Scientific Research Publishing

Open Journal of Veterinary Medicine, 2026, 16(6), 67-78
https://www.scirp.org/journal/ojvm
ISSN Online: 2165-3364
ISSN Print: 2165-3356

Inclusion of Free-Oxygen Radical Scavengers within the Plastic Matrix of the Collection Vessel Appears to Positively Impact Sperm Biochemical Processes

Melissa Sillivent[1], Lindsay L. Penrose[1,2], Sam Prien[1,2]

[1]Department of Animal and Food Sciences, Texas Tech University. Lubbock, TZ, USA
[2]Department Obstetrics and Gynecology, Texas Tech University Health Sciences Center, Lubbock, TX, USA
Email: samuel.prien@ttuhsc.edu

How to cite this paper: Sillivent, M., Penrose, L.L. and Prien, S. (2026) Inclusion of Free-Oxygen Radical Scavengers within the Plastic Matrix of the Collection Vessel Appears to Positively Impact Sperm Biochemical Processes. *Open Journal of Veterinary Medicine*, **16**, 67-78.
https://doi.org/10.4236/ojvm.2026.166006

Received: May 7, 2026
Accepted: June 20, 2026
Published: June 23, 2026

Abstract

Semen collection has been a mainstay in both human and animal-assisted reproduction for well over half a century. However, until quite recently, little care or concern has been raised about the conditions of the collection environment and their potential effects, not only on sperm quality, but on cycle outcomes as well. Within the last few years, it has become recognized that the abnormal environment of a collection container can lead to membrane disruption, organelle dysfunction, and DNA damage, all of which may limit or totally prevent positive outcomes. Nowhere is this more apparent than in humans and the equine, where intracytoplasmic sperm injection (ICSI) has become the procedure of choice for fertilization during assisted reproductive technology (ART) procedures. Previous work from this laboratory has described a species-specific device for improved semen collection (DISC) that possesses several properties that help maintain sperm quality, including antioxidant scavengers within the plastic matrix. Using an equine model, the objective of the present study was to determine the effectiveness of the original scavenger formulation (DISC) and a modified formulation (DISC+) in mitigating loss of biochemical function in sperm collected and maintained in a non-optimal environment, compared with the traditional collection device, the standard baby bottle.

Keywords

Sperm, Antioxidant Scavengers, Biochemical Maintenance, Cell Function

1. Introduction

Over the last half-century, the use of assisted reproductive technologies (ARTs) has increased substantially, especially in the human and equine fields [1]. As in the human field, intracytoplasmic sperm injection (ICSI) has become the standard method for fertilizing oocytes *in vitro* [2]. In fact, it is now almost universally accepted that ICSI is essential for efficient application of ARTs in the horse [3] [4]. While this shift has significantly improved outcomes in controlled reproductive settings [3] [5], it has also—perhaps unintentionally—reduced interest in optimizing the male and his gametes. The prevailing philosophy suggests that because ICSI requires only a few sperm cells and each ejaculate contains millions to billions, further refinement of male-side factors is unnecessary.

However, recent work has demonstrated that the collection and storage environments can lead to significant degradation of sperm quality, by disruption of cell membranes [6], dysfunction of mitochondria [7] and fragmentation of DNA [8]. Further, there is increasing evidence that these disruptions are associated, at least in part due to the buildup of reactive oxygen species (ROS) within the sample during the *ex vivo* period if the cells are actively metabolizing, and induced by the mechanical and chemical stress in frozen/thawed samples [9].

These findings have triggered a renewed interest in improving the quality of the male gamete contribution in both ART and artificial insemination procedures. Work from this lab has concentrated on these processes for a number of years. These studies have led to the creation of patented [10], species-specific collection systems referred to as the devices for improved semen collection (DISC, commercially known as Truebreed (animal) and ProteX (human); RSI technologies; Dallas, TX). The systems were designed to mitigate the effects of the collection environment by: 1) decreasing the exposed surface area to volume ratio, thus slowing changes in temperature and exposure to rapid increases in oxygen, 2) insulating the sample from extremes in the external environment further slowing temperature shifts, 3) decreasing osmotic pressures and pH shifts by including a measured amount of a nutrient media within the collection vessel, and 4) reducing the ROS buildup within the sample using the properties of the container's plastic matrix to maintain ROS levels at a constant rate [11] [12].

Recently, the DISC matrix was redesigned to maximize the ROS scavenger properties of the plastic matrix. Using an equine model, the objective of the study was to determine the effects of this reformulation on the sample's standard semen parameters, as well as to measure shifts in specific physiological and biochemical processes.

2. Materials and Methods

2.1. Selecting a Animals and Experimental Design

Semen was collected from 10 mature, reproductively sound Quarter Horse stallions at a commercial breeding facility (6666's Ranch, Guthrie, TX). Because collections were performed as part of routine clinical procedures (stallions were col-

lected on a routine schedule with unneeded specimens scheduled to be discarded handed to the research team) and no changes to animal handling were required. The study was therefore determined to be exempt by the Texas Tech University Health Sciences Center IACUC. As these collections occurred during the flush out period in the breeding season, it was possible to collect each stallion once per day, every other day, for a total of three ejaculates per stallion. Ejaculates were randomly assigned to one of three collection devices: 1) standard plastic collection baby bottle, 2) the original DISC, or 3) second generation DISC+, which incorporated an updated group of proprietary fixed ROS scavengers. The DISC+ differs from the DISC in the composition and concentration of the antioxidant scavenger incorporated into the plastic matrix. The revised scavenger package was designed in an attempt to more effectively neutralize reactive oxygen species generated during storage, thereby improving preservation of sperm structure and function. Starting device was randomized across stallions. Each stallion was collected three times, and the order of devices was rotated so that every stallion used each device exactly once, with no device appearing in the same position across animals. This balanced the design across day and sequence, preventing confounding between device, stallion, and collection order. The experiment compared semen quality across devices to determine whether the redesigned DISC+ improved cellular function while maintaining cellular biochemistry compared to the previous device.

2.2. Seme Collection and Processing

Stallions were collected using a collection dummy and a Missouri artificial vagina. For each ejaculate, the ranch recorded stallion ID, age, AV type, number of mounts, ejaculate volume, and initial motility and concentration using a CASA system (Hamilton Thorne; Beverly.MA). Immediately after collection, semen was extended 1:1 with INRA-96 (IMV Technologies: Brooklun, MN) directly into the assigned collection device. After the initial on-site semen analysis for station records, lids were secured, and samples were held at room temperature until transport to the TTUHSC reproductive laboratory. Samples were maintained at room temperature in their original collection device throughout the study. It was recognized these suboptimal conditions would increase overall metabolism and induce buildup of ROS compounds at an accelerated rate. Because of the distance between the collection facility and the laboratory, all samples were held until they reached 6 hours post-ejaculation. This ensured that every ejaculate entered the assay at the same 6-hour time point, providing a consistent baseline across all stallions and devices.

2.3. Semen Evaluation

The semen samples were evaluated at 6, 9, 12, 24, 48, 72, and 96 hours, or until motility reached 0%. At each time-point the samples were gently stirred 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, mitochondrial function, and DNA fragmentation.

2.3.1. CASA

Computer-assisted semen analysis quantified motility and kinematic parameters. All CASA measurements from 6 hours were performed on the research laboratory IVOS (Hamilton-Thorne). After gentle mixing, an aliquot was loaded onto an 8-μm fixed slide and analyzed at 20× magnification. 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 five fields or 1000 sperm were evaluated per sample.

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

2.3.2. Morphology

Morphology was assessed using Hematoxylin-Eosin staining. A 4-μL smear was air-dried, 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. Morphology was assessed at all time points except 96 hours, when motility was insufficient.

2.3.3. Acrosome Integrity

Acrosome status was evaluated using the chlortetracycline fluorescence assay [13] [14]. At each time-point, a 4-μL aliquot was mixed with 12.5% glutaraldehyde, smeared, dried, and stained in the dark. Slides were examined using a Biotek Cytation 5 (Agilent Technologies, Inc.; Santa Clara, CA) with 520-nm excitation and 570-nm emission filters. A minimum of 100 sperm were classified as intact acrosome or reacted/non-intact acrosome.

2.3.4. Mitochondrial Function

Mitochondrial activity was assessed using MitoTracker Red CMXRos (Thermo Fisher Scientific; Waltham, MA). A 50-μL aliquot was mixed with 50 μL dye, incubated 15 minutes, centrifuged (500× g, 5 min), and fixed in 4% formalin. A 10-μL smear was prepared and stored protected from light. Slides were examined on the Cytation 5, and ≥100 sperm were categorized as active mitochondria or low/inactive mitochondria (faded or discontinuous staining.

2.3.5. 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 DAPI (diamidino-2-phenylindole) and SYTOX Deep

Red (Thermo Fisher Scientific/Invitrogen). Using the Cytation 5, ≥100 sperm 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. Because of the wide variation between stallions at the 6-hour time point (**Figure 1**), each parameter was normalized to 100% of its own 6-hour value prior to analysis. A General Linear Model with repeated measures was used to evaluate the effects of device, time, and the device × time interaction across the 9, 12, 24, 48, 72, and 96-hour time points.

The study began with N = 10 ejaculates per device (30 total observations) at the 6-hour baseline, and this full sample size was maintained through 48 hours. At 72 hours, one Baby Bottle control sample and one DISC sample dropped out due to 0% motility. By 96 hours, additional samples had reached 0% motility, leaving N = 7 Baby Bottle, N = 8 DISC, and N = 9 DISC+ ejaculates contributing data at the final time point. Statistical significance was set at $P < 0.05$.

3. Result

It is important to note these experiments were conducted during the COVID and the design was shifted to create increased stress on the collected sample to determine if the collection device could mitigate the effects of a suboptimal collection/storage environment. All motility parameters decreased rapidly over time regardless of treatment ($P < 0.001$). This was expected as the samples were stored in suboptimal conditions (room temperature 21˚C - 23˚C), allowing increased cellular metabolism, rapidly depleting nutrients and increasing reactive oxygen species in the media environment. As multiple stallion collections were obtained each trip and there was a minimal 3 hr time delay between the last collection and transport to the processing laboratory where both standard and biochemical processing was possible, true data collection was standardized to 6 hrs after collection. Further, to limit inter- and intra-collection differences within and between stallions all data were normalized to the 6 hr (biochemical) time points.

To demonstrate the baseline variation, all raw 6-hour data are presented in **Figure 1**. For each device, the bar represents the mean of the ten stallion samples, and the overlaid line indicates the full range of observations.

Looking first at motility parameters, observed initial motilities ranged between 55 and 80% and was significant between both collection period ($P < 0.02$) and stallion ($P < 0.005$) but not collection device ($P = 0.112$). Normalized data over time demonstrated maintenance of motility in all collection systems for the first 12 hrs followed by a rapid decrease ($P < 0.01$) at 24 hrs (**Figure 2**), possibly due to exhaustion of nutrients due to the storage conditions

The pattern was repeated for rapid cell movement (**Figure 3**). However, the new version of the DISC (DISC+) maintained rapid cell movement through the first

12 hrs while samples collected in the standard baby bottle or the original DISC demonstrated measurable decreases in cellular activity as soon as 9 hours ($P < 0.03$). All other sperm kinematics demonstrated similar patterns (data not shown).

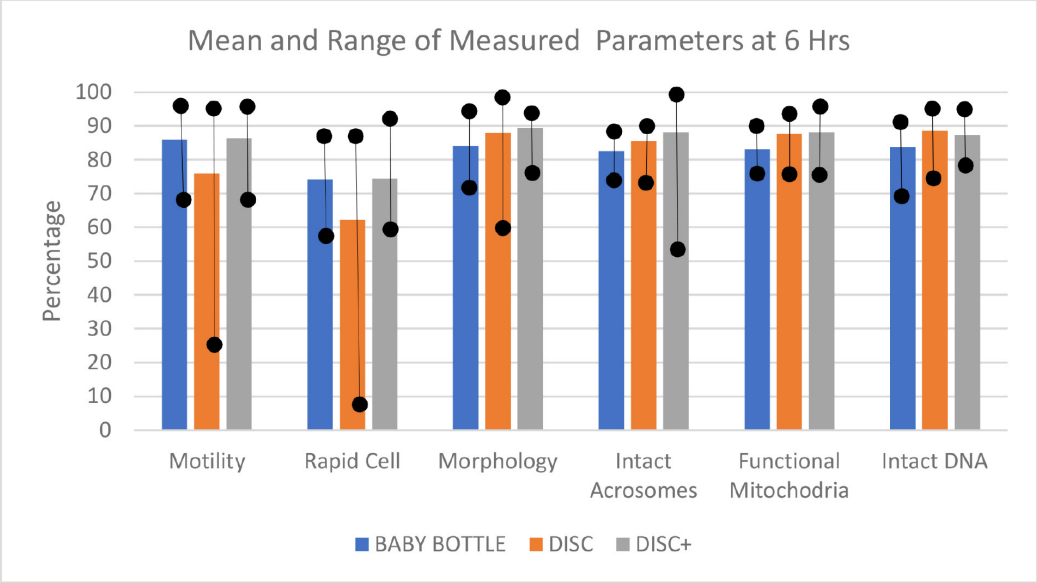

Figure 1. Raw 6-hour measurements showing the mean (bars) and full range (lines) across ten stallions. A significant stallion effect was detected ($P < 0.001$), distributed without pattern across treatment groups. To account for this variability, all outcomes were normalized to each stallion's 6-hour value (set to 100%).

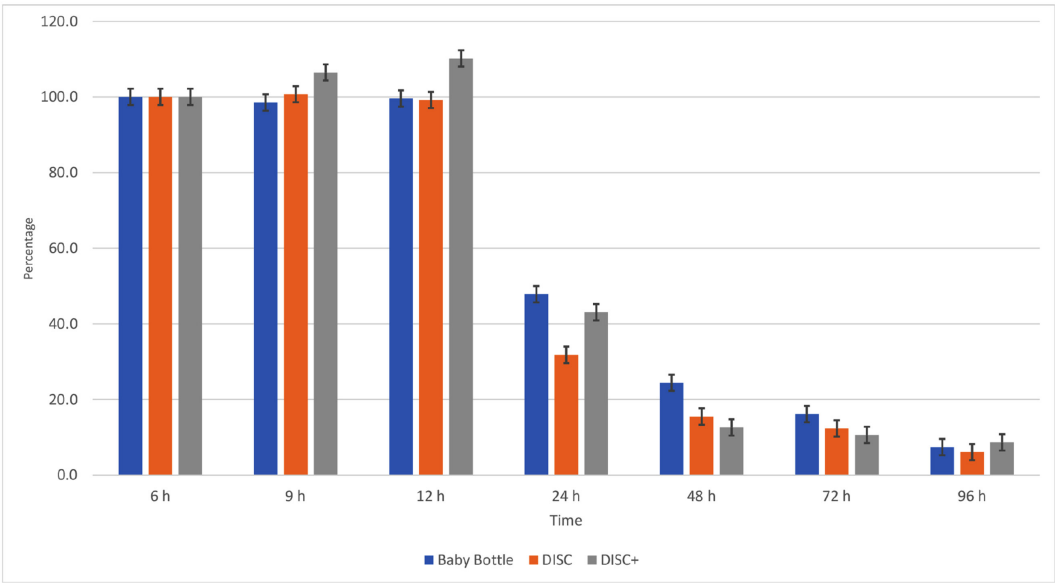

Figure 2. Motility of equine sperm over time following collection in a standard baby bottle, the Device for Improved Semen Collection (DISC), or a modified DISC containing enhanced reactive oxygen species (ROS) scavengers (DISC+). As expected, motility declined rapidly after 12 hours under the intentionally suboptimal storage conditions used to induce oxidative stress ($P < 0.001$). The new antioxidant formulation provided early support but was unable to maintain motility beyond 12 hours ($P < 0.03$).

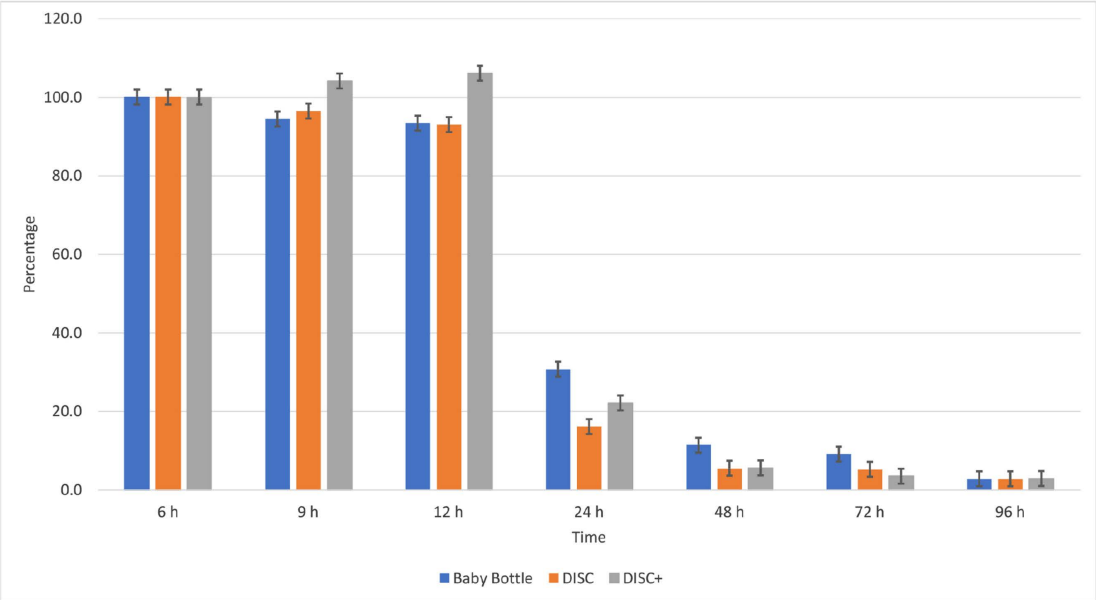

Figure 3. Rapid cell movement of equine sperm over time following collection in a standard baby bottle, the DISC, or the modified DISC+ system. Rapid cell movement declined sharply after 12 hours under the oxidative stress conditions ($P < 0.001$). The DISC+ maintained rapid cell movement through the first 12 hours, whereas samples collected in the baby bottle or original DISC showed measurable declines as early as 9 hours ($P < 0.03$).

While the motility data showed only modest differences between collection systems, the rapid decline across all treatments reflects the intentionally stressful storage conditions. This design imposed a consistent metabolic and oxidative load on the samples, allowing assessment of how each collection environment influenced the biochemical response of the cells. Because elevated ROS is well known to cause loss of membrane integrity and DNA fragmentation, the next series of biochemical and microscopic assays evaluated whether the DISC or modified DISC systems could mitigate these effects and better maintain cellular function.

It is also well documented that loss of membrane integrity can lead to morphological distortion of cell shape. In the case of sperm this could be reflected in numerous changes in the head and tail of the cells. **Figure 4** demonstrates the morphology of the samples over time, while there was an overall decrease in morphology over time ($P < 0.001$), both the DISC and the DISC+ demonstrated better morphology across time. It is important to note that overall morphology in these animals was high due to their selection and active use in a commercial breeding program.

Acrosome intactness was also used as a means of assessing membrane integrity and would also been a good indicator of the start of capacitation. As expected, all samples demonstrated signs of the acrosome reaction after 9 hrs. However, as reported in other studies [12] [15] [16], it was found that both versions of the DISC maintained acrosome intactness at significantly higher levels over time ($P < 0.001$, **Figure 5**).

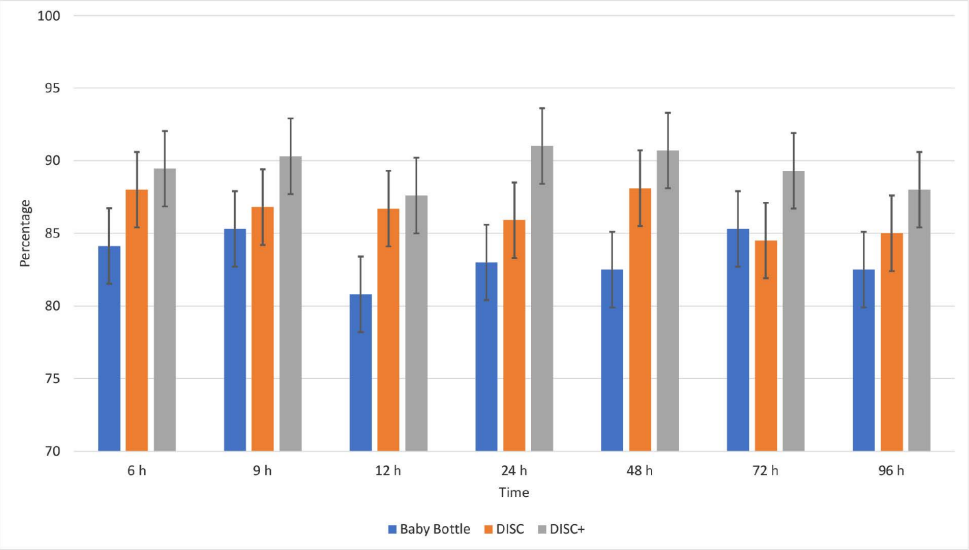

Figure 4. Morphological integrity of equine sperm over time following collection in a standard baby bottle, the DISC, or the DISC+. Both DISC systems maintained significantly higher proportions of morphologically normal cells across most time points ($P < 0.001$). Overall morphology remained high due to the animals' selection and active use in a commercial breeding program.

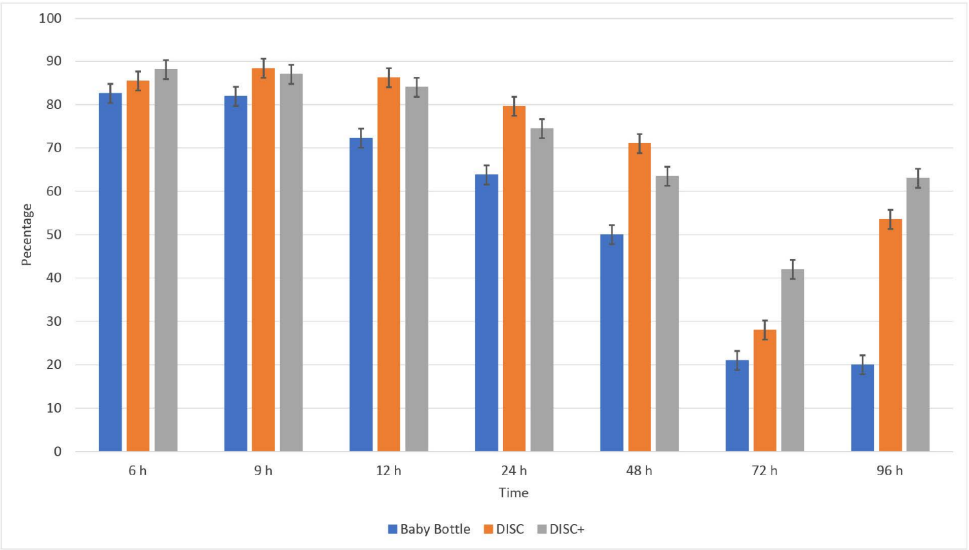

Figure 5. Acrosome-intact sperm decreased over time in all treatments ($P < 0.001$). However, both the DISC and DISC+ maintained significantly higher proportions of acrosome-intact cells at all time-points beyond 9 hours ($P < 0.001$), further supporting improved membrane preservation in these devices.

Obviously, mitochondrial function is essential to sperm function. **Figure 6** shows the integrity of the mitochondria in these collections over time. There was a trend toward higher populations of cells with mitochondrially activity when collected in either version of the DISC through the first 12 hrs post-collect which became significant at 24 hrs through the end of the experimental period. The DISC+ formulation appeared to maintain activity better after 48 hr ($P < 0.001$).

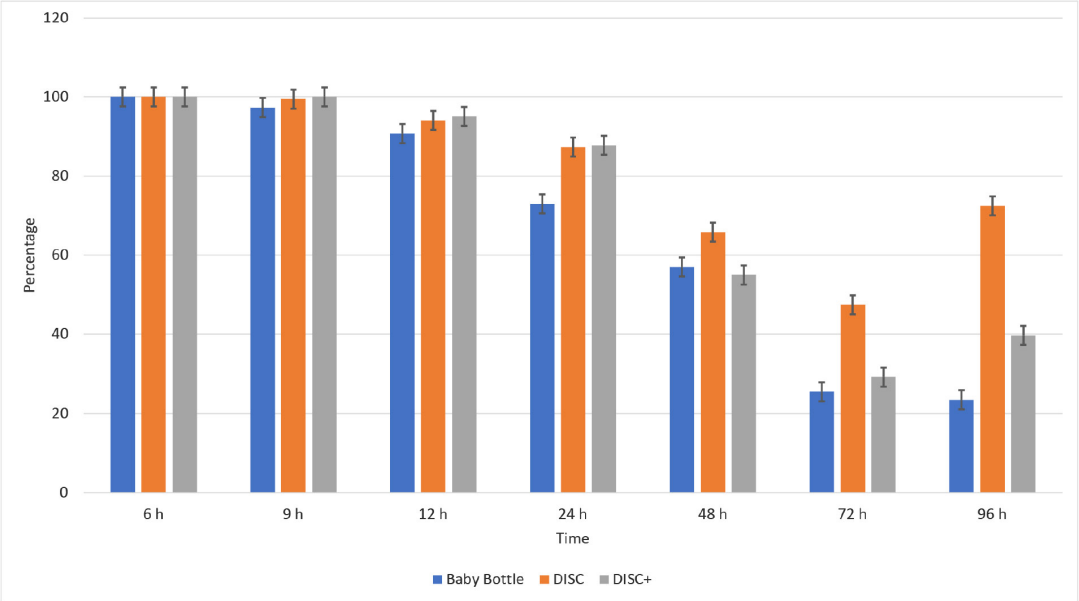

Figure 6. Mitochondrial activity decreased over time in all treatments ($P < 0.001$). Both the DISC and DISC+ maintained higher proportions of sperm with functional mitochondria through 12 hours, with significant differences emerging by 24 hours and persisting through 96 hours ($P < 0.001$). The DISC+ formulation appeared to provide the greatest support after 48 hours.

Finally, DNA fragmentation was assessed using the HALO technique. As would be expected DNA intactness decreased on time ($P < 0.001$, Figure 7). However, results demonstrated the DISC and DISC+ superior to the traditional baby bottle in maintaining intact DNA across the 96 hr time period ($P < 0.001$).

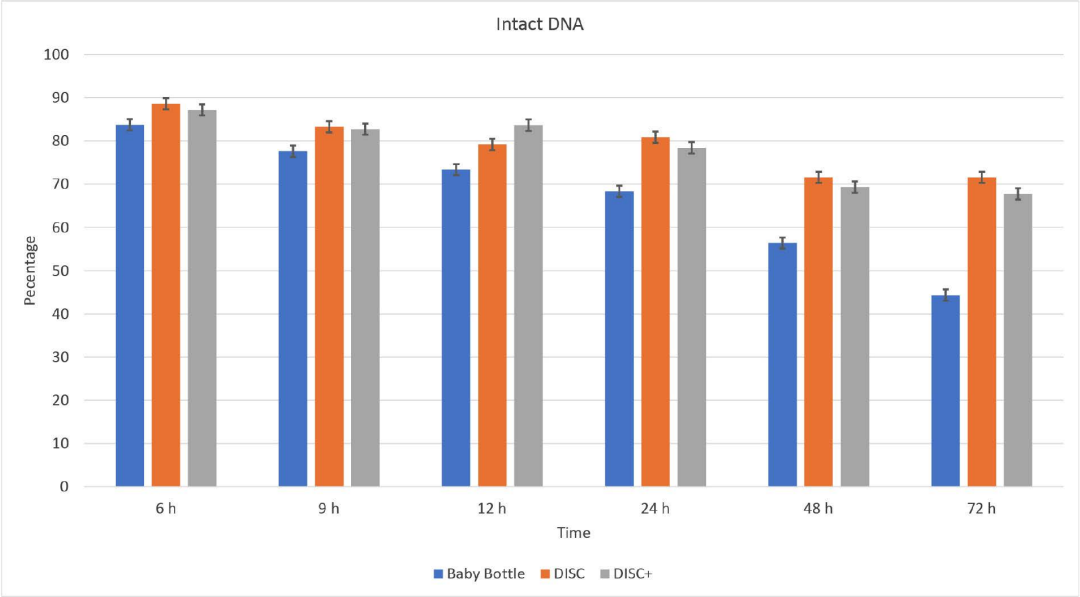

Figure 7. DNA fragmentation increased over time in all treatments ($P < 0.001$). However, sperm collected into the DISC or DISC+ systems maintained significantly higher proportions of DNA-intact cells at all time points beyond 9 hours ($P < 0.001$), indicating improved genomic stability under oxidative stress.

Taken together, these data demonstrate that the DISC and its modified versions preserve sperm health more effectively than traditional collection methods.

4. Discussion

Until fairly recently, the majority of improvements seen in ART have been focused on the female gametes and resulting embryos [14]-[16]. However, there is mounting evidence that the health of the male gametes can have significant impact on procedural outcomes; as the ultimate desire must be measured in healthy live young, not simply positive pregnancies.

Previous studies have demonstrated the DISC to improve the semen parameters across species [10] [11] [17]. Further, blastocyst development rates and pregnancy outcome data are suggestive of improvement in overall embryo health through this simple modification in the collection process [12].

The current study was designed to more fully understand the role of the DISC in this process, focusing on the role of the incorporation of the antioxidant scavengers and a reformation of those scavengers in a second-generation design in the biochemical protection of the sperm cells.

Obviously, the study design, placing the sperm cells in suboptimal storage conditions (room temperature) might be seen as a limitation of this study. However, the reader is reminded of the study took place during the height of the COVID which prompted a rapid shift toward remote sperm collection in both humans and animals.

While the storage conditions resulted in rapid deterioration of motility parameters across treatments, the biochemical data suggests this decrease may have taken place for different reasons. Samples collected and stored in the traditional baby bottle suggested breakdown of membranes, loss of mitochondrial function and disruption of DNA.

Previous studies associate these downstream sperm changes with increasing ROS in the samples [9]. An important limitation, however, is that ROS in the present study were inferred from downstream effects rather than measured directly. Samples collected in either version of the DISC showed better maintenance of membranes, slower progression toward capacitation or apoptosis, a higher proportion of cells with functional mitochondria, and more cells with intact DNA over time. These patterns suggest that the loss of motility may reflect nutrient exhaustion rather than extensive oxidative damage, although this cannot be confirmed without direct ROS measurement.

Future studies—particularly those conducted under optimal storage conditions—should therefore incorporate direct ROS quantification to more precisely evaluate the mechanisms underlying these effects.

Acknowledgements

The authors wish to thank Dr. Clarissa Strieder-Barboza for allowing me access to her lab and equipment and Dr. Oscar Benitez-Rojas for sharing his expertise in

analyzing my samples. We also wish to thank a large number of undergraduate students who assisted in specimen transport and data collection. We would also acknowledge the assistance RSI Reproduction Solutions in manufacture of the DISC+ with the modified scavenger system. Finally, the authors acknowledge the support of the Texas Tech University Innovation HUB for supplying funding used in support of this project.

This work was presented in part at the 2024 Scientific Congress of the American Society for Reproductive Medicine [11].

Conflicts of Interest

SP and LP acknowledge potential conflict of interest due to being inventors of the technology and small percentage stockholders in RSI. SP is also a scientific consultant to RSI.

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