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An Alternate Technique for Use of the ProteX/NovoSort Sperm Isolation System for Use with Low Volume-Low Concentration Cryopreserved Samples

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Abstract

Centrifugation preparation has been the mainstay of ART sperm preparation since the procedure was first introduced in 1978. However, recent studies have suggested that commonly used collection and preparatory techniques, such as centrifugation, may negatively impact sperm cell function. Recently, a number of devices have been introduced to select sperm using a physical barrier. While these barrier techniques show great promise, most have been described as only working with freshly ejaculated sperm. As the less motile cryopreserved cells seem to lack the ability to penetrate the barriers. However, one of the newest systems, the NovoSort (NS; Reproductive Solutions Frisco, TX), uses a different barrier system that appears to harvest a higher percentage of motile cells and allows processing of whole ejaculates. The objective of the present study was to determine whether the system, with its unique barrier design, could be successfully adapted for use with frozen samples, particularly low-volume commercial samples, to enhance the motile population of sperm cells used in ART procedures. By design, the NS is intended for use with a ProteX collection cup (PX; Reproductive Solutions). The NS, containing 0.75 - 1.0 mL of media, is then lowered into the native ejaculate in the PX, allowing cells to migrate through the barrier into the clean media. While the design works well with a normal ejaculate, its design requires a volume of a minimum of 1 mL in the PX in order for it to make contact with the mesh of NS. As most commercial samples are both low volume (0.5 mL) and have relatively low motile concentrations, the standard technique is not an option. Therefore, the objective of the present study was to develop a technique that would allow the use of the NS barrier

method with cryopreserved samples. In a series of preliminary experiments, it was found that the locations of the media and the native sample within the system could be reversed, creating the necessary contact between the sample, mesh, and media to facilitate sperm migration. To test the effectiveness of the modified protocol for motile cell recovery, 8 donated cryopreserved samples were thawed, and 0.5 mL of the sample, to mimic commercial samples, was placed in the NS. The NS was then placed in the PX, which contained 1 mL of culture media, and incubated for 1 hr. The outer media were then sampled at times 0, 5, 15, 30, 45, and 60 minutes to assess the presence and concentration of motile sperm. These data were then compared to the native concentration and forward progression at thaw. Results suggest these modifications allow for the recovery of a highly motile sperm population. There can be no doubt a significant amount of frozen semen is used in ART, especially in FDA-regulated male donor cases. Previous studies have suggested issues with motile cell recovery using other barrier methods due to decreased motility resulting from the freeze-thaw process. The current study suggests that, with modification, the combination of PX and NS can be employed to isolate motile cells from low–volume, low-concentration cryopreserved samples, such as commercial samples, thereby eliminating steps that might further damage compromised cells. Further study is needed to test the limits of this modified isolation technique.

Keywords

Sperm Isolation, Cryopreserved, Non-Centrifuged, Barrier Isolation, Technique

1. Introduction

In vitro fertilization (IVF) has become a cornerstone of modern infertility treatment and now accounts for nearly 2% of all births in the United States [1]. This widespread adoption has been made possible by major advancements in specialized equipment, hormonal therapy regimens, and optimized culture conditions [2]-[6]. One of the longstanding foundational methods in assisted reproductive technology (ART) is sperm preparation via centrifugation, which has remained a standard technique since its introduction. Despite its ubiquity, numerous studies have highlighted significant drawbacks of conventional sperm preparation methods, including DNA fragmentation and structural damage to sperm membranes and organelles; factors that ultimately compromise fertilization outcomes [6] [7].

As global demand for IVF continues to rise, ART laboratories are under increasing pressure to improve procedural efficiency without sacrificing clinical outcomes. On the female side of fertility procedures, this has led to innovations such as reduced media volumes, necessitating oil overlays, advanced incubator designs, tools like the Stripper® for rapid denudation and manipulation of oocytes and embryos, and emerging non-invasive approaches to preimplantation genetic testing that may eliminate the need for biopsy [2] [5] [8]. On the male side, intracytoplas-

mic sperm injection (ICSI) has emerged as a preferred method, particularly beneficial for treating severe male factor infertility, including patients with extremely low sperm counts, poor motility, or the need for surgical sperm retrieval [8] [9].

However, despite its advantages, ICSI is not without limitations [7]-[10]. The technique is costly, labor-intensive, and requires highly trained personnel. Moreover, sperm selection during ICSI is typically limited to assessments of motility and morphology, with few reliable methods available to evaluate the overall health of individual sperm cells. While the development of a comprehensive test to assess every functional aspect of a single sperm cell may not be feasible, there remains a critical need for simpler and more effective selection tools that can enhance outcomes without increasing complexity or cost.

Previous work from this laboratory has suggested a simple, one-step sperm selection method aimed at addressing these persistent challenges [11] [12]. While other barrier methods exist [13] [14], the NovoSort (NS; Reproductive Solutions Technologies, Frisco, TX) device utilizes a unique woven mesh barrier-based system designed to enrich motile sperm populations efficiently, with the added benefit of processing whole ejaculates. The objective of the present study is to evaluate whether the NS system can be effectively adapted for use with frozen, low-volume commercial semen samples. The goal was to determine if this approach offers a cost-effective and streamlined method for improving sperm motility of low-volume, cryopreserved samples.

2. Materials and Methods

2.1. Preliminary Experiments

While the NS has proven an effective barrier to seminal plasma and media, which prevents significant intermixing of fluids on either side of the mesh, it was recognized that sperm cryoprotectants contain several compounds that may have different interactions with the hydrostatic barrier formed at the mesh interface. Therefore, NS were subjected to two experiments prior to the semen experiments. In the first, 1 mL of cryoprotectant (FUJIFilm Biosciences; Santa Ana, CA) was placed in the inner well of the NS, and the NS was placed on the counter and observed for 1 hr. In the second, a dry NS was suspended in a PX containing 3 mL of cryoprotectant and observed for 1 hr. No obvious leakage was observed in either case.

A series of experiments were then conducted to determine what volumes were needed to form an interface between the media on one side of the mesh barrier and the semen sample on the other, using the standard 0.5 mL volume found in commercially available semen samples. All attempts to place the 0.5 mL cryopreserved sample in the PX cup failed to make contact with the mesh of the NS basket. Attempts were then made to increase the volume (*i.e.*, dilute) of the sample with the PX prior to the NS. While it was possible to contact the mesh interface, the minimum volume resulted in a 100% dilution of the frozen sample, leading to poor final yields of motile cells in the NS basket.

Noting that the standard protocol [11] [12] would not work, the decision was made to attempt to reverse the process and place the native sample on the interior of the basket to swim out into the media contained within the PX. It was found that the 0.5 mL of cryopreserved sample made good contact with the NS mesh when the sample was placed in the interior of the NS basket. Furthermore, a minimal volume of 1 mL of media was sufficient in the PX to form the same interface between the fresh media and the mesh. The resulting interaction of sample-mesh-media allowed for a consistent path for motile sperm to exit the basket and swim into the fresh media in the PX. Because the support ring on the NS is solid, it was necessary to cut a small sampling port in the NS to allow sample collection at all time points.

A total of 14 cryopreserved samples were used in these preliminary experiments. All semen used in these and subsequent experiments were obtained via an IRB-approved protocol allowing use of deidentified samples meant to be discarded in non-fertility experiments (L14-177). Due to the ever-changing nature of these trials, these data were excluded from the final analysis.

2.2. Motile Sperm Recover Trials

Once motile sperm were consistently recovered, we conducted a trial to determine recovery rates, motility, and forward progress of recovered sperm versus the native sample. A total of 8 samples were used in these experiments. All samples had previously been frozen in volumes ranging from 0.5 to 2.0 mL in 2 mL cryovials (Nunc, ThermoFisher, Waltham, MA) by hanging the vials in a mist of liquid nitrogen, using standard clinical protocols, and then stored in liquid nitrogen.

Prior to thawing the samples, a PX cup was prepared by adding 1 mL of 37˚C FUJIFILM Multipurpose Handling Media-Complete (FUJIFILM Biosciences) into the center well of a PX collection cup. At thaw, the sample was removed from liquid nitrogen, held at room temperature for 90 sec, then plunged into a 37˚C water bath for 2 min. The samples were thoroughly mixed and underwent an initial semen analysis for concentration, motility, and forward progression using a Hamilton-Thorne IVOS semen analyzer (Hamilton-Thorne, Beverly, MA). A 0.5 mL aliquot, to mimic a commercial sample, was added to the center well of an NS that had been modified as described above to allow intermittent sampling from the PX media, and the NS was lowered into the PX cup. Once the sample was prepared, it was incubated on the countertop at room temperature. At times 0, 15, 30, 45, and 60 mins, a 4 µL aliquot of the media was extracted through the sampling port and assessed for concentration, motility, and forward progression using the IVOS semen analyzer.

The resulting data were analyzed using variance analysis and Tukey's mean separation, comparing the recovered values to those of the native sample.

3. Results

As stated above, a total of 22 samples were used in the development and testing of

this procedure. However, 14 were used in optimizing the protocol and their data were not included in the final analysis. The concept was to establish a continuum between the sample inside the basket of the NS, the mesh, and the collecting media in the PX, allowing cells in the interior of the basket to escape their seminal plasma and cryoprotectant through the mesh and into the media (Figure 1).

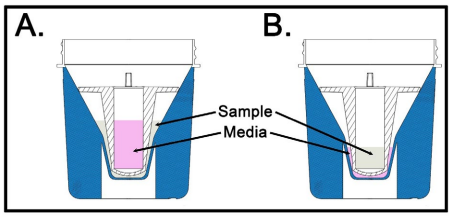

Figure 1. A comparison of the ProteX/NovoSort sperm selection system as designed by the manufacturer. (A) and as reconfigured for the present study; (B) where the sample was placed with the NS inner basket and the cells allowed to migrate into the fresh isolation media within the PX cup.

The remaining eight samples were observed at all five time points. Table 1 demonstrates the average cellular concentration, motility, and forward progression, and their ranges of the eight samples used, demonstrating a wide range of starting parameters.

Table 1. Average post-thaw semen parameters prior to processing using a modified NovoSort/ProteX technique.

	Average	Range
Concentration (mil/mL)	44.9	22 - 89
Motility (%)	48.6	25 - 76
Track speed (mic/sec)	68.9	31 - 112

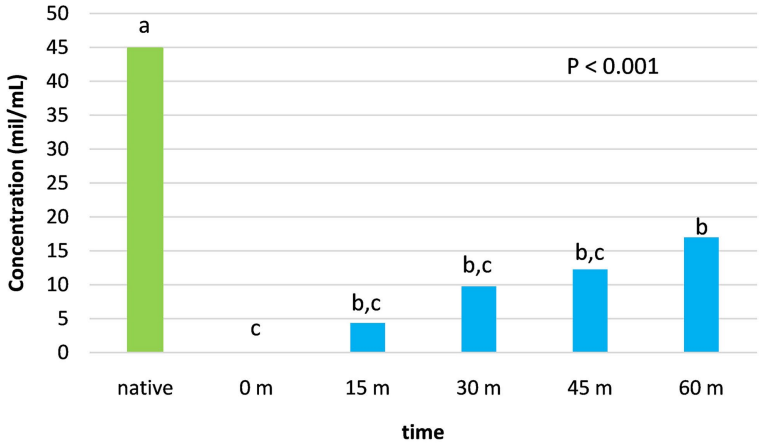

Figure 2. Average concentration across sample processing time using a modified NovoSort/ProteX technique ($P < 0.001$). Means with similar subscripts are statistically nondifferent.

As expected, no cells were observed in the media immediately after placing the NS in the PX. However, also as expected, concentration increased over time as the

motile cells migrated out of the seminal fluid/cryoprotectant inside the NS via the sample-mesh-media interface into the PX (Figure 2). The data suggests sufficient cells at all points past 0 min to perform ICSI or conventional insemination following oocyte retrieval in an assisted reproductive setting.

As demonstrated in Figure 3, the cells migrating out of the post-thaw samples were mostly motile, suggesting the cells are migrating under their own power and that there is little to no flow of liquid materials across the mesh. While there was initially no motility because no cell had yet migrated across the mesh, average motility at all points beyond 0 mins were above 90% and significantly higher than average post-thaw motility ($P < 0.001$). Further, there were no differences in the motility seen between 15 - 60 min. Figure 4 presents the raw data for motility for each of the eight samples demonstrating the consistency of the recovered motility across samples.

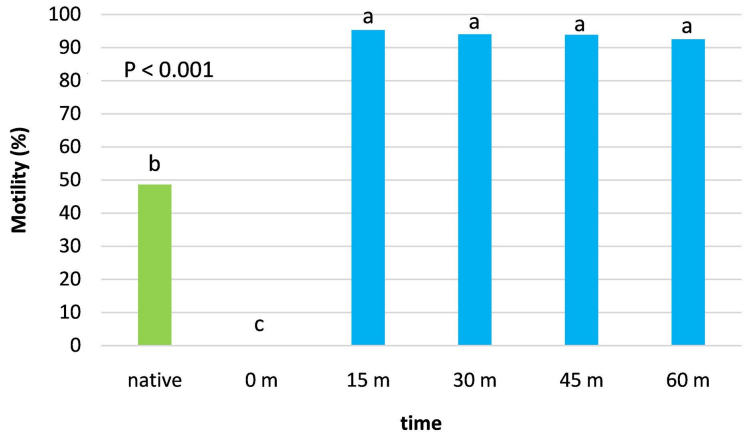

Figure 3. Average recovered motility of eight post-thaw semen samples processed using a modified NovoSort/ProteX technique ($P < 0.001$). Means with similar subscripts are statistically nondifferent.

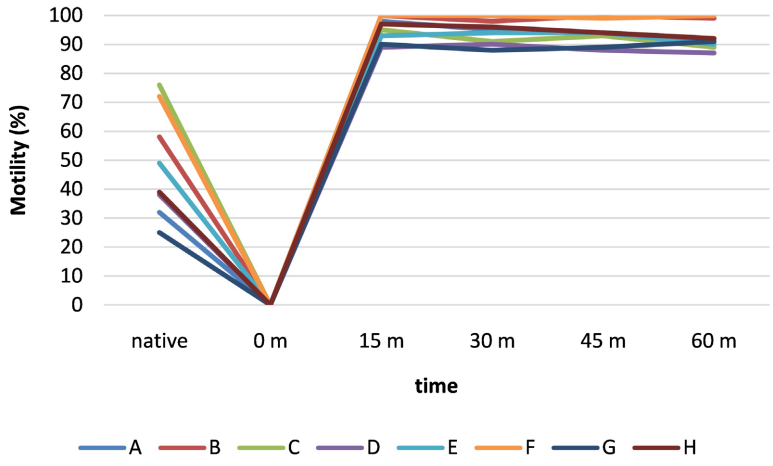

Figure 4. Individual motilities seen in eight post-thawed semen samples over 1 hr when processed using a modified NovoSort/ProteX technique. Note while starting motilities varied between 25% - 76% all processed samples yield motilities of >89% once cells begin migrating into the collection media.

Finally, the average forward progression remained consistent over the one-hour processing time. While there was no forward progression at the 0 time-point when cells had not migrated into the media ($P < 0.001$), all other time points demonstrated forward progressions similar to the non-processed sample immediately post-thaw (Figure 5).

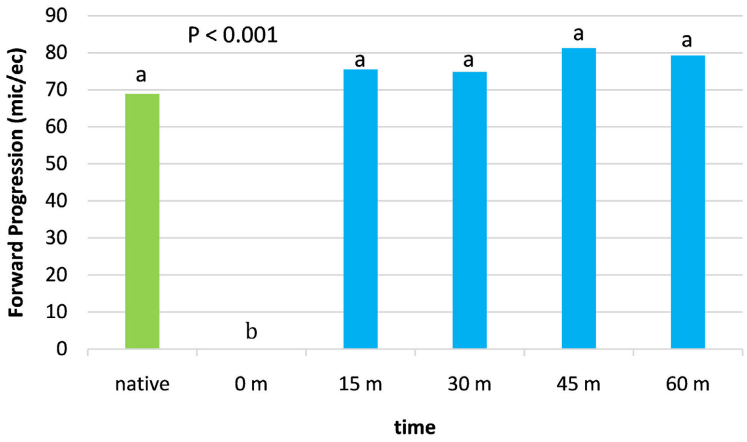

Figure 5. Use of a modified NovoSort/ProteX technique for semen processing appears to have little effect on cell forward progression when preparing sperm for use in assisted reproduction. Means with similar subscripts are statistically nondifferent.

Collectively, these data show that the modified NovoSort/ProteX technique would have produced an average total-motile population (concentration X motility) of between 4.2 and 15.6 million cells, providing sufficient cells for either ICSI or conventional insemination as early as 15 minutes from the start of processing. Further, as the average post-thaw total motile cell count would have been 21.8 million cells, this suggests a minimal recovery rate of 20% - 71% of the motile cells from the thawed samples.

4. Discussion

Assisted reproductive technologies are soon to be 50 years old [15]. Intrauterine insemination with prepared semen is older than that [16]. However, the chief means of harvesting motile cells has changed little beyond the use of centrifugation techniques [16] [17]. This may be due to early attempts at filtration using a fiberglass matrix, which proved to be damaging to sperm cell membranes and potentially resulted in the transfer of fiberglass fibers to the uterus [18]-[20].

Furthermore, with the introduction of intracytoplasmic sperm injection (ICSI) in the early 1990s [6] [9] [10], the general perception was that there was no further need to optimize semen sample quality, as few sperm were required to complete fertilization. However, during this same period, there has been significant evidence that spermatozoa with normal appearing morphology can carry significant damage and the very techniques used to isolate and store motile cell populations can cause damage to membranes, mitochondria, and even DNA [7]. In the case of DNA damage, there is now considerable evidence that if damaged DNA is present

at fertilization, it cannot be repaired and leads to aneuploidy, embryo malformation, and could be related to pregnancy failure [20]-[25].

Previous research from this laboratory [11] [12] [26]-[30] led to the development of the PX and NS. The PX is a specialized semen collection cup designed to maintain the sample's temperature, pH, and osmolarity stability when used with the recommended collection method. Further, the PX is uniquely designed to prevent the accumulation of reactive oxygen species. Together, these studies demonstrated the stability of the collection environment created and its ability to maintain sperm motility parameters [26]-[28], stabilize the cellular biochemical processes [30], which appear to result in improved outcomes [28]. When coupled with the NS device, with its woven mesh barrier, the devices can be used to isolate a highly motile sample from full ejaculates [11]. Preliminary studies suggest better outcomes when sperm are isolated using the combination of the PX and NS devices [12].

Initially, it was thought that the design, which allows harvesting of cells from larger volumes and complete ejaculates, might limit its usefulness. Further, anecdotal reports of other barrier devices have suggested they have limited usefulness with cryopreserved samples. However, as data from the current study demonstrate, it is possible to modify the NS separation procedure by placing the cryopreserved semen in the NS to isolate motile cells into the PX lower chamber, making it a viable option for isolating highly motile sperm from low-volume samples, like those supplied by commercial sperm banks. In a real-world setting, one would simply remove the NS and use cells collected in the fresh media contained in the PX. This preliminary trial demonstrated the good recovery of motile cells from PX well, which would be easily adequate for ICSI or conventional insemination.

As this study was conducted using a single cryoprotectant (egg yolk-glycerol), further studies may be necessary with other semen cryoprotectant combinations to ensure the hydrostatic barrier established at the cryoprotectant-mesh-media interface is maintained during sample processing.

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Conflicts of Interest

This is to acknowledge SP and LP are inventors of the PX/NS technology and shareholders in Reproductive Solutions, and SP serves as a paid consultant for scientific matters. While SP designed this study, it was carried out independently by EC and HD. Further, the study was reviewed and approved by an independent oversight committee as defined in our institutional conflict of interest management program.

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