified from cultured fibroblasts of human skin [120]. The typical sample was first digested by N-proteinase, leaving a specimen called pC-collagen with the C-terminal attached. The specimen was put in buffer composition and temperature close to the physiological conditions with the presence of C-proteinase enzyme, so that the pC-collagen molecules became tropocollagen molecules in our terminology (see **Figure 2** and **Figure 3**). We plot the schematic representation of the rate of tropocollagen folding versus temperature in the above figure based on data of [120], obtained by measuring the turbidity as described in the text. Notice that the slope is positive up to body temperature.

rather extensively. Some groups therefore used collagen III to investigate the thermal stability problem.

A tropocollagen III of bovine, like that of human, consists of three identical α_1(III) chains containing 342 tripeptide units or 1026 amino acids each, extending to a length of about 300 nm (see **Figure 3**).

Type III collagen contains interchain disulfide bonds in its mature processed form, holding the chains at the carboxyl-terminal end. Some pioneering works were done in the 1980s for the stated purpose, applying the Circular Dichroism (CD) or Optical Rotatory Dispersion technique (ORD) [122] [123] to measure the fraction of collagen molecules to be in the helical form. We will leave out the technicality details, and review very briefly a more recent experiment below.

In [124], bovine fetal skin was extracted and denatured by heating at a rate of 1˚C per hour, to a temperature of 45˚C. The sample was then cooled and the (ORD) method was used to measure the fraction of the protein sample that was in the α-helix conformation. The reading of the ORD signal was found to remain stable at the points indicated by the triangles in the original Figure 1 of [124].

We roughly estimate the five temperature points to be at 39˚C, 35.5˚C, 34.5˚C, 33.5˚C and 25˚C. In Figure 12 we followed their heating curve and joined the stated five points in the cooling part, obtaining a hysteresis loop (painted with deep orange color). We interpret the appearance of this loop using the energy concept. In fact, we can draw an analogy to the action of a piston in a car engine. During one cycle of the piston action, the pressure of the gas inside the cylinder compresses and expands in cycles (by the chemical reaction of the burning fuel) and the volume of the cylinder changes from zero to a maximum as limited by the structure. When the piston resumes its original volume, some energy is wasted in friction. This loss of energy also happens in the refolding of the collagen molecules. The larger the area enclosed by the hysteresis loop, the more energy is wasted/stored in refolding, and in general, more time is needed to refold. There are similar results published showing hysteresis loops, but we shall not repeat to review these works. The key issue we point out is that, in these sets of experiment, as temperature increases, the folding rate is not increasing up to body temperature, as found by experiments stated in Sections (5.1) and (5.2); rather, it is negative (unfolding) all the way to the highest temperature designed for the experiment (in Figure 12, it is 45˚C). The hysteresis area is not zero, meaning energy is wasted/stored in refolding and it takes hours for the refolding process to complete during cooling in results where time period was recorded. The contradictory issue, which is very important in medicine, will be followed up in the discussion section.

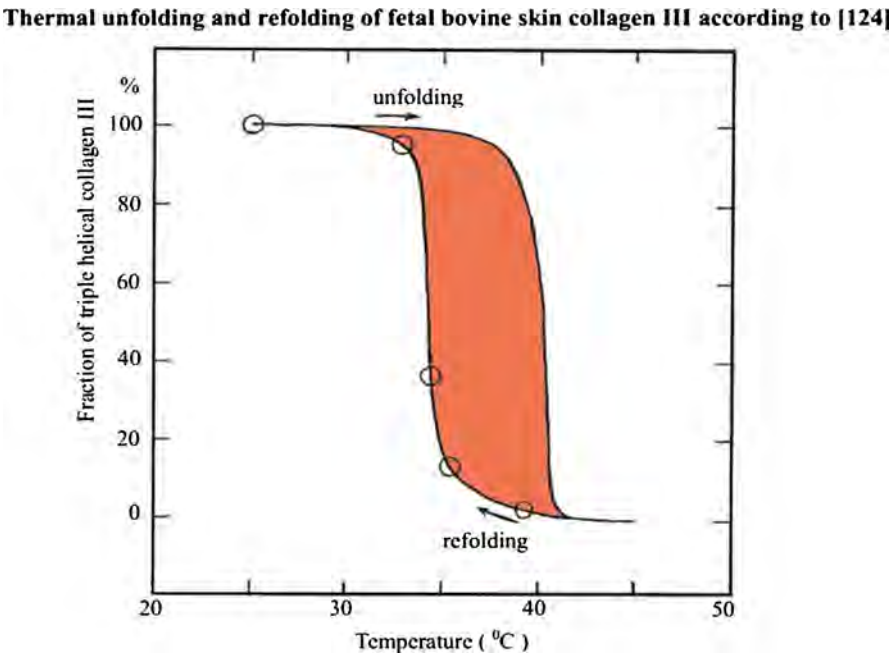


Figure 12. Bovine fetal skin was extracted and denatured by heating at a rate of 1˚C per hour, to a temperature of 45˚C. The sample was then cooled and the ORD method was used to measure the fraction of the protein sample that was in the α-helix conformation. We roughly estimate the data of Fig.1 in [124], and plot their result, showing a hysteresis loop in the fraction of triple helix III-temperature plot.

6. The Presence of Homotrimers in Collagen Types I, II and III and Related Diseases

MMP degradation of damaged domains of collagen molecules is a physiological process, leading to tissue remodeling, homeostasis, and to allow for wound repair. The basic unit, *i.e.*, tropocollagen of collagen I which is the most abundant type in humans, consists of two α_1(I) chains and one α_2(I) chain (**Table 1**). To digest a portion of a collagen fibril, the MMP first unwinds the collagenous cleavage site, so that the individual α chains can be placed (by chemical force) inside the catalytic cleft of the enzyme. The unwinding process is rate limiting. The presence of the α_2(I) chain is to allow efficient unwinding for digestion to take place. Due to genetic mutation or disorders in the folding process at the RER by heat shock proteins/chaperones, the isoform of three α_1(I) chains, forming the homotrimers, is also present in collagen I of various unhealthy tissues of the human body. Investigation of the difference in reactions of the homotrimers and the (native) heterotrimers of collagen I to MMP-1 and other MMPs enzymatic processes during the past three decades indicate that it is the structure of the homotrimers that hinders the exposure of the cleavage sites of such collagen fibrils [125], leading to the phenomenon that type I homotrimers resist cleavage of all tissue MMPs [126] [127] [128]. Accelerated degradation of dermal collagen matrix plus decrease in collagen synthesis lead to skin aging [129], correlated with other diseases such as rheumatoid arthritis [130], hypertensive cardiac [131], hypertrophic cardiomyopathy [132] (both are caused by the predominance of synthesis over degradation), and others. On the other hand, resistance to physiological digestion also leads to many diseases, such as osteogenesis imperfecta (brittle bone disease in mice model) [133] (interpreted to be due to disordered alignment of mineralized collagen fibrils in the bone), weakening in tail tendon of mice model [134]. A type of glomerulopathy was detected in tissues of mouse model with homozygous null for α_2(I) gene [135]. Note that not all the diseases mentioned are analyzed with respect to the presence of homotrimers. There are other factors involved, but for collagen I, the correlation of the progression of the diseases mentioned with the resistance to digestion (because of the presence of homotrimers) is more clearly established. Note that collagen III is a homotrimer in its native form; it is established that during wound healing, the collagen structure is synthesized by stem cells in the injured CTIF site during the earliest phase of wound healing, followed by synthesis of collagen I by the matured fibroblasts [136]. Since collagen III is observed to be replaced by collagen I, MMPs 1, 3, 8 must be able to digest collagen III far more readily than collagen I due to the difference in their structures. Collagen II is also a homotrimer (**Table 1**). The cartilages (with some amount of collagen IX) are turned over very slowly during live time after adulthood, and the common degenerative disorder is arthritis when one ages (see e.g. [137] [138]). We consider the homotrimer structure of the vertebral discs is one factor that causes fibrosis; the loss of gela-

tinous characters of the nucleus pulosus is another important factor that leads to degenerative disorder. The other types of collagen having homotrimer compositions are found in small amounts only, and present in a small part of the fascia.

7. Causes of Tendinopathy and Some Plausible Clinical Treatments

7.1. Manifestation of Tendinopathy in the Fascia

Having explained the "longevity" of tendon cores, it has been a problem to understand the cause of tendinopathy. Tendinopathy is a complex multi-faceted tendon pathology, leading to decline in musculoskeletal function, not only limited to sports medicine. There is usually no symptom at the initial stage. When the disease is recognized, common pain is felt with stretching, together with a certain degree of localized swelling, and palpitation of the pathological area [139] [140]. Under clinical settings, rheumatologists commonly used the term tendonitis to describe painful symptoms as a result of tendon inflammation [141], whereas other clinical experts might consider the painful symptoms as disorders, called tendinosis, reflecting degenerative changes at the microscopic level [142]. The treatments are usually temporary, costing a lot of resources to the society. Tendinopathy refers to overuse tendon injury; the intrinsic pathogenetic mechanism of this disease has been under rather intense research and debate during the past ten years. One key issue is whether inflammation is involved during the pathological process. From the collagen view point, tendinopathy is characterized by the change of collagen production from type I to III, resulting in a decrease in tensile strength that explains the fact that tendinopathy has a higher risk of tendon rupture [143].

7.2. The Evidence that Tendinopathy Starts with Inflammation and the Disease Progresses as a Complex Interplay between the Intrinsic and Extrinsic Compartments

We have in the beginning remarked that there are cells migrating in the CTIF system and the collagen structures were interspersed with various non-collagenous proteins, predominantly proteoglycans and glycoproteins, as matrix molecules. Structural integrity is kept by collagen crosslinks at the molecular and sub-fibrillar levels. In the normal state, the fibroblasts attached to the tendons are called tenocytes, and are found to be residing in rows (via connection protein connexin 43) following the direction of the crimp of collagen fibers [144] [145] [146]. It is not surprising that the resident cells, cells in transit, fibrous collagen constituents, plus the above-mentioned "linkage proteins" are involved in the development of the pathological state, when a tendon is overused. In more details, the concept of complex interplay has been introduced. The progress of the disease is a complex interplay between two compartments: the intrinsic and extrinsic compartments as named in [51]. The intrinsic one is composed of: (i) The fibrous collagen core (mainly collagen I with small amounts

of collagen III, V and VI) [147]. (ii) Fibril-associated collagen with interrupted triple helices (FACITs). (iii) The resident cells (mainly tenocytes, mast cells, some macrophages, plus some stem cells [148]). (iv) Some attached proteins (such as the small leucine-rich proteoglycans (SLRP) which regulate collagen self-assembly to form fibrils [149], plus some glycoproteins). (v) Resident stem cells in the CTIF system (one origin is from hair follicles, because there is no evidence that they migrate from the bone marrow when they are in need) [150] [151].

The extrinsic compartment is composed of: (i) Interstitial fluid. (ii) Nerve fibers/fibrils with various endings. (iii) Blood vessels. (iv) Lymphatic vessels. (v) Cells in transit (immunity cells which migrate to the target when inflammation occurs) as well as some stem cells migrating into the region of interest [148]. Here we emphasize the importance of including the interstitial fluid which is not commonly mentioned in other works in general. In particular, the tenocytes have mechanical sensors in the cell membrane. Tenocyte behaviour, including change of phenotyping, is much affected by all the mechanical forces experienced, including (a) shear force of the interstitial fluid flow, (b) collagen-structure-mediated integrin activity, leading to focal adhesion and other functions as described in Sections (1) and (3). The IF flow shear force is experienced by the known ion-channels as well as the prime cilia in the cell membrane of tenocytes which have only been found recently to be highly oriented along the collagen direction and the long axis of extensor tendon [152]. We postulate that similar structure could be found in other tendons too. Interaction (a) is an example of interaction between the extrinsic and intrinsic compartments. Interaction (b) is an example of interaction among members of the intrinsics compartment.

Since the collagen fibers have long turnover time, microstructure disorder among the fibers, fascicles, sub-tendons are accumulative, for instance, glycation in diabetic patients (details will be treated in another paper). Tendon repair is then a complex interaction between the intrinsic and extrinsic compartments, involving degradation of collagen fragments, synthesis of new procollagens for repair as experienced in wound healing. Overdoing healing leads to scarring, and even fibrosis.

Including the "accumulative factor" just mentioned, we consider that overuse of the tendons could trigger off the very complex interplay between the two mentioned compartments, with inflammation starting the pathological process. **Table 4** lists some special features of typical examples of tendinopathy, suggesting strongly that the disease begins with inflammation. In fact, human tissue biopsy specimens obtained from small rotator cuff tears of 40 patients during surgery operation showed a significant inflammatory infiltrate of macrophages and mast cells in cases of more severe mechanical tears of the tendons [153], supporting the hypothesis that tendinopathy is associated with inflammation at the early stage.

Table 4. Some typical examples of inflammatory response of injured tendons.

Treatment	Genes of cytokines expressed; secreted cytokines detected	Types of cells examined	Models of *in vitro* study	mRNA or proteins found	Expected biomedical consequences	Reference
Cyclic mechanical stretching	Interleukin IL-1β over production—17 times of control	Tenocytes of Achille tendon	Rabbit	↑ of collagenase-1 and stormelysin-1 genes	Over degradation of collagen I; inflammation	[154]
IL-1β added to cell culture	NIL	Tenocytes	Human	mRNA of COX-1, MMP-1, MMP-3 expressed; prostaglandin PGE_2 secreted	Over degradation of collagen I; inflammation	[155]
Torn edges of supraspinatus tendon	Genes expressed: IL-18, IL-15, IL-16, macrophage inhibitory factor (MIF), tumor necrosis factor α (TNF-α)	Tenocytes of supraspinatus tendon	Human	Proteins of IL-18, IL-15, IL-6, MIF, TNF-α, caspases 3 and 8 detected	Apoptosis of tenocytes via the caspase pathway	[26] [156]

7.3. The Very Recent Evidence that a Model System with Tenocytes Cultured on Aligned Biomaterial Scaffolds Would be Less Susceptible to a Catabolic Inflammatory Stimulus than the One with Tenocytes Seeded on Disorganized Topography—We Postulate that Well Aligned Collagen Fibers Have "Anti-Inflammatory Effect"

The anterior cruciate ligament (ACL) and posterior cruciate ligament constitute a pair of cruciate ligaments in the human knee. The hamstring tendons form two groups of tendons at the back of the knee. In [157], fragments of healthy hamstring tendons were collected from patients who underwent surgical reconstruction of the anterior cruciate ligament. Tenocytes were stripped from surrounding tissues in the way described in [158]. Monocytes were differentiated towards the uncommitted macrophages called MO macrophages by stimulation with phorbol 12-myristate 13-acetate. These MO macrophages were chemically stimulated by Interferon-c and Lipopolysaccharide (LPS) to arrive at the M1 phenotype, which is known to be able to induce strong immune response and tumoricidal activity. When MO cells were activated with Interleukin-4 (IL-4), they acquire the M2-polarized phenotype, and they would shut down their TIMP1 gene expression, would also initiate the production of highly angiogenic TIMP-deficient proMMP-9 molecules [159]. All three types of macrophages (MO, M1- and M2-phenotypes) plus human tendon fibroblasts were seeded on two substrates; (i) electrospun polycaprolactone (PCL) mats with either highly aligned, or (ii) randomly oriented fiber structures. The mono-cultured tenocytes on aligned scaffolds served as control. Leaving out the technicality of the analysis, the main findings were: (a) There was crosstalk between the tenocytes with the immune cells (macrophages of two phenotypes) with results dependent on the mechanical conditions of the scaffolds they were seeding. When the substrate, which represented the ECM collagen fibril/fiber network, was well aligned (parallel), such cross-talk led to a result tending to downregulate the expression of MMPs. The result was aberrant collagen matrix turnover. (b) As expected with the result of (a), highly aligned tendon cell scaffolds would promote tendon

matrix synthesis. (c) The tenocytes would be less sensitive to stimulation of immunity cells with substrate (i) because they were involved in the synthesis activity as a postulate. In other words, we infer that there is anti-inflammatory effect when the tenocytes are seeding on highly aligned scaffold [157].

7.4. Application of Synthetic Mechano-Growth Factor to Cultured Rat Achille Tendon Tenocytes Would Lead to Enhancement of MMP-2 Activity, a Condition Necessary for Tendon Repair

Directional migration is a fundamental cellular process essential for embryonic development, wound healing, immune response, and tissue development. We use the term pseudopodia for physiological migration to distinguish migration during cancer invasion (called invadosome, to be treated in the next paper).

To repair an injured tendon, tenocytes need to move to the repair site, require remodelling of the ECM, giving room/way for migration, to begin with. After that, synthesis of collagen and other proteins follow, which might be considered as the second stage of wound healing. The major part of collagen III is eventually replaced by collagen I in the tissue, and the healing is complete. Employing cultured tenocytes from rat's Achille tendon, it has been shown in [160] that treatment of these tenocytes with a new synthetic growth factor, called the mechano-growth factor (MGF), would lead to enhanced MMP-2 activity (but not the MMP-9 activity) via the activation of the FAK-ERK1/2 signalling pathway. Whereas both MMP-2 and 9 can degrade collagen IV, V (as constituents of the basal membrane), MMP-2 also degrades elastin. It has been found that elastic fibers are broadly distributed in tendon and localized around tenocytes [161], though the total amount is small [162]. It therefore makes sense that MGF would expedite tendon repair. However, we also note that elastin has an extremely slow turnover rate. It is therefore desirable to find out later whether tendon repair itself would cause elastin degeneration.

7.5. Proteomic Profile Analysis in the Fascicle Matrix (FM) and Inter-Fascicle Matrix (IFM) and Its Relevance to Development of Future Clinical Treatment for Tendinopathy

We have noted that the "core" of a tendon has a very slow turnover rate. This core of a tendon is referred to the collagen fibrils/fibers forming aligned (along the direction of force transmission) structure for obvious physical demand—to sustain tensile force. A fascicle is composed of many fibrils and enclosed by a sheath called endotenon as shown in **Figure 4**. The blue region inside a fascicle contains proteoglycans and glycoproteins of which only several of each class have been named in Section (1.2). These proteins are embedded in the interstitial fluid, and the blue region may be called a fascicle matrix (FM) in **Figure 4**. Likewise, in a hierarchy way, a sub-tendon contains fascicles embedded in a blue domain, enclosed by a layer called epitenon; this blue domain is called inter-fascicle matrix (IFM). Resident cells and cells in transit can reach both FM and IFM. The abundance of proteins in FM and IFM may differ according to

functional demands. In a recent investigation, superficial digital flexor tendons (SDFT) of the forelimbs of horses, euthanased based on an incurable disease rather than that related to the tendons, were taken as samples [163]. Using laser-capture microdissection and mass spectrometry, it has been demonstrated that majority of the proteins found in FM were ECM proteins, such as collagen types I and XII, thrombospondins-1 and-4, COMP, fibromodulin and decorin. Both Thrombospondin 1 and 4 are adhesive glycoproteins that mediates cell–cell and cell–matrix interactions [164]. Thrombospondin-1 binds fibrinogen, fibronectin, laminin, type V collagen and integrins $\alpha_V\beta_1$, and is inferred to participate in angiogenesis and platelet aggregation. Thrombospondin-4 binds heparin (a glycosaminoglycan having anti-coagulant property) and calcium. It is involved in local signaling in the nervous system. The protein COMP has been thought to be more involved in the cartilage structure, but it has other functions: binding to bone morphogenetic protein (BMP), binding to heparan sulfate proteoglycan, playing a role in collagen fibril organization, as an ECM structural constituent [165]. Fibromodulin is involved in collagen fibrillogenesis, keratan sulfate (major glycosaminoglycan in the cornea) biosynthesis, transforming growth factor β receptor complex assembly [166]. Decorin is a leucine-rich proteoglycan mainly involved in collagen fibril assembly [167]. Thus from protein analysis, the FM's functions are mainly fibril synthesis and refolding.

The proteins found in higher abundance in the IFM were mainly cellular proteins. The IFM contains more protein fragments (neopeptides), implying that greater matrix degradation has happened in this compartment for homeostasis. This revelation is intuitively understandable because the fascicles, as basic units of the tendon (macroscopically), need to slide along the force direction to absorb external force, causing deformation of the IFM. In addition, the number of these neopeptides was found to reduce with ageing, implying that the turnover rate of the IFM decreases in aged tendons, offering less resistance to cyclic load. Collagen type III concentration, perlecan (a basement membrane-specific heparan sulfate proteoglycan core protein), and 5 glycoproteins (3 laminin subunits, transforming growth factor β-induced protein and adiponectin) are enriched in the IFM. The laminin is a major component of the basal lamina, a protein network. The transforming growth factor-β (TGF-β) protein is involved in regulating and mediating processes at the cellular level, including cell proliferation, differentiation, motility, adhesion and apoptosis, as well as processes of tissue organism. The adiponectin protein, being secreted by adipocyte, is a novel adipocyte-specific protein. It is suggested to play a role in the development of insulin resistance and atherosclerosis.

Summing up, these data suggest that reduced turnover of the IFM with increasing age may result in damage accumulation and alterations in IFM mechanical properties, increasing the risk of tendon injury, and hence tendinopathy, in aged individuals [168]. Since the tendon inserts into the bone and joins the muscle, it will be a fruitful future project to study the functions of the FM

and IFM proteins during the development of tendinopathy, including that due to aging. We might even consider the development of local application of deficient proteins when a pathological state emerges.

7.6. Enhancement of Collagen Synthesis via Growth Factors Derived from Platelet-Rich Plasma: Can It Be a Therapeutic Measure to Treat Tendinopathy?

Platelet-rich plasma (PRP), obtained simply by centrifugation of whole blood, is a portion of the plasma fraction that has a platelet concentration above that of whole blood. Being activated by activator such as thrombin, the PRP forms a gel. Platelets enclose different types of granules that can perform different functions. The three main types of granules are α-granules, dense granules, and lysosomes. In particular, the α-granules store up some grow factors such as platelet-derived growth factor (PDGF), transforming growth factor- (TGF-) β, insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) [169]. Analyzing the relevant growth factors, it was shown in [170] that intermediate concentration of platelet-rich plasma within the range of (0.5×10^6, 1×10^6 plt/μL) strongly enhanced the processes of proliferation, migration, collagen production, and MMPs production; these processes are needed for tendon repair. Such a result suggests that further experimentation is encouraged to apply an intermediate concentration of platelet-rich plasma locally to sites of tendon injury.

7.7. A Balance between Reparation and Degeneration in Treating Tendinopathy: Sparing Inflammation-Induced Healing Moieties and Encouraging Robust and Rapid ECM Repair Treatment by Exploiting miRNA-29a

Let us follow on from **Table 4**. Among the cytokines, IL-33, a member of the IL-1 family, plays a major role in innate and acquired immune responses. It is expressed in endothelial cells and fibroblasts, and is released after cellular damage and biomechanical overload; it has the nickname of "alarmin" [171].

On the other hand, we note that a microRNA (miRNA) is a small non-coding RNA molecule that functions in post-transcriptional regulation of gene expression [172]. Along this line the following specimens were obtained in the investigation of [173]: (i) Seventeen supraspinatus (torn) tendon specimens were obtained from 12 patients with rotator cuff tears undergoing shoulder surgery in [173]. (ii) Samples of both the subscapularis part of the tendon (thus representing the onset or early sample of tendinopathy) were also collected from the same patients. Arthroscopic repair, a minimally invasive surgical procedure of the rotator cuff was carried out. Human tenocyte cells were explanted from hamstring tendon tissues; a hamstring tendon is one of the three tendons from the three hamstring muscles in the back of the thigh. These tenocytes were transfected with synthetic mature miRNA for miR-29a and b. The tenocytes were the human *in vitro* cell cultured on two tendon tissues (i) and (ii) stated above. Ana-

lyzing these cultured models, we leave out the details here, the authors reported that IL-33 was secreted from the tenocytes at the early stage of tendinopathy, leading to a transition of collagen I to III, the latter of which is well known to play a key role in collagen repair. The use of synthetic miRNA-29a provides information that miRNA-29a is a post-transcriptional regulator of matrix/inflamematory genes during tendon repair. It has been suggested [173] to exploit miRNA-29a as a future therapy for quick collagen repair in the presence of inflammation, to treat tendinopathy. The reader is referred to a very recent review [174] for the concept of balancing between reparation and degeneration in treating tendinopathy: sparing inflammation-induced healing moieties and encouraging robust and rapid ECM repair. While we have to wait for further experimental results adopting this miRNA-29a therapy, the present authors wish to draw an analogy discovered a few decades ago in treating pain: applying needle acupuncture to initiate a neurogenic inflammation, resulting in quick repair and analgesic effect; the reader is referred to [3] [175] for more details.

7.8. Wound Healing of Tendon is Initiated by Endogenous Nitric Oxide Generation, with All the Three Isoforms iNOS, eNOS, nNOS of the Nitric Oxide Synthases Participating in Time Sequence, Suggesting the Usage of Glyceryl Trinitrate Patches to Treat Tendinopathy Might Be Useful

When NOS catalyzes the substrate L-arginine to L-citrulline, nitric oxide (NO) is produced. Cofactors calmodulin, tetrahydrobiopterin (BH4), heme, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) are working to regulate the production. There are three isoforms of the nitric oxide synthase: eNOS is mainly expressed in endothelial cells; nNOS is expressed in neurons. The third, inducible, isoform iNOS is expressed in macrophages, and is induced by proinflammatory cytokines (such as interleukin-1 and tumor necrosis factor), and cytokines from alien cells. Normally, the amount of NO produced by iNOS is vast. For example, after transplantation of organ (liver, say), the huge amount of NO produced can react with super-oxide, a natural product in ATP generation in the mitochondria, to form peroxynitrite $(ONOO)^-$, a biochemical molecule being detrimental to all cells, and the transplanted organ will be readily damaged. The inhibitors of iNOS can be used as anti-rejection drug after organ transplantation, such as myco-phynolate mofetil, which was further developed to treat renal ischemia-reperfusion injury [176]. The enzyme iNOS is doing its immunity duties, some of which are involved in physiological processes, but the degree of activation has to be strictly regulated.

Using *in vivo* mouse models, it was demonstrated in [177] that nitric oxide is an important mediator of normal wound healing, resulting in collagen synthesis and accumulation at the wound site. Injection of S-methyl isothiouronium sulphate (S-Methyl-ITU), a potent inhibitor of nitric oxide synthase NOS, was shown to inhibit the healing process, as a check.

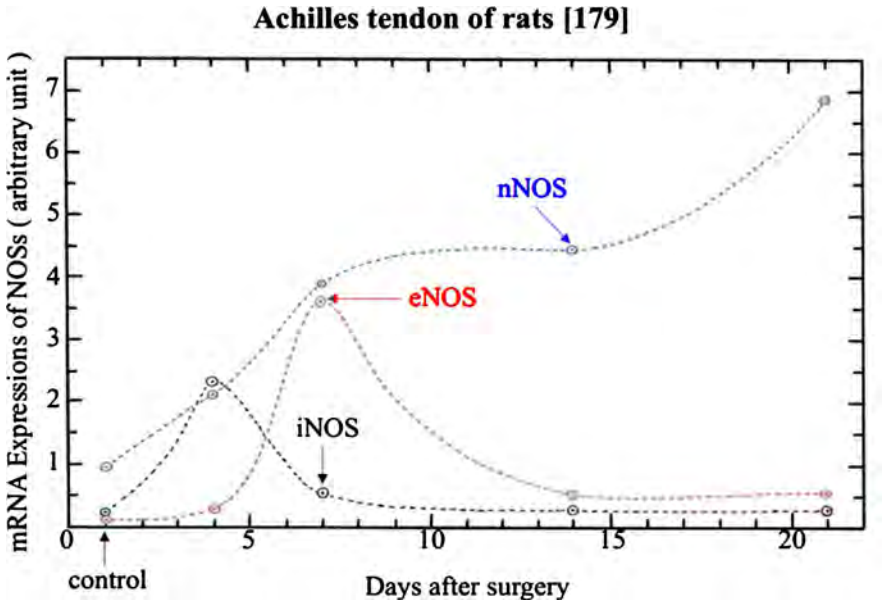


Figure 13. Surgery injury was introduced to the right Achille tendons of 44 rat models and the left Achille tendons were uninjured, as control specimens. The mRNA expressions of the inducible nitric oxide synthase iNOS, the endothelial NO synthase eNOS, the neuronal NO synthase nNOS were detected in the injured tendons. Taking the histogram values in Fig.2 of [179], we show that the wound healing process was initiated by iNOS, an immunity action, followed by enhanced expression of eNOS, causing blood vessel dilation. The neuronal NOS expression was activated from the beginning of injury, and was enhanced in general during the healing process.

Note that there is also evidence that NO induces collagen synthesis in cultured human tendon cells [178]. In another study [179], Achilles tendon and plantaris of the right hind limb (of the animal model with 44 rats) were dissected free from the surrounding fascia. The Achilles tendon was transected with a scalpel from its calcaneal insertion. The tendinous portion of the plantaris was removed to prevent any possible action as an internal splint. RNA expressions of eNOS, iNOS, nNOS were investigated at 4, 7, 14 and 21 days following the stated surgery by the usual RT-PCR procedure. Based on the values of the histogram published in Fig.2 of [179], we plot three lines in **Figure 13**, representing respectively the activity of the three stated enzymes, after the stated surgery operation was carried out to induce injury of the Achille tendon. The data points (circles in black, red and blue) at day 1 are the relative mRNA expressions of the control samples. The other circles with three different colors are taken from the maximum values of the histogram in Fig.2 of [179]. The dotted lines are only the speculated data points if more data of the expressions of mRNA had been measured. The purpose of this figure is to show, qualitatively, that iNOS (black dotted line) is responsible for initiating the healing process, substantiating the result of [177]. After the first 7 days, the expression of iNOS remained at a very low level. Such an initiation is not unreasonable, as the damaged tissues would have been broken down, and carried away (by the interstitial fluid). Significant expression of eNOS was observed at day 7. We interpret that result as representing dilation

of the blood vessel for better blood supply to the wound site occurred after the immunity act was about to complete. Here NO (from eNOS) acted purely as a vessel dilator. NO is also a potent neuro-transmitter. Right from the start, the neurons at the wound site were informed, and have been playing roles to regulate the healing process, all the way to over 21 days after injury. The uninjured Achille tendon was found not to express any significant isoform of nitric oxide synthase [179]. There are other studies published on the application of supplying NO to treat tendinopathy, and the reader is referred to the review of [180].

More recently, glyceryl trinitrate patches, which can release nitric oxide, have been developed to treat tendinopathy, but noting that an exercise program must accompany the patch treatment [181]. Such a consequence is in line with the analysis presented in Section (7.3). Without a certain amount of mechanical tension, the tenocytes are in the dormant state and would not synthesize much collagen. At this stage, we do not have quantified data as to the magnitude of tension required for the injured tendon for certain type of tendinopathy.

7.9. Effects of Taking Antibiotic Ciprofloxacin on the Strength of Tendon Based on Explant Experiment According to Reference [182]

Tendon fragments were obtained from 6 healthy subjects, undergoing surgical procedures to treat anterior cruciate ligament rupture, excluding cases of tendinopathy [182]. Ciprofloxacin (CPX), an antibiotic to treat possible infection (which might have been acquired during surgical operations), was applied with different dosages, to three groups of tenocyte cultures obtained from the mentioned tendon explants. The following measurements were carried out on the treated and untreated tenocytes: (i) MMP-1,2 protein expressions; (ii) mRNA expressions of collagen I; (iii) LH2b (protein for cross-linking of newly synthesized collagen); (iii) mRNA expressions of TIMP-1,2; (iv) mRNA expressions of Cx43 & N-cadherin (as intercellular connexin proteins). The levels of these expressions of three groups associated with different dosage of the medicine, plus that of the control group receiving no treatment, were measured. Analyzing the difference of these levels, the authors of [182] conclude that application of CPX could lead to "toxicity" that weakens the regularity of the tendon structure, causes over degradation of collagen, and reduces the ability of the tenocytes to repair the tendon tissues. The result implies that the application of CPX may lead to failure of the tendon's resistance to mechanical loading *in vivo*. Such investigation suggests that the effects of medicines during the repairing process needs to be monitored with care.

8. Discussion

8.1. The Fascia Is an Active Organ with Cells Interacting Continuously with the Protein Network via Mechanotransduction

The fascia is not a passive organ. The resident cells and the cells in transit when inflammation occurs are continuously communicating with the collagenous and

non-collagenous proteins (28 types, Table 1), the interstitial fluid via mechano-transduction. Characteristics of the communications are different in different CTIF domains that make up the whole fascia, because the constituents of the irregular-shaped fascia embed all internal organs, even as small as nerve fibers, lymphatic and the smallest blood vessels. The forces involved include fluid shear force exerted by the viscous IF on the cilia and mechano-sensors (integrins) at the cell membrane of the resident cells (such as fibroblast, mast cell), as well as organ cells (such as hepatocytes). Mechanical forces are also exerted by collagen fibers/fibrils/basal membranes, elastic fibers, reticular fibers on these sensors on any cell in contact. On reception of these forces, focal adhesion complexes are built, attached to the inner sides of the integrins. Cytoskeletal elements like F-actin, myocin-II, microfilaments, microtubules, intermediate filaments are induced to form "bridges" bringing the mechanical signals directly to the nucleus surface. Depending on the features of the signals, the associated genes are expressed, splitting into mRNA. The corresponding peptide chains are synthesized at the ribosomes which are attached to the surface of the RER. Passing through the RER-Gorgi apparatus "factory", sugars are added and the peptides are folded with the help of heat shock proteins (HSP). The misfolded proteins (beyond repair) are sent to apoptosis pathways [26]. For any type of collagen, three peptide chains with the proper combinations (Table 1) form the procollagen molecules which exit to the CTIF domain. Thus the types of proteins synthesized by the cells depend much on the type and intensity of the mechano-transduction signals received; these signals are transmitted through certain parts of the fascia. In short, the constituents of the fascia are communicating continuously with the cells in contact. Note that cells migrate according to the rule of durotaxis (towards collagen network of higher gradient of mechanical tension [10]) and chemotaxis (attracted by biochemicals released by other cells or from the stores in the associated CTIF domain, a rather well-known process). The migrating cells therefore react on the collagen fibers and other protein structure during their crawling action; at the same time the tension of the collagen structure is increased. The resident cells, cells in transit, and the non-cellular network form the largest active organ of the body. Moreover, the mechanical tension of the collagen network can change the fate of the stem cells and fibroblasts crawling via durotaxis [52]. The concept of the CTIF system being a dynamic microenvironment for stem cell niche has been introduced [35]. The six main functions of the ground substance, or interstitial fluid have been stated in Section (1.3), and also described in more details in our previous work [11]. This fascia organ therefore also forms the largest communication network of the body. In short, since all transport of materialistic constituents and energy signals pass back and forth between the blood circulation and organs, it is easy to understand that malfunctions of such transportation, in any form, are causes of the pathological states; and these states manifest with certain characteristics in the fascia.

The fascia is also affecting the functions of internal organs. For example, during cyclic heart beating, the heart cells do the least amount of work for the same output (the best possible efficiency) if the heart cells and the ECM around the heart (which is part of the fascia) play the "push and pull" game in synchrony; details are described in [11].

8.2. Homeostasis of the Fibrous and Non-Fibrous Collagen Structures is Maintained by a Balance of the Following Two Main Processes: (a) An Intricate Process of Degradation and Its Inhibition; (b) A Proper Rate of Synthesis of Collagen

Let us consider three scenarios concerning the synthesis of collagen. (i) If the tension of the collagen network is low at a CTIF domain, the main resident cells, *i.e.*, the fibroblasts, are in the dormant state, leading to low collagen output. (ii) If the mechanical tension of the collagen network is "properly high", the fibroblasts are in the activated state, stretching to form cell-cell connection, and there is a good communication network. The fibroblasts themselves form part of the collagen network, and the synthesis rate of collagen is proper. (iii) If the tension of the collagenous network is too high, the fibroblasts are over-activated, and would produce collagen at a rate which is too fast. However, we should note that scenario (i) would lead to the degeneration of the CT, but condition (iii) might not necessarily lead to fibrosis. The digestive enzymes MMPs, (23 classes, 15 are mentioned in **Table 1**) with specificity to types of collagen digested are secreted by the fibroblasts which digest the damaged fibrils after certain collagen structures, such as the tendons, have been loaded (exercise or injury). A clear explanation of degradation processes, with the example of the loading/overloading the bovine tail tendon, is reviewed in Section (4). Degradation actions of the MMPs are regulated by 4 inhibitors TIMP -1 to 4 (**Table 3**). The regulations are carried out in very subtle ways, as some MMPs are bound to the receptors of the cell membrane, whereas some are soluble. Normal physiological remodeling processes requires precise regulation of collagen degradation. We can use the concept of "net degradation rate" to simplify matters in our series. The overall ratio of (synthesis rate)/(net degradation rate) decides whether (A) fibrosis or (B) excessive breakdown occurs. So far, there are not enough parameters for us to quantify the stated ratio, which is specific for the type of collagen concerned.

A number of examples of (A) have been reported; to name a few: hypertensive myocardiac fibrosis [183], heart failure [184], spontaneous abnormal endochondral bone development in mice model [185], liver fibrosis [186]. Examples of (B) include rheumatoid arthritis [187], atherosclerotic heart disease [188], glomerulonephritis [189]. Also, Crohn's disease is a chronic inflammatory disease of the digestive tract, manifesting clinical symptoms such as pain and diarrhea. Recently, analysis of serological biomarkers (P1NP, Pro-C3, Pro-C5, Pro-C6) indicates that the active inflammation in Crohn's disease is characterized by increased formation of type V collagen and increased matrix metalloproteinase mediated breakdown of both type I and III collagen. The penetrating

Crohn's disease is characterized by increased matrix metalloproteinase-9 degraded type III collagen and formation of type V collagen [190].

8.3. On Possible Treatment of Inherited Diseases Which Manifest Pathological States in the Fascia

We have reviewed in Section (6) that collagen I, II, III built of homotrimers would resist the normal degradation by MMPs. In particular, the osteogenesis imperfecta (OI) disease is interpreted to be due to disordered alignment of mineralized collagen fibrils (particular collagen I) in the bone, leading to a brittle structure, as well as weakening of other tissues built of collagen I [113] [191]. In fact, more than 70 mutations in the two structural genes (COL1A1 and COL1A2) for type I procollagen have been found in probands in patients with Ehiers-Danlos syndromes, osteoarihritis, chondrodysplasias, (familial) intracranial aneurysms [192], in addition to OI [193]. The two common Ehiers-Danlos syndromes refer to loose/unstable joints which are prone to dislocations, and fragile skin that tears or bruises easily [194]. Intracranial aneurysms cause subarachnoid hemorrhage (SAH). Osteoarihritis is well-known. These diseases are usually found to have either abnormal collagen triple helices or abnormal (too much or shortage of) proteoglycans (or glycoproteins). We post the following questions: (i) Are all the peptides that are supposed to fold up to become the proper procollagen (or non-collagenous proteins) mutated? If the answer is positive, measures on genetic correction are needed, and would have been considered by genetic experts. (ii) If among the related peptides synthesized, some do have the correct sequences, but are not folded properly in the RER, can activation of the related heat shock proteins help to improve their intracellular refolding processes? We propose to investigate on whether heat shock proteins can help in the issue of folding correction intracellularly in the future.

8.4. Pathological States of Inflammation Often Manifest as Edema in Certain Part of the Fascia

We have already listed the seven key functions of the interstitial fluid in Section (1.3). In the experimental study of [195], fluid shifts were measured in eight subjects employing a "simultaneous, radionuclide dilution technique" for three postures: (a) seating (control), (b) supine, and (c) standing. Technically, radioiodinated serum fibrinogen, radiochromated crythrocytes, radiobromine and tritiated water were used to measure plasma volume, red cell volume, extracellular and total body water volumes. Vascular fluid lost during standing was found to be filtered into the interstitial compartment, while the extracellular and intracellular volumes remaining unaffected. The loss of intravascular fluid during standing was caused by the filtration of plasma into the fascia. On the other hand, in the supine position, intravascular volume was found to increase, indicating that there is IF fluid flow from the fascia back to the blood circulation. Although there is overall balance of fluid volumes in the fascia and blood circulation, since the geometrical structure of the fascia is so irregular, the IF pres-

sures in different CTIF domains of the fascia are in general not equal [196]. It is the difference in pressure that drives the thick IF to flow from sites to sites. In the above statement, it has been assumed that the flow rate of IF into the lymphatic system remains constant. Moreover, it has recently been discovered that a lymphatic system inside the skull exists, and the "glymphatic lymph", which is a mixture of the classical cerebrospinal fluid and the interstitial fluid in the brain parenchyma, will flow from the brain via sheaths of nerve fibers, sheaths and inside the collagen layers of blood vessels to join the IF of the neck and peripheral lymphatic vessels (see Fig.3, 4, 6, 7, 8 and 10 of [11]). The fluids inside the body form a complicated circulation system. Many diseases manifest their pathological states in the fascia which includes properties of these fluids. For example, we infer that blockage of the glymphatic fluid to join the peripheral IF and hence the peripheral lymphatic system will naturally inibit the clearance of the debris of metabolism inside the brain, lead to the accumulation of proteins such as β-Amyloid and τ-protein, which are highly correlated to neurodegenerative diseases such as Alzheimer disease. Peripheral edema, due to disorder of IF-lymph flow is associated with some well-known diseases, details of which will not be discussed in this paper. However, we would like to point out that due to overuse of muscle cells, intracellular edema of these cells is the cause of the exertional compartment syndrome. The largest organ which enclosed many muscle stripes/striations, are partitioned into pulley systems including bones (and bone joints), tendons, ligaments, for efficient locomotion of different parts of the body. Each of these pulley systems is "protected" by sophisticated collagenous connective layers, which are stiffer than the common connective tissues (such as that in the skin). Readers are referred to Fig.2, 3, 4 of [197] for details in some anatomy structures. During the development of the exertion compartment syndrome, the IF pressure in the compartment concerned highly increases, resulting in the augmentation of lyses of the muscle cells (which have been overused). The only way, so far, is to release the IF pressure by surgery procedure, well known to orthopaedics surgeons. We may consider the exertional compartment syndrome as the failure of potent flow of IF from one compartment to another; this restriction is inevitable if we need efficient locomotion of certain muscle-joint systems. It is not out of the blue to say that treating the IF to release pressure, together with clinical measures to repair the overused muscle cells, might help to prevent certain excertion compartment syndromes.

On the other hand, to study the cause of inflammatory edema in the fasica, the *in vivo* burn model with rats was set up some decades ago [198]. The interstitial fluid pressure (IFP) was found to drop from −1.3 mm Hg (preburn) to −5.5 mm Hg after 10% total body surface (BSA) burn. After a 40% BSA, the IFP dropped drastically to −50 mm Hg at 25 min. postburn. This quick exchange of water between the IF and the blood circulation is much stronger than that due to the classically known capillary leakage. Such a leakage was thought to be an imbalance of the transcapillary hydrostatic pressure (P), colloid osmotic pressure (COP) and capillary filtration coefficient (CFC), a theory established before

1980s. After a series of investigations [199] [200], inflammatory edema was considered to originate from the sudden drop (as observed in the burn experiment) of IFP due to interaction of the dermal fibroblasts with the connective tissues, leading to contraction of the IF gel via the integrins, and a new one ($\alpha_{11}\beta_1$) was identified to be involved by 2009 [201]. Treatment on edema was suggested to develop inhibitors of the integrin(s) involved. We shall not discuss the mechanism of the integrin mediated edema here, leaving it to topics on specific diseases related to edema. It suffices to note that inflammation of organs often leads to edema, manifesting as a pathological state in some parts of the fascia. Of course, the noninvasive measure is to drain the stagnant IF via the lymphatic vessels. We would also note that it is well-known that chemo-therapy medicine cannot reach the tumor site because the IFP is high around the tumor.

8.5. From a Half-Century Puzzle on Protein Folding to the Debate on Thermal Stability of the Collagen Triple Helices below and above Body Temperature

A proper process of unfolding and refolding of collagen proteins process must be at work in catering homeostasis of the fascia for survival—understanding this process is crucial to develop therapeutic measures for treating many diseases. We shall briefly list below some lines of thought on the search of the half-century puzzle—why and how a peptide can fold up into the same native three-dimensional conformation, despite there being trillions of choices.

First, mRNA transcription gives only a peptide chain. How does such a chain fold up spontaneously to a three-dimension configuration with extremely high conformation specificity has been a mystery for half a century because there are ways, of astronomical order in number, to fold up this chain. In physics, a photon finds the shortest way to go from one point to another, following the principle of least action. A similar, but more complicated, principle must be at work also in the formation of a protein, for survival, because any deviation of the structure has a similar consequence of a gene mutation, leading to pathology state(s). Note also that when the protein is denatured to become a random coil, it can refold back into the same conformation. After decades of research, in short, experts propose several principles/routes that could lead a peptide to follow, ending up with the same structure every time it folds. We will only list a few below as examples because there are other models. (i) The peptide follows the easiest path (also the quickest) towards a state with the lowest "local free energy"—this route is called the kinetic principle/control. (ii) The peptide "finds" among all the possible paths that also lead towards the lowest possible energy minimum state, but that state pertains to the highest degree of stability (of the native protein with respect to the protein environment *in vivo*) thermodynamically—reaching thermodynamical equilibrium. Both models (i) and (ii) are said to follow the energy landscape [202]. (iii) A protein is built of cooperative unfolding-refolding units called foldons (each with 15 - 35 residues) so that the folding is separated into some defined discrete steps; each step-wise process,

however, has to include also the energy landscape of (i) or/and (ii). Each foldon remains part of the folded native protein configuration [203]. While intensive research on this big puzzle continues, we have to reconsider the issue of thermal stability of collagen fibrils within the context of this paper.

In Section (5), we have presented only three pieces of experimental evidence on folding and unfolding of collagen tropocollagen molecules. Results discussed in Sections (5.1) and (5.2) are in line, contrasting the result of Section (5.3) which is only one example of other *in vitro* experiments showing evidence of unfolding of collagen fibrils as temperature is raised, in general, below and above body temperature. Daily experience tells us that a person practicing long run, gymnastic exercise, playing a full football match would have core body temperature a few degrees higher than 37˚C. If the result of Section (5.3) occurs *in vivo*, we should expect that a person, after a relatively long period of exercise, would have very loose skin, would have lost the ability for basic locomotion because parts of the tendons/ligaments would have been denatured and degraded significantly by MMPs. This is not what we experience. Moreover, as explained in Section (4.4), the ^{14}C dating examination of the 28 forensic specimens of Achille tendon gives strong support to the fact that the tendon core is formed at about 17 years of age, without further significant renewal [116]. However, degradation and replacement of the collagen fibrils by new tropocollagen/fibrils outside the core tendon have been taking place all the time.

The continuous collagen fibril structure, with the end of one fibril connecting another fibril in mouse tendon modelled, has recently been revealed [204]. We infer that collagen fibrils, either newly synthesized or resulting from MMP digestion, can elongate to join other fibrils during the repair process.

We would also emphasize that in the investigation of thermal stability of collagen molecules, the parts played by the proteoglycans and glycoproteins are usually difficult to be incorporated in the specimens of *in vitro* experiments. As demonstrated in atomic force and scanning electron microscopy examination of rat's Achille tendon, the SEM micrograph of Fig. 2A of [205] revealed that the collagen fibrils were interwoven with glycosaminoglycan filament structure. Such inter-fibrillar weaving structure is a factor of keeping the collagen fibrils and hence the triple helices in a tight structure even when the temperature is above body temperature *in vivo*. In view of the analysis stated in this sub-section, we put forth the notion that tropocollagen fibrils will spontaneously fold in a range of temperature (35˚C - 42˚C) and the denatured collagen molecules, e.g., triple helices with unfolded regions, tend to refold via a mechanism satisfying one of the principles still under research briefly mentioned above in this section. There is not enough evidence at present for us to draw a concrete conclusion as to which mechanism is at work for this puzzle, but a collagen system needs to climb through an energy barrier to refold. Understanding more about the nature of the mechanism will help in taking measures to refold fibrils when collagen homeostasis or more collagen fibrils are needed in a certain part of the fascia. On

the other hand, counter-measures may be developed in case pathological fibrosis occurs.

8.6. Limitations of This Paper

We have provided some basic material of different constituents/components of the fascia, so that disorders in certain domains of the fascia can be related to the type of disease analyzed in each paper of the series. The reviewed basics in Sections (1) - (3) are by no means exhaustive. We have not analyzed on the special roles played by the elastic and reticular fibers, because elastin fibers amount to only 1% - 2% by weight of the tendon [4] [162], and the fine reticular fibers play a role mainly in supporting the integrity of various internal organs [5], but there is no information, so far, that they are involved in the pathogenesis of the tendon. We have analyzed only very briefly the functions of the proteoglycans and glycoproteins with respect to diseases of the tendons. For diseases of the knee, for instance, we will analyze the very special roles of proteoglycans in the pathogenesis involved. In other words, more basic material of the fascia will be supplemented as needed for discussion on the manifestation of pathological states in the fascia for the type of diseases concerned in the future.

9. Concluding Remarks

All organ cells need (i) oxygen, (ii) nutrients, (iii) peptides/proteins (such as hormones) from other cells to perform their physiological duties. All (i)-(ii) are carried by the blood stream before they can be used by organs. However, (i)-(iii) are transported by the IF fluid to the organ cells. The CT of various structures enclose all organs, even the small vessels, to maintain the organs' integrity and to provide a platform of communication and durotaxis of cells. The CT must be synthesized and maintained by resident cells (fibroblasts, fibroblast-like cells and stem cells). These cells, together with different CTIF domains, form the largest communication network and organ of the body. To further appreciate the extensivity of this organ, we present in **Figure 14** a simplified painting based on the SEM (Figure 2(C) of [206]) of the three-dimensional collagen network forming the endomysium embracing human cardiomyocytes.

Genetic mutation of collagen and other proteins can lead to disorders in the structure of the CT. A pathological state of the CTIF of a certain domain occurs when there is an imbalance of the digestion and synthesis of collagen triple helices in the CT. Such disorders also affect the function of organ cells. Hence the pathological states of numerous diseases manifest in the fascia.

Tendinopathy is initiated by inflammation due to overuse or blockage of the IF, developed by a complex display between the extrinsic and intrinsic compartments, the mechanisms of which are still under research. Development of different groups on plausible treatments of this disease is in process.

Pathological states of inflammation often manifest as edema in certain parts of the fascia. Serological biomarkers (P1NP, Pro-C3, Pro-C5, Pro-C6) have been

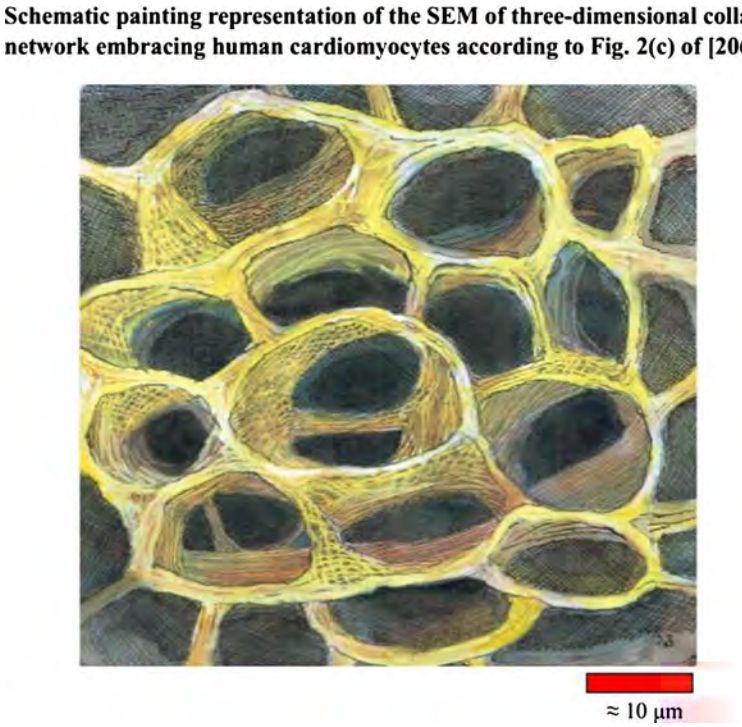


Figure 14. A collagen network forming the endomysium connects human cardiomyocytes was reported in the SEM (Figure 2(C) of [206]). The collagen fibrils grow in multi-directions, allowing movement of the heart cells with three degrees of freedom. The "caves" hold the cardiomyocytes *in vivo*. This diagram is a hand-painted diagram by author PCWF based on the stated SEM micrograph.

found for the Crohn's disase, targeting disorders of collagen V. Another practical example is the recent usage of the "flash glucose monitoring system" (attached to the upper arm) to detect sugar concentration in the IF any time at the wish of the subject (particular for diabetic patient), rather than basing on blood test. We hypothesize other biomarkers related to the fascia are to be found in the future. Since nerve fibers are also inside the fascia, stimulating the sensory nerves would lead to profound biomedical consequences as explained in [207].

Acknowledgements

The authors wish to express their gratitude to Mr. Benjamin Fung, brother of PCWF, for his unfailing assistance during the preparation of the manuscript. PCWF wishes to dedicate his work of this series of studies to his late parents Mr. Joseph Wah Hei Fung and Mrs. Helen Woi Suet Lau Fung. All diagrams have been hand-painted by author PCWF.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Ingber, D.E. (1997) Tensegrity: The Architectural Basis of Cellular Mechanotrans-

duction. *Annual Review of Physics*, **59**, 575-599. https://doi.org/10.1146/annurev.physiol.59.1.575

[2] Fung, P.C.W. (2009) Probing the Mystery of Chinese Medicine Meridian Channels with Special Emphasis on the Connective Tissue Interstitial Fluid System, Mechanotransduction, Cells Durotaxis and Mast Cell Degranulation. *Chinese Medicine*, **4**, 10. https://doi.org/10.1186/1749-8546-4-10

[3] Fung, P.C.W. (2013) Chapter 5. Plausible Biomedical Consequences of Acupuncture Applied at Sites Characteristic of Acupoints in the Connective-Tissue-Interstitial-Fluid System. In: Chen, L.L. and Cheng, T.O., Eds., *Acupuncture in Modern Medicine*, IntechOpen, Rijeka, 95-131. https://doi.org/10.5772/53901

[4] Liu, X., Zhao, Y., Gao J, Pawlyk, B., Starcher, B., Spemcer, J.A., Yanagisawa, H., Zuo, J. and Li, T. (2004) Elastic fiber Homeostasis Requires Lysyl Oxidase-Like 1 Protein. *Nature Genetics*, **36**, 178-182. https://doi.org/10.1038/ng1297

[5] Ushiki, T. (2002) Collagen Fibers, Reticular Fibers and Elastic Fibers. A Comprehensive Understanding from a Morphological Viewpoint. *Archives of Histology and Cytology*, **65**, 109-126.

[6] Page, K.E. (1952) The Role of the Fascia in the Maintenance of Structural Integrity Newark. Academy of Applied Osteopathy Yearbook, Newark, 70.

[7] Yuan, L., Yao, D.W., Tang, L., Huang, W.H., Jiao, P.F., Lu, Y.T., Dai, J.X., Zhang, H., He, Z.Q. and Zhong, S.Z. (2004) A Study on Morphological Basis of Chinese Acupuncture and Moxibustion from Digital Human Body. *Acta Anatomica Sinica*, **35**, 337-343.

[8] Ross, R. (1975) Connective Tissue Cells, Cell Proliferation and Synthesis of Extracellular Matrix—A Review. *Philosophical Transactions of the Royal Society of London. Series B*, **271**, 247-259. https://doi.org/10.1098/rstb.1975.0049

[9] Wilke, J., Schleip, R., Yucesoy, C.A. and Banzer, W. (2017) Not Merely a Protective Packing Organ? A Review of Fascia and Its Force Transmission Capacity. *Journal of Applied Physiology*, **124**, 234-244. https://doi.org/10.1152/japplphysiol.00565.2017

[10] Lo, C.M., Wang, H.B., Dembo, M. and Wang, Y.L. (2000) Cell Movement Is Guided by the Rigidity of the Substrate. *Biophysical Journal*, **79**, 144-152. https://doi.org/10.1016/S0006-3495(00)76279-5

[11] Fung, P.C.W. and Kong, R.K.C. (2016) The Integrative Five-Fluid Circulation System in the Human Body. *Open Journal of Molecular and Integrative Physiology*, **6**, 45-97. https://doi.org/10.4236/ojmip.2016.64005

[12] Sastry, S.K. and Burridge, K. (2000) Focal Adhesions: A Nexus for Intracellular Signaling and Cytoskeletal Dynamics. *Experimental Cell Research*, **261**, 25-36. https://doi.org/10.1006/excr.2000.5043

[13] Katsum, A., Naoe, T., Matsushita, T., Kaibuchi, K. and Schwartz, M.A. (2006) Integrin Activation and Matrix Binding Mediate Cellular Responses to Mechanical Stretch. *The Journal of Biological Chemistry*, **280**, 16546-16549. https://doi.org/10.1074/jbc.C400455200

[14] Wang, N., Tytell, J.D. and Ingber, D.E. (2009) Mechanotransduction at a Distance: Mechanically Coupling the Extracellular Matrix with the Nucleus. *Nature Reviews|Molecular Cell Biology*, **10**, 75-82. https://doi.org/10.1038/nrm2594

[15] Bershadsky, A.D., Balaban, N.Q. and Geiger, B. (2003) Adhesion-Dependent Cell Mechanosensitivity. *Annual Review of Cell & Development Biology*, **19**, 677-695. https://doi.org/10.1146/annurev.cellbio.19.111301.153011

[16] Nicholson, B.J. (2003) Gap Junctions—From Cell to Molecule. *Journal of Cell*

Science, **116**, 4479-4481. https://doi.org/10.1242/jcs.00821

[17] Kumar, N.M. and Gilula, N.B. (1996) The Gap Junction Communication. *Cell*, **84**, 381-388. https://doi.org/10.1016/S0092-8674(00)81282-9

[18] Delma, M., Coombs, W., Sorgen, P., Duffy, H.S. and Taffet, S.M. (2004) Structural Bases for the Chemical Regulation of Connexin43 Channels. *Cardiovascular Research*, **62**, 268-275. https://doi.org/10.1016/j.cardiores.2003.12.030

[19] Boot-Handford, R.P. and Tuckwell, D.S. (2003) Fibrillar Collagen: The Key to Vertebrate Evolution? A Tale of Molecular Incest. *BioEssays*, **25**, 142-151. https://doi.org/10.1002/bies.10230

[20] Exposito, J.Y., Cluzel, C., Garrone, R. and Lethias, C. (2002) Evolution of Collagens. *The Anatomical Record*, **268**, 302-316. https://doi.org/10.1002/ar.10162

[21] Aumailley, M. and Gayraud, B. (1998) Structure and Biological Activity of the Extracellular Matrix. *Journal of Molecular Medicine*, **76**, 253-265. https://doi.org/10.1007/s001090050215

[22] Linsenmayer, T.F. (1987) Collagen. In: Hay, E.D., Ed., *Cell Biology of the Extracellular Matrix*, 2nd Edition, Plenum Press, New York, 7-44.

[23] Wilson, S.L., Guilbert, M., Sulé-Suso, J., Torbet, J., Jeansson, P., Sockalingum, G.D. and Yang, Y. (2012) The Effect of Collagen-Ageing on Its Structure and Cellular Behavior. *Proceedings of SPIE*, **8222**, Article ID: 822210. https://doi.org/10.1117/12.908749

[24] Kuhn, K. (1987) The Classical Collagens: Types I, II and III. In: Mayne, R. and Burgeson, R.E., Eds., *Structure and Function of Collagen Types*, Academic Press, Orlando, 1-42. https://doi.org/10.1016/B978-0-12-481280-2.50005-2

[25] Kielty, C.M. and Grant, M.E. (2003) Chapter 2. The Collagen Family: Structure, Assembly, and Organization in the Extracellular Matrix. In: Royce, P.M. and Steinmann, B., Eds., *Connective Tissue and Its Heritable Disorders*, Wiley, New York, 159-221.

[26] Fung, P.C.W. and Kong, R.K.C. (2017) The Heat Shock Protein Story—From Taking mTORC1,2 and Heat Shock Protein Inhibitors as Therapeutic Measures for Treating Cancers to Development of Cancer Vaccines. *Journal of Cancer Therapy*, **8**, 962-1029. https://doi.org/10.4236/jct.2017.811086

[27] Buevich, A.V., Silva, T., Brodsky, B. and Baum, J. (2004) Transformation of the Mechanism of Triple-Helix Peptide Folding in the Absence of a C-Terminal Nucleation Domain and Its Implications for Mutations in Collagen Disorders. *The Journal of Biochemistry*, **279**, 46890-46895.

[28] Nagata, K. (1996) Hsp47: A Collagen-Specific Molecular Chaperone. *Trends in Biochemical Sciences*, **21**, 23-26. https://doi.org/10.1016/S0968-0004(06)80023-X

[29] Silver, F.H. (2009) The Importance of Collagen Fibers in Vertebrate Biology. *Journal of Engineered Fibers and Fabrics*, **4**, 9-17. https://doi.org/10.1177/155892500900400203

[30] Veis, A. and Yuan, L. (1975) Structure of the Collagen Microfibril. A Four-Strand Overlap Model. *Biopolymers*, **14**, 895-900. https://doi.org/10.1002/bip.1975.360140418

[31] Liu, J-.F. and He, J.-H. (2010) Hierarchical Structure and Fractal Dimensions of Tendon. *Materials Science and Technology*, **26**, 1317-1319. https://doi.org/10.1179/026708310X12798718274232

[32] Werner, S. and Grose, R. (2003) Regulation of Wound Healing by Growth Factors and Cytokines. *Physiological Reviews*, **83**, 835-870.

https://doi.org/10.1152/physrev.2003.83.3.835

[33] Ge, Z., Goh, J.C.H. and Lee, E.H. (2005) The Effects of Bone Marrow-Derived Mesenchymal Stem Cells and Fascia Wrap Application to Anterior Cruciate Ligament Tissue Engineering. *Cell Transplantation*, **14**, 763-773. https://doi.org/10.3727/000000005783982486

[34] Mistriotis, P. and Andreadis, S.T. (2013) Hair Follicle: A Novel Source of Multipotent Stem Cells for Tissue Engineering and Regenerative Medicine. *Tissue Engineering Part B*: *Reviews*,**19**, 265-278. https://doi.org/10.1089/ten.teb.2012.0422

[35] Gattazzo, F., Urciuolo, A. and Bonaldo, P. (2014) Extracellular Matrix: A Dynamic Microenvironment for Stem Cell Niche. *Biochimica et Biophysica Acta* (*BBA*)-*General Subjects*, **1840**, 2506-2519. https://doi.org/10.1016/j.bbagen.2014.01.010

[36] http://www.histology.leeds.ac.uk/bone/cartilage.php

[37] Seeman, E. (2006) Chapter 13. Bone Structure and Strength. In: Seibel, M.J., Robins, S.P. and Bilezikian, J.P., Eds., *Dynamics of Bone and Cartilage Metabolism*: *Principles and Clinical Applications*, 2nd Edition, Academic Press, London, 213-220.

[38] Histology Guide © Faculty of Biological Sciences, University of Leeds. http://www.mhhe.com/biosci/ap/histology_mh/cartilag.html

[39] Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S. and Marshak, D.R. (1999) Multilineage Potential of Adult Human Mesenchymal Stem Cells. *Science*, **284**, 143-147. https://doi.org/10.1126/science.284.5411.143

[40] Arana-Chavez, V.E., Soares, A.M. and Katchburian, E. (1995) Junctions between Early Developing Osteoblasts of Rat Calvaria as Revealed by Freeze-Fracture and Ultrathin Section Electron Microscopy. *Archives of Histology and Cytology*, **58**, 285-292.

[41] Blair, H.C., Sun, L. and Kohanski, R.A. (2007) Balanced Regulation of Proliferation, Growth, Differentiation, and Degradation in Skeletal Cells. *Annals of the New York Academy of Sciences*, **1116**, 165-173. https://doi.org/10.1196/annals.1402.029

[42] Gurish, M.F., Tao, H., Abonia, J.P., Arya, A., Friend, D.S., Parker, C.M. and Austen, K.F. (2001) Intestinal Mast Cell Progenitors Require CD49dβ7 (α4β7 Integrin) for Tissue-Specific Homing. *The Journal of Experimental Medicine*, **194**, 1243-1252. https://doi.org/10.1084/jem.194.9.1243

[43] Rodewald, H.R., Dessing, M., Dvorak, A.M. and Galli, S.J. (1996) Identification of a Committed Precursor for the Mast Cell Lineage. *Science*, **271**, 818-822. https://doi.org/10.1126/science.271.5250.818

[44] Metcalfe, D.D., Baram, D. and Mekori, Y.A. (1997) Mast Cells. *Physiological Reviews*, **77**, 1033-1079. https://doi.org/10.1152/physrev.1997.77.4.1033

[45] Artuc, M., Steckelings, U.M. and Henz, B.M. (2002) Mast Cell-Fibroblast Interactions: Human Mast Cells as Source and Inducers of Fibroblast and Epithelial Growth Factors. *Journal of Investigative Dermatology*, **118**, 391-395. https://doi.org/10.1046/j.0022-202x.2001.01705.x

[46] Dioguardi, N., Grizzi, F., Bossi, P. and Roncalli, M. (1999) Fractal and Spectral Dimension Analysis of Liver Fibrosis in Needle Biopsy Specimens. *Analytical and Quantitative Cytology and Histology*, **21**, 262-266.

[47] Magnusson, S.P., Qvortrup, K., Larsen, J.O., Rosager, S., Hanson, P., Aagaard, P., Krogsgaard, M. and Kjaer, M. (2002) Collagen Fibril Size and Crimp Morphology in Ruptured and Intact Achilles Tendons. *Matrix Biology*, **21**, 369-377. https://doi.org/10.1016/S0945-053X(02)00011-2

[48] Silver, F.H., Freeman, J.W. and Gwinder, P.S. (2003) Collagen Self-Assembly and the Development of Tendon Mechanical Properties. *Journal of Biomechanics*, **36**, 1529-1553. https://doi.org/10.1016/S0021-9290(03)00135-0

[49] Ho, M.W. and Saunders, P.T. (1994) Liquid Crystalline Mesophases in Living Organisms. In: Ho, M.W., Popp, F.A. and Warnke, U., Eds., *Bioelectrodynamcis and Biocommunications*, World Scientific, Singapore, 213-227. https://doi.org/10.1142/9789814503822_0008

[50] Martin, R., Farjanel, J., Eichenberger, D., Colige, A., Kessler, E., Hulmes, D.J.S. and Giraud-Guille, M.M. (2000) Liquid Crystalline Ordering of Procollagen as a Determinant of Three-Dimensional Extracellular Matrix Architecture. *Journal of Molecular Biology*, **301**, 11-17. https://doi.org/10.1006/jmbi.2000.3855

[51] Snedeker, J.G. and Foolen, J. (2017) Tendon Injury and Repair—A Perspective on the Basic Mechanisms of Tendon Disease and Future Clinical Therapy. *Acta Biomaterialia*, **63**, 18-36. https://doi.org/10.1016/j.actbio.2017.08.032

[52] Guilak, F., Cohen, D.M., Estes, B.T., Gimble, J.M., Liedtke, W. and Chen, C.S. (2009) Control of Stem Cell Fate by Physical Interactions with the Extracellular Matrix. *Cell Stem Cell*, **5**, 17-26. https://doi.org/10.1016/j.stem.2009.06.016

[53] Itoh, M., Lin, Y., Yang, Z., Nguyen, J., Liang, F., Morris, R.J. and Cotsarelis, G. (2005) Stem Cells in the Hair Follicle Bulge Contribute to Wound Repair but Not to Homeostasis of the Epidermis. *Nature Medicine*, **11**, 1351-1354. https://doi.org/10.1038/nm1328

[54] Liu, C.-F., Aschbacher-Smith, L., Barthelery, N.J., Dyment, N., Butler, D. and Wylie, C. (2011) What We Should Know before Using Tissue Engineering Techniques to Repair Injured Tendons: A Developmental Biology Perspective. *Tissue Engineering Part B*: *Reviews*, **17**, 165-176. https://doi.org/10.1089/ten.teb.2010.0662

[55] Ackermann, P.W., Franklin, S.L., Dean, B.J., Carr, A.J., Salo, P.T. and Hart, D.A. (2014) Neuronal Pathways in Tendon Healing and Tendinopathy—Update. *Frontiers in Bioscience* (*Landmark Ed*), **19**, 1251-1278. https://doi.org/10.2741/4280

[56] Ackermann, P.W., Salo, P. and Hart, D.A. (2016) Tendon Innervation. In: Ackermann, P. and Hart, D., Eds., *Metabolic Influences on Risk for Tendon Disorders*. *Advances in Experimental Medicine and Biology*, Vol. 920, Springer, Cham, 35-51. https://doi.org/10.1007/978-3-319-33943-6_4

[57] Franchi, M., Fini, M., Quaranta, M., Pasquale, V.D., Raspanti, M., Giavaresi, G., Ottani, V. and Ruggeri, A. (2007) Crimp Morphology in Relaxed and Stretched Rat Achilles Tendon. *Journal of Anatomy*, **210**, 1-7. https://doi.org/10.1111/j.1469-7580.2006.00666.x

[58] Franchi, M., Ottani, V., Stagni, R. and Ruggeri, A. (2010) Tendon and Ligament Fibrillar Crimps Give Rise to Left-Handed Helices of Collagen Fibrils in Both Planar and Helical Crimps. *Journal of Anatomy*, **216**, 301-309. https://doi.org/10.1111/j.1469-7580.2009.01188.x

[59] Icardo, J.M., Elvira Colvee, E. and Revuelta, J.M. (2013) Structural Analysis of Chordae Tendineae in Degenerative Disease of the Mitral Valve. *International Journal of Cardiology*, **167**, 1603-1609. https://doi.org/10.1016/j.ijcard.2012.04.092

[60] Xu, J., Rodriguez, D., Petitclerc, E., Kim, J.J., Hangai, M., Yuen, S.M., Davis, G.E. and Brooks, P.C. (2001) Proteolytic Exposure of a Cryptic Site within Collagen Type IV Is Required for Angiogenesis and tumor Growth *in Vivo*. *The Journal of Cell Biology*, **154**, 1069-1079. https://doi.org/10.1083/jcb.200103111

[61] MMP1 Matrix Metallopeptidase 1 [Homo Sapiens (Human)], Gene ID: 4312, updated on 25-Nov-2018, Full Report, NCBI.

[62] MMP2 Matrix Metallopeptidase 2 [Homo Sapiens (Human)], Gene ID: 4313, updated on 2-Dec-2018, Full Report, NCBI.

[63] MMP3 Matrix Metallopeptidase 2 [Homo Sapiens (Human)], Gene ID: 4314, updated on 22-Nov-2018, Full Report, NCBI.

[64] MMP8 Matrix Metallopeptidase 8 [Homo Sapiens (Human)], Gene ID: 4317, updated on 22-Nov-2018, Full Report, NCBI.

[65] MMP9 Matrix Metallopeptidase 9 [Homo Sapiens (Human)], Gene ID: 4318, updated on 2-Dec-2018, Full Report, NCBI.

[66] MMP10 Matrix Metallopeptidase 10 [Homo Sapiens (Human)], Gene ID: 4319, updated on 22-Nov-2018, Full Report, NCBI.

[67] MMP13 Matrix Metallopeptidase 13 [Homo Sapiens (Human)], Gene ID: 4322, updated on 24-Nov-2018, Full Report, NCBI.

[68] MMP14 Matrix Metallopeptidase 14 [Homo Sapiens (Human)], Gene ID: 4323, updated on 24-Nov-2018, Full Report, NCBI.

[69] Itoh, Y. (2015) Membrane-Type Matrix Metalloproteinases: Their Functions and Regulations. *Matrix Biology*, **44-46**, 207-223. https://doi.org/10.1016/j.matbio.2015.03.004

[70] MMP15 Matrix Metallopeptidase 15 [Homo Sapiens (Human)], Gene ID: 4324, updated on 22-Nov-2018, Full Report, NCBI.

[71] Ramachandran, G.N. and Kartha, G. (1955) Structure of Collagen. *Nature*, **176**, 593-595. https://doi.org/10.1038/176593a0

[72] Orgel, J.P., Irving, T.C., Miller, A. and Wess, T.J. (2006) Microfibrillar Structure of Type I Collagen *in Situ*. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 9001-9005. https://doi.org/10.1073/pnas.0502718103

[73] Overall, C.M. (2002) Molecular Determinants of Metalloproteinase Substrate Specificity: Matrix Metalloproteinase Substrate Binding Domains, Modules, and Exosites. *Molecular Biotechnology*, **22**, 51-86. https://doi.org/10.1385/MB:22:1:051

[74] Rosenblum, G., Van den Steen, P.E., Cohen, S.R., Bitler, A., Brand, D.D., Opdenakker, G. and Sagi, I. (2010) Direct Visualization of Protease Action on Collagen Triple Helical Structure. *PLoS ONE*, **5**, e11043. https://doi.org/10.1371/journal.pone.0011043

[75] Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. (1997) Membrane Type 1 Matrix Metalloproteinase Digests Interstitial Collagens and Other Extracellular Matrix Macromolecules. *The Journal of Biological Chemistry*, **272**, 2446-2451. https://doi.org/10.1074/jbc.272.4.2446

[76] Li, J., Brick, P., O'Hare, M.C., Skarzynski, T., Lloyd, L.F., Curry, V.A., Clark, I.M., Bigg, H.F., Hazleman, B.L., Cawston, T.E. and Blow, D.M. (1995) Structure of Full-Length Porcine Synovial Collagenase Reveals a C-Terminal Domain Containing a Calcium-Linked, Four-Bladed β-Propeller. *Structure*, **3**, 541-549. https://doi.org/10.1016/S0969-2126(01)00188-5

[77] Murphy, G. and Nagase, H. (2011) Localizing Matrix Metalloproteinase Activities in the Pericellular Environment. *The FEBS Journal*, **278**, 2-15. https://doi.org/10.1111/j.1742-4658.2010.07918.x

[78] Manka, S.W., Carafoli, F., Visse, R., Bihan, D., Raynal, N., Farndale, R.W., Murphy, G., Enghild, J.J., Hohenester, E. and Nagase, H. (2012) Structural Insights into Triple-Helical Collagen Cleavage by Matrix Metalloproteinase 1. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 12461-12466. https://doi.org/10.1073/pnas.1204991109

[79] Welgus, H.G., Stricklin, G.P., Eisen, A.Z., Bauer, E.A., Cooney, R.V. and Jeffrey, J.J. (1979) A Specific Inhibitor of Vertebrate Collagenase Produced by Human Skin Fibroblasts. *The Journal of Biological Chemistry*, **254**, 1938-1943.

[80] Vater, C.A., Mainardi, C.L. and Harris, E.D. (1979) Inhibitor of Human Collagenase from Cultures of Human Tendon. *Journal of Biological Chemistry*, **254**, 3045-3053.

[81] TIMP1 TIMP Metallopeptidase Inhibitor 1 [Homo Sapiens (Human)], Gene ID: 7076, updated on 7-Dec-2018, Full Report, NCBI.

[82] Taraboletti, G., Morbidelli, L., Donnini, S., Parenti, A., Granger, H.J., Giavazzi, R. and Ziche, M. (2000) The Heparin Binding 25 kDa Fragment of Thrombospondin-1 Promotes Angiogenesis and Modulates Gelatinase and TIMP2 Production in Endothelial Cells. *The FASEB Journal*, **14**, 1674-1676. https://doi.org/10.1096/fj.99-0931fje

[83] TIMP2 TIMP Metallopeptidase Inhibitor 2 [Homo Sapiens (Human)], Gene ID: 7077, updated on 7-Dec-2018, Full Report, NCBI.

[84] Arris, C.E., Bevitt, D.J., Mohamed, J., Li, Z., Langton, K.P., Barker, M.D., Clarke, M.P. and McKie, N. (2003) Expression of Mutant and Wild-Type TIMP3 in Primary Gingival Fibroblasts from Sorsby's Fundus Dystrophy Patients. *Biochimica et Biophysica Acta* (*BBA*)-*Molecular Basis of Disease*, **1638**, 20-28. https://doi.org/10.1016/S0925-4439(03)00036-X

[85] Thomas Fréour, T., Jarry, A., Bach-Ngohou, K., Dejoie, T., Bou-Hanna, C., Denis, M.G., Mosnier, J.F., Laboisse, C.L. and Masson, D. (2009) TACE Inhibition Amplifies TNF-α-Mediated Colonic Epithelial Barrier Disruption. *International Journal of Molecular Medicine*, **23**, 41-48.

[86] Qi, J.H., Ebrahem, Q., Moore, N., Murphy, G., Claesson-Welsh, L., Bond, M., Andrew Baker, A. and Anand-Apte, B. (2003) A Novel Function for Tissue Inhibitor of Metalloproteinases-3 (TIMP3): Inhibition of Angiogenesis by Blockage of VEGF Binding to VEGF Receptor-2. *Nature Medicine*, **9**, 407-415. https://doi.org/10.1038/nm846

[87] TIMP3 TIMP Metallopeptidase Inhibitor 3 [Homo Sapiens (Human)], Gene ID: 7078, updated on 8-Dec-2018, Full Report, NCBI.

[88] Casagrande, V., Mauriello, A., Bischetti, S., Mavilio, M., Federici, M. and Menghin, R. (2017) Hepatocyte Specific TIMP3 Expression Prevents Diet Dependent Fatty Liver Disease and Hepatocellular Carcinoma. *Scientific Reports*, **7**, Article No. 6747. https://doi.org/10.1038/s41598-017-06439-x

[89] TIMP4 TIMP Metallopeptidase Inhibitor 4 [Homo Sapiens (Human)], Gene ID: 7079, updated on 7-Dec-2018, Summary, NCBI.

[90] Lizarraga, F., Espinosa, M., Ceballos-Cancino, G., Vazquez-Santillan, K., Bahena-Ocampo, I., Schwarz-Cruzy Celis, A., Vega-Gordillo, M., Garcia Lopez, P., Maldonado, V. and Melendez-Zajgla, J. (2016) Tissue Inhibitor of Metalloproteinases-4 (TIMP-4) Regulates Stemness in Cervical Cancer Cells. *Molecular Carcinogenesis*, **55**, 1952-1961. https://doi.org/10.1002/mc.22442

[91] Lizarraga, F., Ceballos-Cancino, G., Espinosa, M., Vazquez-Santillan, K., Maldonado, V. and Melendez-Zajgla, J. (2015) Tissue Inhibitor of Metalloproteinase-4 Triggers Apoptosis in Cervical Cancer Cells. *PLoS ONE*, **10**, e0135929. https://doi.org/10.1371/journal.pone.0135929

[92] Haerian, B.S., Sha'ari, H.M., Fong, C.Y., Tan, H.J., Wong, S.W., Ong, L.C., Raymond, A.A., Tan, C.T. and Mohamed, Z. (2015) Contribution of *TIMP*4 rs3755724 Polymorphism to Susceptibility to Focal Epilepsy in Malaysian Chinese. *Journal of Neuroimmunology*, **278**, 137-143. https://doi.org/10.1016/j.jneuroim.2014.12.016

[93] Groft, L.L., Muzik, H., Rewcastle, N.B., Johnston, R.N., Knäuper, V., Lafleur, M.A., Forsyth, P.A. and Edwards, D.R. (2001) Differential Expression and Localization of TIMP-1 and TIMP-4 in Human Gliomas. *British Journal of Cancer*, **85**, 55-63. https://doi.org/10.1054/bjoc.2001.1854

[94] Kannus, P. and Józsa, L. (1991) Histopathological Changes Preceding Spontaneous Rupture of a Tendon. A Controlled Study of 891 Patients. *The Journal of Bone and Joint Surgery*, **73**, 1507-1525. https://doi.org/10.2106/00004623-199173100-00009

[95] Veres, S.P. and Lee, J.M. (2012) Designed to Fail: A Novel Mode of Collagen Fibril Disruption and Its Relevance to Tissue Toughness. *Biophysical Journal*, **102**, 2876-2884. https://doi.org/10.1016/j.bpj.2012.05.022

[96] Veres, S.P., Harrison, J.M. and Lee, J.M. (2014) Mechanically Overloading Collagen Fibrils Uncoils Collagen Molecules, Placing Them in a Stable, Denatured State. *Matrix Biology*, **33**, 54-59. https://doi.org/10.1016/j.matbio.2013.07.003

[97] Baldwin, S.J., Kreplak, L. and Lee, J.M. (2016) Characterization via Atomic Force Microscopy of Discrete Plasticity in Collagen Fibrils from Mechanically Overloaded Tendons: Nano-Scale Structural Changes Mimic Rope Failure. *Journal of the Mechanical Behavior of Biomedical Materials*, **60**, 356-366. https://doi.org/10.1016/j.jmbbm.2016.02.004

[98] Medalia, O. and Geiger, B. (2010) Frontiers of Microscopy-Based Research into Cell-Matrix Adhesions. *Current Opinion in Cell Biology*, **22**, 659-668. https://doi.org/10.1016/j.ceb.2010.08.006

[99] Knight, D.P. and Vollrath, F. (2002) Biological Liquid Crystal Elastomers. *Philosophical Transactions of the Royal Society B*, **357**, 155-163. https://doi.org/10.1098/rstb.2001.1030

[100] Hulmes, D.J.S. (2002) Building Collagen Molecules, Fibrils, and Suprafibrillar Structures. *Journal of Structural Biology*, **137**, 2-10. https://doi.org/10.1006/jsbi.2002.4450

[101] Hamley, I.W. (2010) Liquid Crystal Phase Formation by Biopolymers. *Soft Matter*, **6**, 1863-1871. https://doi.org/10.1039/b923942a

[102] Diamant, J., Keller, A., Baer, E., Litt, M. and Arridge, R.G. (1972) Collagen; Ultrastructure and Its Relation to Mechanical Properties as a Function of Ageing. *Proceedings of the Royal Society B: Biological Sciences*, **180**, 293-315. https://doi.org/10.1098/rspb.1972.0019

[103] Järvinen, T.A., Järvinen, T.L., Kannus, P., Józsa, L. and Järvinen, M. (2004) Collagen Fibres of the Spontaneously Ruptured Human Tendons Display Decreased Thickness and Crimp Angle. *Journal of Orthopaedic Research*, **22**, 1303-1309. https://doi.org/10.1016/j.orthres.2004.04.003

[104] Bass, E.C., Wistrom, E.V., Diederich, C.J., Nau, W.H., Pellegrino, R., Ruberti, J. and Lotz, J.C. (2004) Heat Induced Changes in Porcine Annulus Fibrosus Biomechanics. *Journal of Biomechanics*, **37**, 233-240. https://doi.org/10.1016/j.jbiomech.2003.07.002

[105] Nabeshima, Y., Grood, E.S., Sakurai, A. and Herman, J.H. (1996) Uniaxial Tension Inhibits Tendon Collagen Degradation by Collagenase *in Vitro*. *Journal of Orthopaedic Research*, **14**, 123-130. https://doi.org/10.1002/jor.1100140120

[106] Ruberti, J.W. and Hallab, N.J. (2005) Strain-Controlled Enzymatic Cleavage of Collagen in Loaded Matrix. *Biochemical and Biophysical Research Communications*, **336**, 483-489. https://doi.org/10.1016/j.bbrc.2005.08.128

[107] Wyatt, K.E., Bourne, J.W. and Torzilli, P.A. (2009) Deformation-Dependent En-

zyme Mechanokinetic Cleavage of Type I Collagen. *Journal of Biomechanical Engineering*, **131**, Article ID: 051004. https://doi.org/10.1115/1.3078177

[108] Bhole, A.P., Flynn, B.P., Liles, M., Saeidi, N., Dimarzio, C.A. and Ruberti, J.W. (2009) Mechanical Strain Enhances Survivability of Collagen Micronetworks in the Presence of Collagenase: Implications for Load-Bearing Matrix Growth and Stability. *Philosophical Transactions of the Royal Society A*: *Mathematical, Physical and Engineering Sciences*, **367**, 3339-3362. https://doi.org/10.1098/rsta.2009.0093

[109] Flynn, B.P., Bhole, A.P., Saeidi, N., Liles, M., DiMarzio, C.A. and Ruberti, J.W. (2010) Mechanical Strain Stabilizes Reconstituted Collagen Fibrils against Enzymatic Degradation by Mammalian Collagenase Matrix Metalloproteinase 8 (MMP-8). *PLoS ONE*, **5**, e12337. https://doi.org/10.1371/journal.pone.0012337

[110] Wu, H., Byrne, M.H., Stacey, A., Goldring, M.B., Birkhead, J.R., Jaenisch, R. and Krane, S.M. (1990) Generation of Collagenase-Resistant Collagen by Site-Directed Mutagenesis of Murine Pro Alpha 1(I) Collagen Gene. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 5888-5892. https://doi.org/10.1073/pnas.87.15.5888

[111] Flynn, B.P., Tilburey, G.E. and Ruberti, J.W. (2013) Highly Sensitive Single-Fibril Erosion Assay Demonstrates Mechanochemical Switch in Native Collagen Fibrils. *Biomechanics and Modeling in Mechanobiology*, **12**, 291-300. https://doi.org/10.1007/s10237-012-0399-2

[112] Welgus, H.G., Jeffrey, J.J., Stricklin, G.P., Roswit, W.T. and Eisen, A.Z. (1980) Characteristics of the Action of Human Skin Fibroblast Collagenase on Fibrillar Collagen. *The Journal of Biological Chemistry*, **255**, 6808-6813.

[113] Toledano, M., Aguilera, F.S., Yamauti, M., Ruiz-Requena, M.E. and Osorio, R. (2013) *In Vitro* Load-Induced Dentin Collagen-Stabilization against MMPs Degradation. *Journal of the Mechanical Behavior of Biomedical Materials*, **27**, 10-18. https://doi.org/10.1016/j.jmbbm.2013.06.002

[114] Tonge, T.K., Ruberti, J.W. and Nguyen, T.D. (2015) Micromechanical Modeling Study of Mechanical Inhibition of Enzymatic Degradation of Collagen Tissues. *Biophysical Journal*, **109**, 2689-2700. https://doi.org/10.1016/j.bpj.2015.10.051

[115] Chithra, P., Sajithlal, G.B. and Chandrakasan, G. (1998) Influence of *Aloe vera* on Collagen Characteristics in Healing Dermal Wounds in Rats. *Molecular and Cellular Biochemistry*, **181**, 71-76. https://doi.org/10.1023/A:1006813510959

[116] Heinemeier, K.M., Schjerling, P., Heinemeier, J., Magnusson, S.P. and Kjaer, M. (2013) Lack of Tissue Renewal in Human Adult Achille Tendon Is Revealed by Nuclear Bomb ^{14}C. *The FASEB Journal*, **27**, 2074-2079. https://doi.org/10.1096/fj.12-225599

[117] Leikina, E., Mertts, M.V., Kuznetsova, N. and Leikin, S. (2002) Type I Collagen Is Thermally Unstable at Body Temperature. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 1314-1318. https://doi.org/10.1073/pnas.032307099

[118] Bruckner, P. and Eikenberry, E.F. (1984) Procollagen Is More Stable *in Cellulo* than *in Vitro*. *European Journal of Biochemistry*, **140**, 397-399. https://doi.org/10.1111/j.1432-1033.1984.tb08115.x

[119] Steinmann, B., Bruckner, P. and Superti-Furga, A. (1991) Cyclosporin A Slows Collagen Triple-Helix Formation *in Vivo*: Indirect Evidence for a Physiologic Role of Peptidyl-Prolyl Cis-Trans-Isomerase. *The Journal of Biological Chemistry*, **266**, 1299-1303.

[120] Kadler, K.E., Hojima, Y. and Prockop, D.J. (1988) Assembly of Type I Collagen Fi-

brils *de Novo*. Between 37 and 41 Degrees C the Process Is Limited by Micro-Unfolding of Monomers. *Journal of Biological Chemistry*, **263**, 10517-10523.

[121] Bell, M.L. and Engvall, E. (1982) The Specific Detection of Collagenous Proteins after Electrophoresis Using Enzyme-Conjugated Collagen-Binding Fibronectin Fragments. *Analytical Biochemistry*, **123**, 329-335. https://doi.org/10.1016/0003-2697(82)90454-7

[122] Bachinger, H.P., Bruckner, P., Timpl, R., Prockop, D.J. and Engel, J. (1980) Folding Mechanism of the Triple Helix in Type-III Collagen and Type-III pN-Collagen: Role of Disulfide Bridges and Peptide Bond Isomerization. *The FEBS Journal*, **106**, 619-632. https://doi.org/10.1111/j.1432-1033.1980.tb04610.x

[123] Bruckner, P. and Prockop, D.J. (1981) Proteolytic Enzymes as Probes for the Triple-Helical Conformation of Procollagen. *Analytical Biochemistry*, **110**, 360-368. https://doi.org/10.1016/0003-2697(81)90204-9

[124] Davis, J.M. and Bachinger, H.P. (1993) Hysteresis in the Triple Helix-Coil Transition of Type III. *The Journal of Biological Chemistry*, **268**, 25965-25972.

[125] Nerenberg, P.S. and Stultz, C.M. (2008) Differential Unfolding of α1 and α2 Chains in Type I Collagen and Collagenolysis. *Journal of Molecular Biology*, **382**, 246-256. https://doi.org/10.1016/j.jmb.2008.07.009

[126] Miles, C.A., Sims, T.J., Camacho, N.P. and Bailey, A.J. (2002) The Role of α2 Chain in the Stabilization of the Collagen Type I Heterotrimer: A Study of the Type I Homotrimer in *oim* Mouse Tissues. *Journal of Molecular Biology*, **321**, 797-805. https://doi.org/10.1016/S0022-2836(02)00703-9

[127] Han, S., Makareeva, E., Kuznetsova, N.V., DeRidder, A.M., Sutter, M.B., Losert, W., Phillips, C.L., Visse, R., Nagase, H. and Leikin, S. (2010) Molecular Mechanism of Type I Collagen Homotrimer Resistance to Mammalian Collagenases. *The Journal of Biological Chemistry*, **285**, 22276-22281. https://doi.org/10.1074/jbc.M110.102079

[128] Makareeva, E., Han, S., Vera, J.C., Sackett, D.L., Holmbeck, K., Phillips, C.L., Visse, R., Nagase, H. and Leikin, S. (2010) Carcinomas Contain a Matrix Metalloproteinase-Resistant Isoform of Type I Collagen Exerting Selective Support to Invasion. *Cancer Research*, **70**, 4366-4374. https://doi.org/10.1158/0008-5472.CAN-09-4057

[129] Huang, K.A., Yi, B.R. and Choi, K.C. (2011) Molecular Mechanisms and *in Vivo* Mouse Models of Skin Aging Associated with Dermal Alterations. *Laboratory Animal Research*, **27**, 1-8. https://doi.org/10.5625/lar.2011.27.1.1

[130] Hakala, M., Risteli, L., Manelius, J., Nieminen, P. and Risteli, J. (1993) Increased Type I Collagen Degradation Correlates with Disease Severity in Rheumatoid Arthritis. *Annals of the Rheumatic Diseases*, **52**, 866-869. https://doi.org/10.1136/ard.52.12.866

[131] González, A., López, B., Querejeta, R. and Díez, J. (2002) Regulation of Myocardial Fibrillar Collagen by Angiotensin II. A Role in Hypertensive Heart Disease? *Journal of Molecular and Cellular Cardiology*, **34**, 1585-1593. https://doi.org/10.1006/jmcc.2002.2081

[132] Lombardi, R., Betocchi, S., Losi, M.A., Tocchetti, C.G., Aversa, M., Miranda, M., Alessandro, G.D., Cacace, A., Ciampi, Q. and Chiariello, M. (2003) Collagen Turnover in Hypertrophic Cardiomyopathy. *Circulation*, **108**, 1455-1460. https://doi.org/10.1161/01.CIR.0000090687.97972.10

[133] Misof, K., Landis, W.J., Klaushofer, K. and Fratzl, P. (1997) Collagen from the Osteogenesis Imperfecta Mouse Model (*oim*) Shows Reduced Resistance against Tensile Stress. *Journal of Clinical Investigation*, **100**, 40-45.

https://doi.org/10.1172/JCI119519

[134] McBride, D.J., Choe, V., Shapiro, J.R. and Brodsky, B. (1997) Altered Collagen Structure in Mouse Tail Tendon Lacking the *α*2(I) Chain. *Journal of Molecular Biology*, **270**, 275-284. https://doi.org/10.1006/jmbi.1997.1106

[135] Phillips, C.L., Pfeiffer, B.J., Luger, A.M. and Franklin, C.L. (2002) Novel Collagen Glomerulopathy in a Homotrimeric Type I Collagen Mouse (*oim*). *Kidney International*, **62**, 383-391. https://doi.org/10.1046/j.1523-1755.2002.00451.x

[136] Gay, S., Vijanto, J., Raekallio, J. and Penttinen, R. (1978) Collagen Types in Early Phases of Wound Healing in Children. *Acta Chirurgica Scandinavica*, **144**, 205-211.

[137] Wooley, P.H., Luthra, H.S., Stuart, J.M. and David, C.S. (1981) Type II Collagen-Induced Arthritis in Mice. I. Major Histocompatibility Complex (I Region) Linkage and Antibody Correlates. *The Journal of Experimental Medicine*, **154**, 688-700. https://doi.org/10.1084/jem.154.3.688

[138] Maroudas, A., Palla, G. and Gilav, E. (1992) Racemization of Aspartic Acid in Human Articular Cartilage. *Connective Tissue Research*, **28**, 161-169. https://doi.org/10.3109/03008209209015033

[139] Maffulli, N., Khan, K.M. and Puddu, G. (1998) Overuse Tendon Conditions: Time to Change a Confusing Terminology. *Arthroscopy*, **14**, 840-843. https://doi.org/10.1016/S0749-8063(98)70021-0

[140] Xu, Y. and Murrell, G.A. (2008) The Basic Science of Tendinopathy. *Clinical Orthopaedics and Related Research*, **466**, 1528-1538. https://doi.org/10.1007/s11999-008-0286-4

[141] Khan, K., Cook, J., Kannus, P., Maffulli, N. and Bonar, S. (2002) Time to Abandon the "Tendinitis" Myth: Painful, Overuse Tendon Conditions Have a Non-Inflammatory Pathology. *BMJ*, **324**, 626-627. https://doi.org/10.1136/bmj.324.7338.626

[142] Riley, G. (2005) Chronic Tendon Pathology: Molecular Basis and Therapeutic Implications. *Expert Reviews in Molecular Medicine*, **7**, 1-25. https://doi.org/10.1017/S1462399405008963

[143] Sharma, P. and Maffulli, N. (2005) Tendon Injury and Tendinopathy: Healing and Repair. *The Journal of Bone and Joint Surgery*, **87**, 187-202.

[144] Ralphs, J.R. (2002) Cell Biology of Tendons. *European Cells & Materials Journal*, **4**, 39-40.

[145] Wall, M.E., Otey, C., Qi, J. and Banes, A.J. (2006) Connexin 43 Is Localized with Actin in Tenocytes. *Cell Motility*, **64**, 121-130. https://doi.org/10.1002/cm.20170

[146] Maeda, E., Pian, H. and Ohashi, T. (2017) Temporal Regulation of Gap Junctional Communication between Tenocytes Subjected to Static Tensile Strain with Physiological and Nonphysiological Amplitudes. *Biochemical and Biophysical Research Communications*, **482**, 1170-1175. https://doi.org/10.1016/j.bbrc.2016.12.007

[147] Wenstrup, R.J., Florer, J.B., Brunskill, E.W., Bell, S.M., Chervoneva, I. and Birk, D.E. (2004) Type V Collagen Controls the Initiation of Collagen Fibril Assembly. *The Journal of Biological Chemistry*, **279**, 53331-53337. https://doi.org/10.1074/jbc.M409622200

[148] da Silva, M.L., Caplan, A.I. and Nardi, N.B. (2008). In Search of the *in Vivo* Identity of Mesenchymal Stem Cells. *Stem Cells*, **26**, 2287-2299. https://doi.org/10.1634/stemcells.2007-1122

[149] Kalamajski, S. and Oldberg, Å. (2010) The Role of Small Leucine-Rich Proteoglycans in Collagen Fibrillogenesis. *Matrix Biology*, **29**, 248-253. https://doi.org/10.1016/j.matbio.2010.01.001

[150] Perryman, S.V. and Sylvester, K.G. (2006) Repair and Regeneration: Opportunities for Carcinogenesis from Tissue Stem Cells. *Journal of Cellular and Molecular Medicine*, **10**, 292-308. https://doi.org/10.1111/j.1582-4934.2006.tb00400.x

[151] Smith, A.A., Li, J., Liu, B., Hunter, D., Pyles, M., Gilletle, M., Dhamdhere, G.R., Abo, A., Oro, A., Helms, J.A. (2016) Activating Hair Follicle Stem Cells via R-spondin2 to Stimulate Hair Growth, *J. Investigative Dermatology*, **136**(8):1549-1558. https://doi.org/10.1016/j.jid.2016.01.041

[152] Donnelly, E., Ascenzi, M.G. and Farnum, C. (2010) Primary Cilia Are Highly Oriented with Respect to Collagen Direction and Long Axis of Extensor Tendon. *Journal of Orthopaedic Research*, **28**, 77-82.

[153] Matthews, T.J.W., Hand, G.C., Rees, J.L., Athanasou, N.A. and Carr, A.J. (2006) Pathology of the Torn Rotator Cuff Tendon, Reduction in Potential for Repair as Tear Size Increases. *The Bone and Joint Journal*, **88**, 489-495.

[154] Archambault, J., Tsuzaki, M., Herzog, W. and Banes, A.J. (2006) Stretch and Interleukin-1β Induce Matrix Metalloproteinases in Rabbit Tendon Cells *in Vitro*. *Journal of Orthopaedic Research*, **20**, 36-39. https://doi.org/10.1016/S0736-0266(01)00075-4

[155] Tsuzaki, M., Guyton, G., Garrett, W., Archambault, J.M., Herzog, W., Almekinders, L., Bynum, D., Yang, X. and Banes, A.J. (2003) IL-1β Induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1β and IL-6 in Human Tendon Cells. *Journal of Orthopaedic Research*, **21**, 256-264. https://doi.org/10.1016/S0736-0266(02)00141-9

[156] Millar, N.L., Wei, A.Q., Molloy, T.J., Bonar, F. and Murrell, G.A. (2009) Cytokines and Apoptosis in Supraspinatus Tendinopathy. *The Bone & Joint Journal*, **91B**, 417-424. https://doi.org/10.1302/0301-620X.91B3.21652

[157] Schoenenberger, A.D., Foolen, J., Moor, P., Silvan, U., Jess, G. and Snedeker, J.G. (2018) Substrate Fiber Alignment Mediates Tendon Cell Response to Inflammatory Signalling. *Acta Biomaterialia*, **71**, 306-317. https://doi.org/10.1016/j.actbio.2018.03.004

[158] Phelan, K. and May, K.M. (2015) Basic Techniques in Mammalian Cell Tissue Culture. *Current Protocols in Cell Biology*, **66**, 1.1.1-1.1.22.

[159] Zajac, E., Schweighofer, B., Kupriyanova, T.A., Juncker-Jensen, A., Minder, P., Quigley, J.P. and Deryugina, E.I. (2013) Angiogenic Capacity of M1- and M2-Polarized Macrophages Is Determined by the Levels of TIMP-1 Complexed with Their Secreted proMMP-9. *Blood*, **122**, 4054-4067. https://doi.org/10.1182/blood-2013-05-501494

[160] Zhang, B., Luo, Q., Sun, J., Xu, B., Ju, Y., Yang, L. and Song, G. (2015) MGF Enhances Tenocyte Invasion through MMP-2 Activity via the FAK-ERK1/2 Pathway. *Wound Repair and Regeneration*, **23**, 394-402. https://doi.org/10.1111/wrr.12293

[161] Grant, T.M., Thompson, M.S., Urban, J. and Yu, J. (2013) Elastic Fibres Are Broadly Distributed in Tendon and Highly Localized around Tenocytes. *Journal of Anatomy*, **222**, 573-579. https://doi.org/10.1111/joa.12048

[162] Kannus, P. (2000) Structure of the Tendon Connective Tissue. *Scandinavian Journal of Medicine & Science in Sports*, **10**, 312-320. https://doi.org/10.1034/j.1600-0838.2000.010006312.x

[163] Thorpe, C.T., Peffers, M.J., Simpson, D., Halliwell, E., Screen, H.R.C. and Clegg, P.D. (2016) Anatomical Heterogeneity of Tendon: Fascicular and Interfascicular Tendon Compartments Have Distinct Proteomic Composition. *Scientific Reports*, **6**, Article No. 20455. https://doi.org/10.1038/srep20455

[164] THBS4 Thrombospondin 4 [Homo Sapiens (Human)], Gene ID: 7060, updated on 7-Dec-2018, Full Report, NCBI.

[165] UniProtKB-P49747 (COMP_HUMAN) 2018.

[166] UniProtKB Q06828 (FMOD_HUMAN) 2018.

[167] DCN Decorin [Homo Sapiens (Human)] Gene ID: 1634, updated on 7-Dec-2018, Full Report, NCBI.

[168] Svensson, R.B., Smith, S.T., Moyer, P.J. and Magnusson, S.P. (2018) Effects of Maturation and Advanced Glycation on Tensile Mechanics of Collagen Fibrils from Rat Tail and Achilles Tendons. *Acta Biomaterialia*, **70**, 270-280. https://doi.org/10.1016/j.actbio.2018.02.005

[169] de Mos, M., van der Windt, A.E., Jahr, H., van Schie, H.T.M., Weinans, H., Verhaar, J.A.N. and van Osch, G.J.V.M. (2008) Can Plateletrich Plasma Enhance Tendon Repair? A Cell Culture Study. *The American Journal of Sports Medicine*, **36**, 1171-1178. https://doi.org/10.1177/0363546508314430

[170] Giusti, I., Sandra D'Ascenzo, S.D., Mancò, A., Stefano, G.D., Francesco, M.D., Rughetti, A., Mas, A.D., Properzi, G., Calvisi, V. and Dolo, V. (2014) Platelet Concentration in Platelet-Rich Plasma Affects Tenocyte Behavior *in Vitro*. *BioMed Research International*, **2014**, Article ID: 630870. https://doi.org/10.1155/2014/630870

[171] Lamkanfi, M. and Dixit, V.M. (2009) IL-33 Raises Alarm. *Immunity*, **31**, 5-7. https://doi.org/10.1016/j.immuni.2009.06.011

[172] Bartel, D.P. (2009) MicroRNAs: Target Recognition and Regulatory Functions. *Cell*, **136**, 215-233. https://doi.org/10.1016/j.cell.2009.01.002

[173] Millar, N.L., Gilchrist, D.S., Akbar, M., Reilly, J.H., Kerr, S.C., Campbell, A.L., Murrell, G.A.C., Liew, F.Y., Kurowska-Stolarska, M. and Mclnnes, L.B. (2015) MicroRNA29a Regulates IL-33-Mediated Tissue Remodelling in Tendon Disease. *Nature Communications*, **6**, Article No. 6774.

[174] Millar, N.L., Murrell, G.A.C. and McInnes, I.B. (2017) Inflammatory Mechanisms in Tendinopathy—Towards Translation. *Nature Reviews*, **13**, 110-112. https://doi.org/10.1038/nrrheum.2016.213

[175] Gee, M.D., Lynn, B. and Cotsell, B. (1997) The Relationship between Cutaneous C Fiber Type and Antidromic Vasodilatation in the Rabbit and the Rat. *Journal of Physiology*, **503**, 31-44. https://doi.org/10.1111/j.1469-7793.1997.031bi.x

[176] Lui, S.L., Chan, L.Y., Zhang, X.H., Zhu, W., Chan, T.M., Fung, P.C.W. and Lai, K.N. (2001) Effect of Mycophenolate Mofetil on Nitric Oxide Production and Inducible Nitric Oxide Synthase Gene Expression during Renal Ischaemia-Reperfusion Injury. *Nephrology Dialysis Transplantation*, **16**, 1577-1582. https://doi.org/10.1093/ndt/16.8.1577

[177] Schäffer, M.R., Tantry, U., Gross, S.S., Wasserkrug, H.L. and Barbul, A. (1996) Nitric Oxide Regulates Wound Healing. *Journal of Surgical Research*, **63**, 237-240. https://doi.org/10.1006/jsre.1996.0254

[178] Xia, W., Szomor, Z., Wang, Y. and Murrell, G.A.C. (2006) Nitric Oxide Enhances Collagen Synthesis in Cultured Human Tendon Cells. *Journal of Orthopaedic Research*, **24**, 159-172. https://doi.org/10.1002/jor.20060

[179] Lin, J.H., Wang, M.X., Wei, A., Zhu, W., Diwan, A.D. and Murrell, G.A.C. (2001) Temporal Expression of Nitric Oxide Synthase Isoforms in Healing Achilles Tendon. *Journal of Orthopaedic Research*, **19**, 136-142. https://doi.org/10.1016/S0736-0266(00)00019-X

[180] Murrell, G.A.C. (2007) Using Nitric Oxide to Treat Tendinopathy. *British Journal*

of Sports Medicine, **41**, 227-231. https://doi.org/10.1136/bjsm.2006.034447

[181] Bokhari, A.R. and Murrell, G.A. (2012) The Role of Nitric Oxide in Tendon Healing. *Journal of Shoulder and Elbow Surgery*, **21**, 238-244. https://doi.org/10.1016/j.jse.2011.11.001

[182] Menon, A., Pettinari, L., Martinelli, C., Colombo, G., Portinaro, N., Dalle-Donne, I., Agostino, M.C. and Gagliano, N. (2013) New Insights in Extracellular Matrix Remodeling and Collagen Turnover Related Pathways in Cultured Human Tenocytes after Ciprofloxacin Administration. *Muscles, Ligaments and Tendons Journal*, **3**, 122-131.

[183] Brilla, C.G., Matsubara, L.S. and Weber, K.T. (1993) Antifibrotic Effects of Spironolactone in Preventing Myocardial Fibrosis in Systemic Arterial Hypertension. *The American Journal of Cardiology*, **71**, A12-A16. https://doi.org/10.1016/0002-9149(93)90239-9

[184] Horn, M.A., Graham, H.K., Richards, M.A., Clarke, J.D., Greensmith, D.J., Briston, S.J., Hall, M.C.S., Dibb, K.M. and Trafford, A.W. (2012) Age-Related Divergent Remodeling of the Cardiac Extracellular Matrix in Heart Failure: Collagen Accumulation in the Young and Loss in the Aged. *Journal of Molecular and Cellular Cardiology*, **53**, 82-90. https://doi.org/10.1016/j.yjmcc.2012.03.011

[185] Stickens, D., Behonick, D.J., Ortega, N., Heyer, B., Hartenstein, B., Yu, Y., Fosang, A.J., Schorpp-Kistner, M., Angel, P. and Werb, Z. (2004) Altered Endochondral Bone Development in Matrix Metalloproteinase 13-Deficient Mice. *Development*, **131**, 5883-5895. https://doi.org/10.1242/dev.01461

[186] Bataller, R. and Brenner, D.A. (2005) Liver Fibrosis. *Journal of Clinical Investigation*, **115**, 209-218. https://doi.org/10.1172/JCI24282

[187] Riley, G.P., Harrall, R.L., Watson, P.G., Cawston, T.E. and Hazleman, B.L. (1995) Collagenase (MMP-1) and TIMP-1 in Destructive Corneal Disease Associated with Rheumatoid Arthritis. *Eye*, **9**, 703-718. https://doi.org/10.1038/eye.1995.182

[188] Barnes, M.J. and Farndale, R.W. (1999) Collagens and Atherosclerosis. *Experimental Gerontology*, **34**, 513-525. https://doi.org/10.1016/S0531-5565(99)00038-8

[189] Akiyama, K., Shikata, K., Sugimoto, H., Matsuda, M., Shikata, Y., Fujimoto, N., Obata, K., Matsui, H. and Makino, H. (1997) Changes in Serum Concentrations of Matrix Metalloproteinases, Tissue Inhibitors of Metalloproteinases and Type IV Collagen in Patients with Various Types of Glomerulonephritis. *Research Communications in Molecular Pathology and Pharmacology*, **95**, 115-128.

[190] Van Haaften, W.T., Mortensen, J.H., Karsdal, M.A., Bay-Jensen, A.C., Dijkstra, G. and Olinga, P. (2017) Misbalance in Type III Collagen Formation/Degradation as a Novel Serological Biomarker for Penetrating (Montreal B3) Crohn's Disease. *Alimentary Pharmacology & Therapeutics*, **46**, 26-39. https://doi.org/10.1111/apt.14092

[191] Gautieri, A., Uzel, S., Vesentini, S., Redaelli, A. and Buehler, M.J. (2009) Molecular and Mesoscale Mechanisms of Osteogenesis Imperfecta Disease in Collagen Fibrils. *Biophysical Journal*, **97**, 857-865. https://doi.org/10.1016/j.bpj.2009.04.059

[192] Chang, S.W., Shefelbine, S.J. and Buehler, M.L. (2012) Structural and Mechanical Differences between Collagen Homo- and Heterotrimers: Relevance for the Molecular Origin of Brittle Bone Disease. *Biophysical Journal*, **102**, 640-648. https://doi.org/10.1016/j.bpj.2011.11.3999

[193] Lee, J.S., Park, I.S., Park, K.B., Kang, D.H., Lee, C.H. and Hwang, S.H. (2008) Familial Intracranial Aneurysms. *Journal of Korean Neurosurgical Society*, **44**, 136-140. https://doi.org/10.3340/jkns.2008.44.3.136

[194] Steinmann, B., Royce, P.M. and Superti-Furga, A. (1993) Chapter 9. The Ehlers-Danlos Syndrome. In: Royce, P.M. and Steinmann, B., Eds., *Connective Tissue and Its Heritable Disorders*, Wiley, New York, 431-523.

[195] Maw, G.J., Mackenzie, I.L. and Taylor, N.A.S. (1995) Redistribution of Body Fluids during Postural Manipulations. *Acta Physiologica Scandinavica*, **155**, 157-163. https://doi.org/10.1111/j.1748-1716.1995.tb09960.x

[196] Husmann, M., Barton, M., Amann-Vesti, B. and Franzock, U.K. (2006) Postural Effects on Interstitial Fluid Pressure in Humans. *Journal of Vascular Research*, **43**, 321-326. https://doi.org/10.1159/000093197

[197] Fung, P.C.W. and Kong, R.K.C. (2018) Relationship among the Meridians, Sinew Channels and Integrative Five Fluid Circulation System. *Traditional Chinese Medicine*, **7**, 74-92. https://doi.org/10.12677/TCM.2018.71013

[198] Lund, T., Wiig, H., Reed, R.K. and Aukland, K. (1987) A "New" Mechanism for Oedema Generation: Strongly Negative Interstitial Fluid Pressure Causes Rapid Fluid Flow into Thermally Injured Skin. *Acta Physiologica Scandinavica*, **129**, 433-435. https://doi.org/10.1111/j.1365-201X.1987.tb10610.x

[199] Wiig, H., Rubin, K. and Reed, R.K. (2003) New and Active Role of the Interstitium in Control of Interstitial Fluid Pressure: Potential Therapeutic Consequences. *Acta Anaesthesiologica Scandinavica*, **47**, 111-121. https://doi.org/10.1034/j.1399-6576.2003.00050.x

[200] Lidén, Å. (2006) Integrin $\alpha_V\beta_3$-Directed Contraction by Connective Tissue Cells Role in Control of Interstitial Fluid Pressure and Modulation by Bacterial Proteins. Ph.D. Thesis, Faculty of Medicine, Uppsala University, Uppsala.

[201] Svendsen, Ø.S., Barczyk, M.M., Popova, S.N., Lidén, Å., Gullberg, D. and Wiig, H. (2009) The $\alpha 11\beta 1$ Integrin Has a Mechanistic Role in Control of Interstitial Fluid Pressure and Edema Formation in Inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **29**, 1864-1870. https://doi.org/10.1161/ATVBAHA.109.194308

[202] Finkelstein, A.V., Badretdin, A.J., Galzitskaya, O.V., Ivankov, D.N., Bogatyreva, N.S. and Garbuzynskiy, S.O. (2017) There and Back Again: Two Views on the Protein Folding Puzzle. *Physics of Life Reviews*, **21**, 56-71. https://doi.org/10.1016/j.plrev.2017.01.025

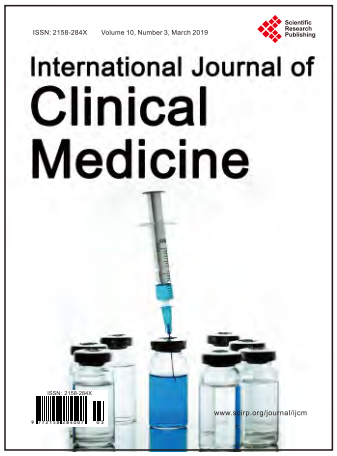
[203] Englandera, S.W. and Maynea, L. (2017) The Case for Defined Protein Folding Pathways. *Proceedings of the National Academy of Sciences of the United States of America*, **114**, 8253-8258. https://doi.org/10.1073/pnas.1706196114

[204] Svensson, R.B., Herchenhan, A., Starborg, T., Larsen, M., Kadler, K.E., Qvortrup, K. and Magnusson, S.P. (2017) Evidence of Structurally Continuous Collagen Fibrils in Tendons. *Acta Biomaterialia*, **50**, 293-301. https://doi.org/10.1016/j.actbio.2017.01.006

[205] Raspanti, M., Congiu, T. and Guizzardi, S. (2002) Structural Aspects of the Extracellular Matrix of the Tendon: An Atomic Force and Scanning Electron Microscopy Study. *Archives of Histology and Cytology*, **65**, 37-43. https://doi.org/10.1679/aohc.65.37

[206] Kanzaki. Y., Terasaki, F., Okabe, M., Fujita, S., Katashima, T., Otsuka, K. and Ishizaka, N. (2010) Three-Dimensional Architecture of Cardiomyocytes and Connective Tissue in Human Heart Revealed by Scanning Electron Microscopy. *Circulation*, **122**, 1973-1974. https://doi.org/10.1161/CIRCULATIONAHA.110.979815

[207] Fung, P.C.W. and Kong, R.K.C. (2018) New Insights on Stimulating the Lung Meridian Based on Modern Neurophysiology. *Chinese Medicine*, *Scientific Research*, **9**, 75-117.



Call for Papers

International Journal of Clinical Medicine

ISSN: 2158-284X (Print) ISSN: 2158-2882 (Online)
http://www.scirp.org/journal/ijcm

International Journal of Clinical Medicine (IJCM) is a peer reviewed journal dedicated to the latest advancement of clinical medicine. The goal of this journal is to keep a record of the state-of-the-art research and to promote study, research and improvement within its various specialties.

Subject Coverage

The journal publishes original papers including but not limited to the following fields:

- Allergy and Clinical Immunology
- Cancer Research and Clinical Oncology
- Clinical Anaesthesiology
- Clinical Anatomy
- Clinical and Applied Thrombosis/Hemostasis
- Clinical and Experimental Allergy
- Clinical and Experimental Dermatology
- Clinical and Experimental Hypertension
- Clinical and Experimental Immunology
- Clinical and Experimental Medicine
- Clinical and Experimental Metastasis
- Clinical and Experimental Nephrology
- Clinical and Experimental Ophthalmology
- Clinical and Experimental Optometry
- Clinical and Experimental Otorhinolaryngology
- Clinical and Experimental Pathology
- Clinical and Experimental Pharmacology and Physiology
- Clinical and Molecular Allergy
- Clinical and Translational Oncology
- Clinical Anesthesia
- Clinical Apheresis
- Clinical Autonomic Research
- Clinical Biochemistry and Nutrition
- Clinical Biomechanics
- Clinical Cardiology
- Clinical Case Studies
- Clinical Child Psychology and Psychiatry
- Clinical Chiropractic
- Clinical Densitometry
- Clinical Effectiveness in Nursing
- Clinical Endocrinology and Metabolism
- Clinical Epidemiology
- Clinical Forensic Medicine
- Clinical Gastroenterology and Hepatology
- Clinical Genetics
- Clinical Haematology
- Clinical Hypertension
- Clinical Imaging
- Clinical Immunology
- Clinical Implant Dentistry and Related Research
- Clinical Interventions in Aging
- Clinical Laboratory Analysis
- Clinical Linguistics & Phonetics
- Clinical Lipidology
- Clinical Microbiology and Antimicrobials
- Clinical Microbiology and Infection
- Clinical Microbiology and Infectious Diseases
- Clinical Molecular Pathology
- Clinical Monitoring and Computing
- Clinical Neurology and Neurosurgery
- Clinical Neurophysiology
- Clinical Neuropsychology
- Clinical Neuroradiology
- Clinical Neuroscience
- Clinical Nursing
- Clinical Nutrition
- Clinical Obstetrics and Gynaecology
- Clinical Oncology and Cancer Research
- Clinical Ophthalmology
- Clinical Oral Implants Research
- Clinical Oral Investigations
- Clinical Orthopaedics and Related Research
- Clinical Otolaryngology
- Clinical Pathology
- Clinical Pediatric Emergency Medicine
- Clinical Periodontology
- Clinical Pharmacology & Toxicology
- Clinical Pharmacy and Therapeutics
- Clinical Physiology and Functional Imaging
- Clinical Practice and Epidemiology in Mental Health
- Clinical Psychology and Psychotherapy
- Clinical Psychology in Medical Settings
- Clinical Radiology
- Clinical Rehabilitation
- Clinical Research and Regulatory Affairs
- Clinical Research in Cardiology
- Clinical Respiratory
- Clinical Rheumatology
- Clinical Simulation in Nursing
- Clinical Sleep Medicine
- Clinical Techniques in Small Animal Practice
- Clinical Therapeutics
- Clinical Toxicology
- Clinical Transplantation
- Clinical Trials
- Clinical Ultrasound
- Clinical Virology
- Complementary Therapies in Clinical Practice
- Consulting and Clinical Psychology
- Contemporary Clinical Trials
- Controlled Clinical Trials
- Diabetes Research and Clinical Practice
- Evaluation in Clinical Practice
- Fundamental & Clinical Pharmacology
- Hereditary Cancer in Clinical Practice
- Human Psychopharmacology: Clinical and Experimental
- Innovations in Clinical Neuroscience
- Laboratory and Clinical Medicine
- Neurophysiologie Clinique/Clinical Neurophysiology
- Nutrition in Clinical Practice
- Pacing and Clinical Electrophysiology
- Psychiatry in Clinical Practice
- Therapeutics and Clinical Risk Management
- Veterinary Clinical Pathology

We are also interested in short papers (letters) that clearly address a specific problem, and short survey or position papers that sketch the results or problems on a specific topic. Authors of selected short papers would be invited to write a regular paper on the same topic for future issues of the ***IJCM***.

Notes for Intending Authors

All manuscripts submitted to IJCM must be previously unpublished and may not be considered for publication elsewhere at any time during IJCM's review period. Paper submission will be handled electronically through the website. All papers are refereed through a peer review process. Additionally, accepted ones will immediately appear online followed by printed in hard copy. For more details about the submissions, please access the website.

Website and E-Mail

http://www.scirp.org/journal/ijcm Email: ijcm@scirp.org

What is SCIRP?

Scientific Research Publishing (SCIRP) is one of the largest Open Access journal publishers. It is currently publishing more than 200 open access, online, peer-reviewed journals covering a wide range of academic disciplines. SCIRP serves the worldwide academic communities and contributes to the progress and application of science with its publication.

What is Open Access?

All original research papers published by SCIRP are made freely and permanently accessible online immediately upon publication. To be able to provide open access journals, SCIRP defrays operation costs from authors and subscription charges only for its printed version. Open access publishing allows an immediate, worldwide, barrier-free, open access to the full text of research papers, which is in the best interests of the scientific community.

- High visibility for maximum global exposure with open access publishing model
- Rigorous peer review of research papers
- Prompt faster publication with less cost
- Guaranteed targeted, multidisciplinary audience

Scientific Research Publishing

Website: http://www.scirp.org

Subscription: sub@scirp.org

Advertisement: service@scirp.org