A Cross-species Comparison of a Specimen Collection Container Designed to Harvest Oxygen Radical Species

A. White[1], B. Smith[2], M. Sillivent[2], S.D. Prien[1,2], and L.L. Penrose[1,2]

Department of Obstetrics and Gynecology, Texas Tech University Health Sciences Center; Lubbock, Texas 79430[1]
and Department of Animal and Food Sciences, Texas Tech University; Lubbock, Texas 79409[2]

Abstract

Objective: Previous research has demonstrated reactive oxygen species (ROS) can significantly impact semen quality. Work from this laboratory has suggested a redesign of the Device for Improved Semen Collection (DISC – trade name ProteX+), may not only improve semen parameters but also mitigate the effects of ROS on sperm-cell function by limiting their buildup in culture media. In the present study, human and equine semen samples were collected using the new system to determine its ability to harvest ROS and maintain semen quality.

Materials and Methods: In the human trial, semen samples were obtained from 10 donors collecting three samples in a standard specimen cup (SSC), the original DISC (PRO), or the redesigned DISC (PRO+), and then prepared for Intrauterine Insemination (IUI) using standard washing techniques with final incubation in a standard polystyrene test tube. The samples were incubated at 37oC, in room-air, and 95% relative humidity to induce ROS formation. At times 0, 1, 3, 6, 9, 12, and 24 hrs, slides were prepared for semen analysis, acrosome reaction, and DNA fragmentation. Similarly, equine samples were collected from 10 studs. Each stud was collected one time in a standard container, the original equine DISC (trade name TrueBreed—TB) and the DISC modified for antioxidant properties (TB+) using standard equine extension techniques. Once prepared, the samples were incubated at room temperature in the collection device. Similar to the human study, semen analysis, acrosome reaction, and DNA fragmentation slides were prepared at times 6, 9, 12, 24, 48, 72, and 96 hrs. Semen analyses were performed on a Hamilton Thorn IVOS unit, and acrosome and DNA fragmentation was determined using standard species-appropriate techniques. The resulting data were analyzed using ANOVA with repeated measures.

Result: As expected, due to the study design to increase ROS generation, all semen analysis parameters decreased over time ($P < 0.001$). However, cells stored in the devices with ROS savaging properties demonstrated increases in various semen parameters at 6, 9 and 12 in the human and equine samples, including maintaining rapid cell activity, progressive velocity, beat-cross frequency and straightness ($P < 0.05$). The collection of biochemical data for mitochondrial activity, DNA fragmentation, and acrosomes are ongoing and will be presented at the meeting.

Discussion: The data suggest it is possible to scavenge ROS from an aqueous cell culture media using a fixed scavenging system. In theory, lowering ROS may maintain semen quality longer ex vivo, potentially without increased risk of DNA damage. However, further study is needed to determine the effects of the fixed scavengers under optimum culture conditions.

Impact: Lessening ROS and resulting sperm cellular damage could result in higher rates of pregnancy and improve pregnancy outcomes.

Introduction

It is well documented that high free-oxygen radical concentration within the fluid environment (native seminal fluid, media, or any combination) can lead to biochemical damage, including disruption of membrane integrity, loss of organelle function, and DNA fragmentation. Therefore maintaining a stable concentration of free-oxygen radical is necessary for normal sperm cell function.

Previous research from this laboratory resulted in creating a new collection system, referred to as the ProteX (Reproductive Solutions, Inc., Dallas, TX). This system provided a superior semen sample by providing a collection environment that minimizes cellular stress, through 1) minimizing exposed surface area maximizing the volume to surface area ratio, 2) Providing a thermal barrier to the outside environment that allows the sample to drive its own temperature and 3) when used as recommended, lessening osmotic shock. These factors combine to produce an optimal culture environment supporting normal physiological and biochemical processes which result in more motile, healthy population of sperm cells for infertility procedures.

One year ago, at this meeting (Prien et. al. Fert Steril 2020; 114:e117-118), we presented the first data from an updated ProteX design, termed the ProteX+, which added a “fixed” scavenger molecule within the container to harvest excess radicals from the media. By design, the scavengers stabilize the sperm environment by not allowing large shifts in free oxygen radical content, stabilizing membranes, organelles, and possibly DNA. The data generated from frozen-thawed Bovine semen demonstrated increased motility and rapid cell movement over time from cells stored in the new device (Figure1).

The present study represents the first experiments where semen was collected in the device. Human and equine semen was collected in the specie specific Protex, ProteX+, or traditional collection container prepped appropriated for the species, placed in an environment to induce free oxygen radical formation, and followed over time.

Materials & Methods

Human Studies

Ten donors were recruited with normal semen parameters at testing using the World Health Organization version 5 parameters for a normal semen analysis. Each Donor then supplied 4 samples, a minimum of 3 days apart and a maximum of 7 days apart. The treatments were as follows

1. A standard semen cup with 1 mL of Fuji Irvine Multipurpose Handling Media (MHM)
2. An original ProteX with 1 mL MHM
3. A ProteX+ with 1 mL MHM

Donors were assigned randomly to which treatment they started with i.e., if assigned to start with treatment 3 they proceed to 4, 1, and finished with 2.

Samples were collected via masturbation. Once collected, the sample was allowed 10-30 minutes to liquefy before undergoing a semen analysis. The semen analysis consisted of manual volume measurement before being evaluated for concentration, motility, rapid cells, average pathway velocity (VAP), straight line velocity (VSL), track speed (TS), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) on a Hamilton Thorn IVOS computer assisted semen analyzer (CASA).

Samples were then prepared using a standard sperm washing technique. The collected sample (semen plus 1 mL of MHM) were transfer to 15 mL conical test tubes. Two additional milliliters of fesh media was added to the tube, and the samples were centrifuged at 600 RPM for 6 minutes. The supernatant was removed and replaced with 5 mL of media of fresh MHM. After mixing sperm into media, CASA measurements were repeated and the samples were incubated at 37ºC.

Samples were reanalyzed at 1,3,6,9, 12, 24 and 48 hrs or until there was no remaining motility. Additionally, slides were prepared at each time point for morphology using a rapid H&E staining technique, mitochondrial intactness using a standard mitotracker red technique, DNA fragmentation using Halosperm® G2 test kit from Halotech® , and acrosomes were evaluated using a standard saturated chlortetracycline technique.

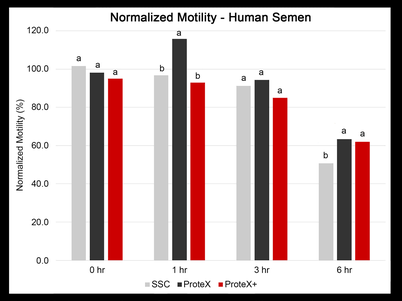


Figure 2. A comparison of normalized motility over time in human semen samples collected in three devices and cultured in an environment designed to induce free oxygen radical species.

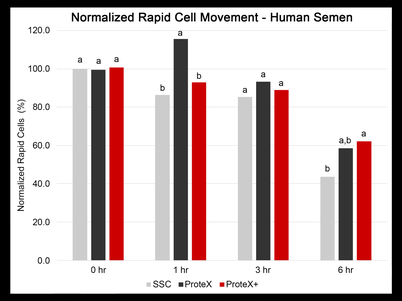


Figure 3. A comparison of normalized rapid cell movement over time in human semen samples collected in three devices and cultured in an environment designed to induce free oxygen radical species.

Semen Movement Parameters - Human Semen				
Parameter	**SSC**	**ProteX**	**ProteX+**	**P VALUE**
Path Velocity	52.9333	54.7000	56.2897	0.500
Progression	44.3974	45.7725	46.9179	0.690
Track Speed	84.6308	86.3100	90.2744	0.330
Elongation	66.2051	65.5250	65.3333	0.530
Lateral Displacement	4.2103	4.0900	4.266667	0.680
Beat Cross Frequency	25.7667	24.6675	26.1590	0.570
Straightness	81.8462	81.8000	82.5517	0.600
Linearity	51.8974	52.9750	51.64103	0.657

Table 1. Human sperm movement parameters after collection in one of three devices and cultured in an environment designed to induce free oxygen radical species.

Figure 4. A comparison of normalized motility over time in equine semen samples collected in three devices and cultured in an environment designed to induce free oxygen radical species.

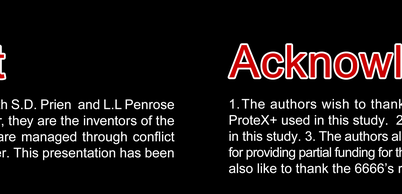
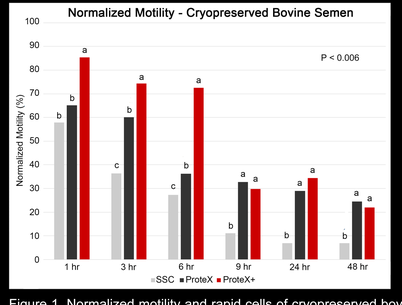


Figure 5. A comparison of normalized rapid cell movement over time in equine semen samples collected in three devices and cultured in an environment designed to induce free oxygen radical species.

Semen Movement Parameters - Equine Semen				
Parameter	**Baby Bottle**	**TrueBreed**	**TrueBreed+**	**P VALUE**
Path Velocity	99.1924	94.7350	101.6128	0.243
Progression	60.7256	53.9425	61.1641	0.005
Track Speed	196.2795	186.2750	193.8128	0.500
Elongation	59.6923	59.7250	60.3590	0.663
Lateral Displacement	7.8821	8.1725	8.2872	0.205
Beat Cross Frequency	32.1667	30.1650	29.2077	0.008
Straightness	59.1186	58.0250	59.7436	0.657
Linearity	31.8475	30.5750	32.5897	0.191

Table 2. Equine sperm movement parameters after collection in one of three devices and cultured in an environment designed to induce free oxygen radical species.



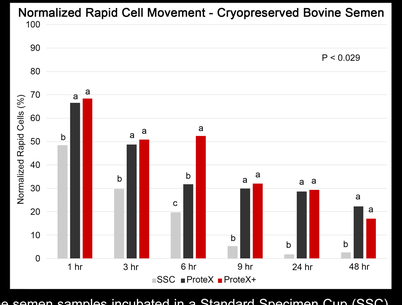


Figure 1. Normalized motility and rapid cells of cryopreserved bovine semen samples incubated in a Standard Specimen Cup (SSC), ProteX or ProteX+ over 48 hrs, demonstrated higher motility in the ProteX+ for at least the first 6 hrs post-thaw ($P < 0.006$). Individual means with different superscripts are different within a time point.

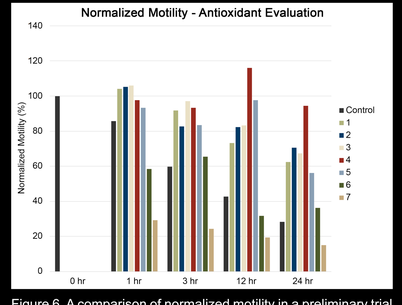


Figure 6. A comparison of normalized motility in a preliminary trial of various formulations of antioxidants used to scavenge free oxygen radicals in a sperm culture environment

Conflicts of Interest

The authors wish to acknowledge potential conflicts of interest as both S.D. Prien and L.L Penrose are founders, shareholders and scientific advisors to RSI. Further, they are the inventors of the technologies used to develop ProteX and ProteX+. Such conflicts are managed through conflict management plans at Texas Tech University Health Sciences Center. This presentation has been reviewed per the authors conflict of interest management plans.

Acknowledgments

1.The authors wish to thank Reproductive Solutions Inc. (RSI) for supplying the ProteX and ProteX+ used in this study. 2.The authors want to thank Fuji-Irvine for supplying the media used in this study. 3. The authors also wish to thank RSI and the Innovation HUB at Texas Tech University for providing partial funding for this study via the Innovation HUB Prototyping Grant. 4.The authors would also like to thank the 6666’s ranch for providing collections from the breeding herd for this study.

Materials & Methods Cont

Equine Studies

Access was obtained to semen samples collected from 10 stallions that are used actively in the donor program at the 6666s Ranch in Guthrie, Texas. Each stallion provided three sample collections on an M, W, F in groups of 5 stallions at a time over two weeks. The treatments were as follows

1. A standard baby bottle (industry standard) with 10 mL of INRA-96 extender (IVM Technologies)
2. An original TrueBreed, the animal version of ProteX, with 10 mL of INRA-96 extender
3. A TrueBreed+, the animal version of ProteX+ with 10 mL of INRA-96 extender

Studs were assigned randomly to which treatment they started with i.e., if assigned to start with treatment 3 they proceed to 1, and finished with 2.

Samples were collected using a Missouri Artificial Vagina. Once collected, the sample underwent a semen analysis consisting of manual volume measurement followed by concentration, motility, rapid cells, average pathway velocity (VAP), straight line velocity (VSL), track speed (TS), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) measured on a Hamilton Thorn IVOS computer-assisted semen analyzer (CASA).

Samples were then transported to the lab and further extended to final concentrations of 20 million cells/mL. CASA measurements were repeated at 6, 9, 12, 24, 48, 72, 96 hrs or until there was no remaining motility. Additionally, slides were prepared at each time point for morphology using a rapid H&E staining technique, mitochondrial intactness using a standard mitotracker red, technique, DNA fragmentation using Halosperm® equine test kit from Halotech® , and acrosomes were stained using a standard saturated chlortetracycline technique.

Statistical Analysis

While data were collected at all time points listed, analysis was truncated to the last time with 50% normalized motility in all three treatments (6 hrs in the human and 24 hrs in the horse) to avoid undue influence of motility on other motion factors. All data analysis was performed using ANOVA and a general linear model, including device and time. In addition, mean seperations were performed at individual time points using Tukey’s mean separation test. All Statistical analysis was performed using SPSS version 25.

Results

1. As expected all semen parameters tended to decrease over time regardless of the collection device.
2. Data from the human samples suggested the ProteX and ProteX+ maintained equal or better motility over the analyzed time period compared to control (Figures 3; $P < 0.05$ and Figure 4; $P < 0.01$)
3. While none of the individual motion parameters in the human trial where better in the ProteXs verses the control, collective all were higher in the ProteX+, Table 1.
4. Similar results were seen in the equine were the Protex+ maintained equal or better motility and rapid cell movement 12 hrs while stored under suboptimal conditions (Figures 5 and Figure 6; $P < 0.05$)
5. Similarly, equine samples collected and stored in the Protex+ collectively had better motion parameters than the ProteX or baby bottle control.
6. All biochemical analyses are ongoing

Discussion

1. It is important to note these experiments were conducted in environments meant to induce accelerated free oxygen radical development.
2. Data from these trials support previous work that suggests the ProteX is a better system for semen collection.
3. Further, data suggest the ProteX+, with its redesign to include antioxidant properties, is superior to either traditional methods or the ProteX even under extreme oxidative stress conditions.
4. Further study in a more controlled environment will be needed to verify these observations.
5. Further study is still needed to identify the optimum scavengers. A recent trial with seven candidates suggests wide variability in their abilities to remove free oxygen radicals from the culture environment (Figure 6)

Presented at the 77th Annual Scientific Congress of the American Society for Reproductive Medicine - October 2021 and the Gender-Specific Medicine & Women's Health Symposium - October 2021