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Specimen Collection: Improved Human Sperm Biochemical and Cellular Parameters Associated with the Use of Device for Improved Semen Collection

Samuel Prien[1,2*], Khaliq Ahmad[1], Bo Suh[1], Lindsay Penrose[1,2]

[1]Department of Obstetrics and Gynecology, Texas Tech University Health Sciences Center, Lubbock, USA
[2]Department of Animal and Food Sciences, Texas Tech University, Lubbock, USA
Email: *samuel.prien@ttuhsc.edu, khaliq.ahmad@ttuhsc.edu, bo.suh@ttuhsc.edu, Lindsay.penrose@ttuhsc.edu

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Abstract

Semen collection is an essential step in assisted reproduction, yet little attention has been given to the conditions of the collection environment and the potential effects these conditions may have on sperm quality and clinical outcomes. Recent evidence suggests that the physical and chemical environment of standard collection containers may contribute to membrane disruption, organelle dysfunction, and DNA damage, all of which can limit or prevent successful fertilization. Previous work from this laboratory described a device for improved semen collection (DISC) that incorporates structural features and antioxidant scavengers within the plastic matrix to help maintain sperm biochemical stability. The objective of the present study was to evaluate the effectiveness of the original DISC scavenger formulation and a second-generation formulation (DISC+) designed to optimize reactive oxygen species scavenging, in mitigating loss of biochemical function in human sperm collected and maintained over time. These devices were compared with the specimen collection cup routinely used in most infertility clinics.

Keywords

Semen Collection Environment, Sperm Biochemical Integrity, Reactive Oxygen Species, Acrosome Integrity, DNA Fragmentation

1. Introduction

Despite shifts in conception patterns and maternal age, global infertility rates have remained relatively stable at approximately 1 in 8 to 1 in 12 couples over the past

five decades [1]-[3]. However, recent global surveys suggest that male factor infertility has increased over this same time interval [1]-[5]. Together, these observations imply a shifting landscape in assisted reproductive technologies (ARTs) procedures to treat human infertility, where up to 50% or more of infertility cases now include a male-factor component [6] [7].

While significant progress has been made in ARTs over the last fifty years, relatively few advances have directly improved the treatment of male infertility since the introduction of ICSI in the early 1990s. For much of this period, the presence of motile sperm in an ejaculate was assumed to be the primary requirement for successful fertilization [8] [9]. However, recent studies demonstrate that motility—or even motility combined with normal morphology—cannot reliably predict positive outcomes or ultimate take-home baby rates [10]. These findings suggest that greater attention should be directed towards underlying biochemical and cellular processes, including DNA fragmentation [11]. [12], mitochondrial function [13] [14], and membrane integrity [15], as well as towards techniques that preserve or enhance these functions during natural or assisted fertilization [10] [16]-[18].

For the last twenty years, this laboratory has focused on the specimen-collection environment and its potential impact on sperm function [19] [20]. A number of studies on this subject have led to the development of a collection system referred to as the Device for Improved Semen Collection (DISC); marketed as (ProteX) [RSI, Frisco, TX]. Several clinical trials have suggested improvements in semen parameters and clinical outcomes associated with the use of DISC for specimen collection [21] [22]. The device incorporates physical design features that limit environmental extremes [19]-[22] and chemical components intended to maintain cellular physiological and biochemical properties. These chemical components were recently modified in an effort to further improve the collection environment. A recent equine study, conducted under extreme environmental conditions, demonstrated that both the original DISC and the updated DISC+ better maintained cellular biochemical function compared to traditional specimen collection methods [23]. The objective of the present study was to extend this equine evaluation to human samples under more controlled, clinically comparable conditions.

2. Materials and Methods

2.1. Experimental Design and Donor Recruitment

The objective of this study was to compare six collection environments utilizing three devices—the DISC, the modified DISC (DISC+), and a standard specimen cup (SSC)—each used with or without 1 mL of Irvine Multi-Purpose Handling Media (C-MPHM; Irvine Scientific; Santa Ana, CA). The target design was to obtain five usable ejaculates per treatment combination (30 total).

Paid donors were recruited under an IRB-approved protocol and provided a single semen sample after a minimum two-day and maximum seven-day absti-

nence period. To avoid collection bias, all 30 device-media combinations were randomized into a schedule before the first donor was enrolled. As donors entered the study, each was assigned to the next available position on the randomization table and used the corresponding device-media combination.

After collection, samples were allowed to stand for 20 minutes to liquefy before initial analysis. Baseline evaluation included motility parameters, and slides were prepared for later assessment of morphology, acrosome integrity, and DNA fragmentation, as described below.

To enter the study, ejaculates were required to meet the following inclusion criteria:

- Original (pre-media) volume: ≥2.5 mL
- Motility: ≥50%
- Concentration: ≥20 million cells/mL

Samples that met all inclusion criteria proceeded to the study, and the initial analysis was considered the 0-hour observational time point. Samples failing to meet any requirement were discarded, and the associated treatment slot was reassigned to the next eligible donor.

2.2. Semen Collection Devices

As noted above, the study incorporated three different collection vessels, each used with or without 1.0 mL of handling media. The standard specimen cup (SSC) was a 150-mL polypropylene container commonly used in fertility clinics [Fisher Scientific; Waltham, MA]. The DISC is a commercially available device specifically designed for semen collection (ProteX). Its design minimizes exposed surface area, maximizes the internal volume of the ejaculate, provides a thermal barrier to the external environment, and incorporates a proprietary set of free-oxygen-radical scavengers into the plastic matrix. The intent is to maintain, rather than eliminate, reactive oxygen species (ROS) at levels compatible with normal cellular signaling and function. When used as originally intended [20] [21], the DISC also includes 1.0 mL of media to supply nutrients, maintain pH, and gradually activate cellular pumps to prevent osmotic stress.

The DISC+ was created specifically for these experiments (RSI). It contains a second-generation scavenger package incorporated into the plastic matrix in an attempt to improve cellular maintenance during storage.

A media arm was included for all three devices to ensure that the effects previously described with the DISC were not simply due to the presence of media alone, yielding six treatments:

1) SSC (standard specimen cup—device alone)

2) SSCm (standard specimen cup—with 1.0 mL media)

3) DISC (original device for improved semen collection—device alone)

4) DISCm (original device for improved semen collection—with 1.0 mL media)

5) DISC+ (reformulated device for improved semen collection—device alone), and

6) DISC + m (reformulated device for improved semen collection – with 1.0 mL media)

2.3. Semen Evaluation

Once collected, study samples were maintained within the collection device for 48 hrs at room temperature. Each semen sample was evaluated at 0, 0.25, 0.5, 0.75, 1, 3, 6, 9, 12, 24, and 48 hours. At each time point, the samples were gently swirled to remix the solution, and a 0.5 mL aliquot was removed for analysis. Parameters assessed included volume (initial only), concentration, total and progressive motility, CASA-derived kinematic parameters, morphology, acrosome integrity, and DNA fragmentation.

2.3.1. CASA

Computer-assisted semen analysis quantified motility and kinematic parameters using an IVOS system (Hamilton-Thorne; Beverly, MA). After gentle mixing, an aliquot was loaded onto a 6-μm fixed slide (Leja Products B.V.; The Netherlands) and analyzed. CASA settings included: 30 frames, 60 Hz frame rate, minimum contrast 80, minimum cell size 5 pixels, VAP cutoff 50 μm/s, progressive cutoff 20 μm/s, VSL cutoff 1 μm/s, cell intensity 120, and magnification factor 1.58. A minimum of two fields or 200 sperm were evaluated per sample.

While all CASA parameters were recorded, the main focus of the research was the biochemical markers, so the motility parameters reported here were limited to percent motility and percent rapid cells.

2.3.2. Morphology

Morphology was assessed using Hematoxylin-Eosin staining (Fisher). Four microliters of the sample were transferred to a slide, smeared, and air-dried. Once all samples were collected, the slides were stained using standard laboratory procedures and examined under 100X oil immersion. At least 100 sperm were evaluated per time point for head, midpiece, and tail defects.

2.3.3. Acrosome Integrity

Acrosome status was evaluated using the chlortetracycline fluorescence assay [24]. At each time point, a 4-μL aliquot was mixed with an equal volume of 12.5% glutaraldehyde, smeared, dried, and stained in the dark. Slides were examined manually using a Nikon Alphaphot Microscope with Fluorescent Optics and 520-nm excitation and 570-nm emission filters (Nikon Instruments; N.Y., N.Y.). A minimum of 100 sperm were classified as intact acrosome or reacted/non-intact acrosome.

2.3.4. DNA Fragmentation

DNA fragmentation was evaluated using Halosperm kits (Halotech; Madrid, Spain). Agarose was warmed to 37˚C, mixed with 50 μL semen, and placed under a coverslip to solidify at 4˚C. Slides were lysed, washed, dehydrated in graded alcohols, and stained with the kit's hematoxylin and eosin stain. Slides were exam-

ined manually using a Nikon Alphaphot Microscope; a total of 100 sperm/slide were classified as intact DNA with halo formation or fragmented DNA, which lacked halo formation.

2.4. Statistical Analysis

Data were analyzed using SPSS v25 (IBM; Armonk, NY). All variables met assumptions for parametric analysis. Two primary questions were assessed: 1) whether the collection environments influenced semen physiology and biochemical quality over a 48-hour period, and 2) whether any differences observed over 48 hours could be correlated with differences within the first hour of collection. Data were first analyzed across the storage period using a General Linear Model (GLM) with repeated measures, comparing 0, 1, 3, 6, 9, 12, 24, and 48 hours. The sub-hour time points (0.25, 0.5, 0.75 hours) were collected for descriptive visualization of early kinetics and were analyzed separately using a GLM that included 0, 0.25, 0.5, 0.75, and 1 hour. These early time points were not included in the larger 0 - 48-hour GLM to prevent overweighting the first hour relative to later observations. Statistical significance was set at $P < 0.05$.

3. Results

A total of 45 donor samples were recruited; fifteen failed one or more inclusion criteria and were excluded, leaving the 30 samples required for the 6 × 5 design. Figure 1 shows the baseline averages and ranges for motility (total motility and rapid cells), morphology, and biochemical measures (acrosome-intact and DNA-intact cells). Although the experimental design minimized major differences between treatment groups ($P = 0.52$), natural variation in semen quality resulted in uneven distribution of high- and low-parameter samples across the six

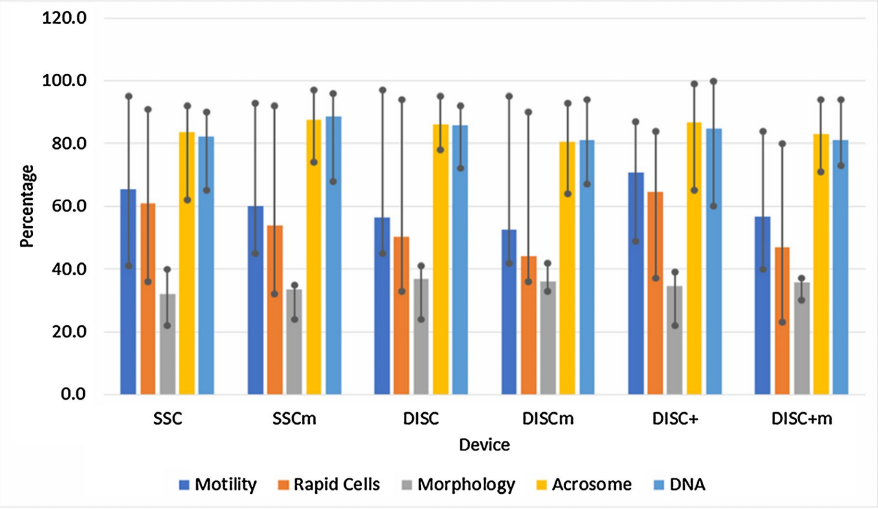

Figure 1. Starting values of samples across the six treatment conditions: standard specimen cup (SSC), SSC with media (SSCm), device for improved semen collection (DISC), DISC with media (DISCm), reformulated DISC (DISC+), and reformulated DISC with media (DISC + m). Bars represent starting means; lines indicate the corresponding ranges.

treatments. Therefore, all subsequent analyses were performed on data normalized to each sample's own zero-hour value. This approach reduces individual-sample effects and allows treatment-related differences to be evaluated more accurately.

It is important to note that these samples were not processed after collection and were maintained at room temperature for the full 48 hours. Focusing first on motility, Figure 2 shows the normalized motility over time ($P < 0.002$). As described in earlier studies, the DISCm combination extended motility longer than all other treatments. While the DISC+ maintained motility well for the first 9 hours, it declined rapidly thereafter (Figure 2).

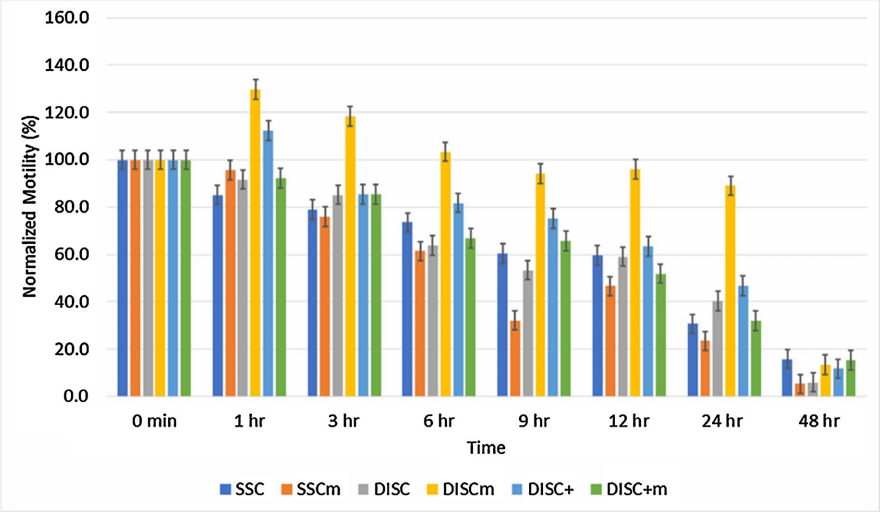

Figure 2. Motility of semen samples over time collected in one of six treatment conditions: standard specimen cup (SSC), SSC with media (SSCm), device for improved semen collection (DISC), DISC with media (DISCm), reformulated DISC (DISC+), and reformulated DISC with media (DISC + m) ($P < 0.002$). Bars represent mean ± SEM.

Similar patterns were observed for rapid cells (Figure 3; $P < 0.006$). However, cells stored in either the DISCm or DISC + m maintained higher rapid cell number than either SSC devices between 1 - 9 hrs but all groups except the SSCm were similar at all times points after 12 hrs.

Unlike a previous study in the equine [23], no differences or changes in morphology were observed in the present study. Using WHO-4 criteria [25], all samples had morphologies within one standard deviation of the mean (32.8%), regardless of treatment ($P = 0.112$) or time ($P = 0.439$).

Acrosome integrity and DNA intactness were used as indicators of biochemical activity. Previous studies have suggested that the DISC environment maintains acrosome-intact cells for longer periods compared to the traditional SSC environment. Data from the present study support this: samples collected in either DISC or DISC+, particularly those with media, maintained intact acrosomes significantly longer than samples collected in the SSC (Figure 4; $P < 0.001$). Interestingly, SSCm also maintained acrosome integrity in almost 50% of cells until ap-

proximately 24 hours.

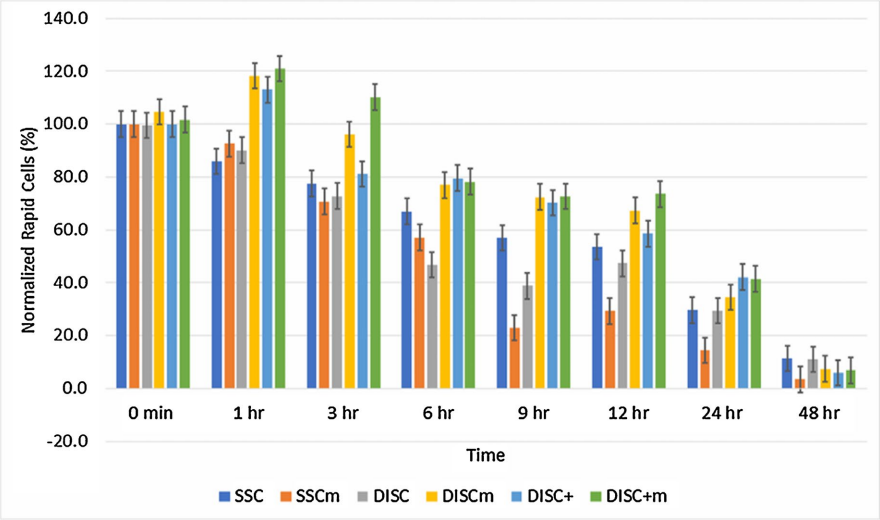

Figure 3. Normalized Rapid Cell movement seen in semen samples over time collected in one of six treatment conditions: standard specimen cup (SSC), SSC with media (SSCm), device for improved semen collection (DISC), DISC with media (DISCm), reformulated DISC (DISC+), and reformulated DISC with media (DISC + m; $P < 0.006$). Bars represent mean with SEM.

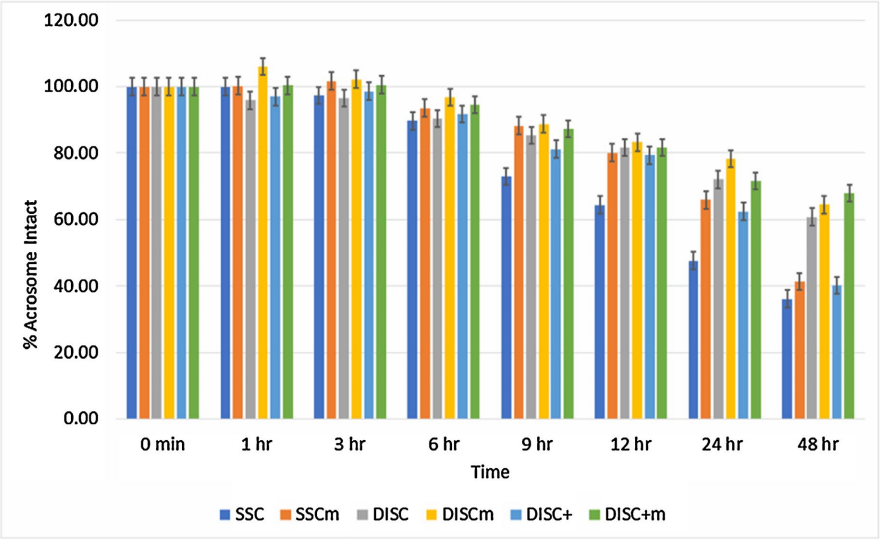

Figure 4. Influence of the collection environment on maintaining intact acrosomes over time in semen samples collected in one of six treatment conditions: standard specimen cup (SSC), SSC with media (SSCm), device for improved semen collection (DISC), DISC with media (DISCm), reformulated DISC (DISC+), and reformulated DISC with media (DISC + m) ($P < 0.001$). Bars represent mean ± SEM.

Finally, the HALO technique was used to assess the degree of DNA fragmentation over time. As described in earlier studies in both humans and other species [19] [23] (**Figure 5**), samples collected in DISC with media maintained a higher proportion of DNA-intact cells after the 6-hour time point compared with the SSC

conditions ($P < 0.001$).

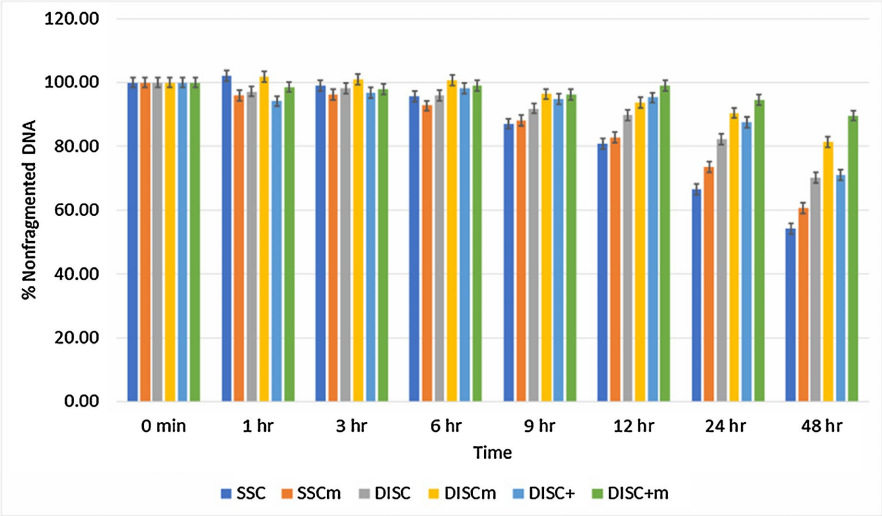

Figure 5. Influence of the collection environment on the maintenance of DNA-intact cells over time in semen samples collected in one of six treatment conditions: standard specimen cup (SSC), SSC with media (SSCm), device for improved semen collection (DISC), DISC with media (DISCm), reformulated DISC (DISC+), and reformulated DISC with media (DISC + m) ($P < 0.001$). Bars represent mean ± SEM.

A second focus of these experiments was to determine whether any differences could be detected within the first hour that might be associated with later manifestations of cell damage. As expected, normalized motility (94% - 103%) and the proportion of rapid cells (95% - 101%) remained very stable during the first hour post-collection regardless of treatment ($P = 0.532$). Morphology parameters were similarly consistent across all groups at these time points ($P = 0.668$). Not surprisingly, acrosome reaction—a process that naturally occurs over 3-7 hours—was also similar ($P = 0.819$), with only 0% - 3% of new cells having undergone the reaction at the zero-hour time point. Further, no meaningful DNA fragmentation was detected during the first hour, ranging from 0% - 5% across all treatments at the five time points ($P = 0.744$).

4. Discussion

Human-assisted reproductive technologies are nearly 50 years old, yet treatment of male infertility has changed surprisingly little over the past three decades. Although male factors contribute to approximately half of all infertility, the introduction of ICSI in the early 1990s largely eliminated the incentive to develop interventions aimed at improving sperm quality itself. Instead, the field focused on overcoming sperm deficiencies rather than addressing their underlying causes. Recent advances in preimplantation genetic testing [26] [27] and growing evidence that lifestyle [28]-[30], environmental [31]-[33], and exposure-related risk factors [34]-[36] influence sperm quality and reproductive outcomes [37] [38] suggest that this paradigm may be shifting. As paternal contributions to fertility

and offspring health become more widely recognized, greater emphasis should be placed on the collection, preservation, and selection of male gametes used in ART.

The DISC device represents one of the first efforts to optimize the sperm collection environment based on the premise that, beyond established patient-level factors, conditions encountered during collection and early storage may influence sperm quality before laboratory processing even begins. To address these factors, the DISC was designed to maintain a stable microenvironment by regulating specimen temperature, supplying nutrients and buffering agents, and incorporating structure-based ROS scavengers intended to preserve, rather than restore, specimen stability.

This study was undertaken to better define the potential benefits of optimizing the sperm collection environment and to evaluate whether those benefits extend beyond conventional semen parameters. A secondary aim was to compare two different combinations of ROS scavengers to determine whether their effects could be further optimized.

Although declines in sperm quality were observed across all study groups over time, the underlying biological processes responsible for these changes may have differed between collection methods. Samples collected using standard approaches demonstrated evidence of progressive cellular dysfunction, including changes consistent with membrane instability and compromised DNA integrity. In contrast, sperm collected with the DISC maintained more favorable measures of cellular function throughout the observation period, suggesting that optimizing the collection environment may help mitigate some of the biochemical stresses experienced immediately after ejaculation.

Because oxidative stress was not measured directly, the contribution of reactive oxygen species can only be inferred from the downstream cellular changes observed in this study. Nevertheless, the preservation of membrane integrity and DNA stability in DISC-collected samples is consistent with the device's proposed mechanism of maintaining a more physiologically stable environment during collection and early storage. The second-generation scavenger package, developed in consultation with plastics experts, appeared superior to both the SSC and the first-generation formulation in protecting biochemical processes. However, the rapid decline in physiological function suggests that this formulation may have been *too* efficient at removing ROS from culture media, potentially reducing ROS levels below those required for normal cellular function. These findings parallel recent equine data demonstrating preservation of membranes, DNA, and mitochondria [23] and are supported by previously described clinical observations [21] [22].

Together, these findings continue to suggest that the semen collection environment may have a direct influence on clinical outcomes. However, the inability to detect changes within the first hour of specimen collection does not support this hypothesis. Because early changes may be subtle and only manifested over time, future studies incorporating more sensitive assays will be required to confirm or

refute the role of the collection environment. Such studies should include direct measurement of ROS within the media at all time points.

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Author Contributions

S.P. and L.P. participated in all aspects of this project from conception to final manuscript preparation. K.A. and B.S. provided help with manuscript 6preparation and independent review of data. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

SP and LP acknowledge potential conflict of interest (COI) due to being inventors of the technology and small percentage stockholders in RSI. SP is also a scientific consultant to RSI. All potential COI was managed in accordance with TTUHSC COI policy.

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