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Quantitative and Qualitative Characteristics of Frozen-Thawed Bovine Spermatozoa Recovered Via a Conventional and a Standardized Swim-Up Technique

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CORREA, J.R., ZARMAKOUPIS-ZAVOS, P.N. and ZAVOS, P.M. *Quantitative and Qualitative Characteristics of Frozen-Thawed Bovine Spermatozoa Recovered Via a Conventional and a Standardized Swim-Up Technique.* Tohoku J. Exp. Med., 1997, **181** (2), 267-274 —— The objective of this study was to use the bovine as a model to evaluate the recovery of frozen-thawed spermatozoa via a conventional and a standardized swim-up technique. Frozen-thawed semen specimens (3 bulls) were washed and reconstituted with 2.9% (w/v) sodium citrate extender containing 20% (v/v) chicken egg yolk (SC-EY). Reconstituted sperm specimens were used for selection via conventional swim-up and the standardized ZSC™ method. The swim-up method consisted of ovarlayering the sperm specimen with 0.7 to 1.0 ml of isolation media (Ham's F-10), followed by 1 hr of incubation. The ZSC™ consisted of a conical cavity on the bottom of a glass column. The sperm specimen was placed into the conical cavity until the surface of the specimen was at the same level as the upper boundaries of the conical cavity. The surrounding periconical and epiconical areas were filled with 0.7 to 1.0 ml of isolation media, followed by 1 hr of incubation. The isolation media was removed (harvesting) from swim-up (80% volume) and ZSC™ specimens (100% volume) at the end of incubation. Recovered specimens were assessed for volume (ml), sperm concentration ($\times 10^6$ spermatozoa/ml), the percentage and grade of motility (0 to 4), the occurrence of osmotic shock and the percentage of spermatozoa reactive to the hypoosmotic swelling (HOS) test. Swim-up and ZSC™ selected specimens were qualitatively similar to each other. However, higher numbers of spermatozoa were recovered when sperm specimens were processed via the ZSC™ method (1.6 fold increase) than with the conventional swim-up technique. Because the ZSC™ method enabled the recovery of up to 100% of the overlayered media, it also enabled the recovery of most of the spermatozoa that migrated from the sperm specimen into the isolation media with no possibility of mixing the two, which was the case with

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the swim-up method, and which could also contaminate the recovered specimen with dead and immotile spermatozoa. Thus, the ZSC™ technique enabled the harvesting of the medium closest to the underlayered sperm specimen, which contributed to maximize the number of sperm recovered. When all assessed parameters were noted and all clinical improvements and efficiency of the method were compared to the swim-up technique, the sperm manipulation procedure of choice was clearly the ZSC™ method. ———— spermatozoa; swim-up techiques; sperm selection

Improvements in sperm quality have involved the use of the swim-up technique based on the swimming abilities of a small percentage of spermatozoa within a sperm population (Lopata et al. 1976; Harris et al. 1981; Russell and Rogers 1987; Zavos 1992). Basically, the swin-up technique consists of overlayering a sperm specimen, such as spermatozoa suspended in raw seminal plasma, culture medium or pellet form, with the same or higher volume of isolation medium of lower density, followed by incubation for 1 to 2 hr (Fulgham and Alexander 1990). During incubation, motile spermatozoa migrate across the interface between the sperm vehicle (culture medium) and isolation medium (Fulgham and Alexander 1990; Zavos 1992). For the most part, direct swim-up allows a highly motile fraction of sperm to be selected. However, the numbers of sperm recovered may be too low to permit clinical use when compared to other sperm selection techniques (Makler et al. 1984; Marrs et al. 1988; Zavos and Centola 1990a; Drobnis et al. 1991; Zavos 1992; Correa and Zavos 1996a). Also, removal of isolation medium and retrieval of higher numbers of motile spermatozoa becomes difficult when attempting to retrieve the isolation medium adjacent to the sperm migration interface (Zavos 1992). Sperm recovery via the swim-up technique can be affected if: 1) the volume of the isolation medium is too high in relation to that of the sperm specimen, and 2) the surface area between the two media is too low to enable efficient sperm migration into the isolation medium (Makler et al. 1984). Recently, a standardized self-contained system was designed to harvest almost all overlayered isolation media at the end of incubation and to maximize the sperm harvest (Zavos 1992). The ZSC™ device consists of a conical cavity located on the bottom of a glass column. The sperm specimen is placed into the conical cavity and the surrouding periconical and epiconical areas are filled with isolation media, followed by incubation for 1 hr. The motile spermatozoa rise into the overlayered modium then swim-down into the peripherally situated medium and are subsequently recovered at the end of incubation (Zavos 1992). In a study by Zavos (1992), the quality of human spermatozoa recovered via the ZSC ™ device was similar to those recovered by conventional swim-up. However, the ZSC™ recovered specimens yielded greater numbers of spermatozoa. Improvements in the number of sperm recovered were attributed to the design of the ZSC™ device, which enables the harvesting of up to 100% of the overlayered (isolation) medium compared to only 80% for swim-up techniques

without disturbing the sperm specimen-isolation medium interface (Zavos 1992). Similar studies have not been attempted with cryopreserved semen specimens.

The objective of this study was to use the bovine as a model to evaluate the recovery of frozen-thawed spermatozoa via a conventional and a standardized swim-up technique (ZSC™ method), and to assess the quantitative and qualitative characteristics of the recovered spermatozoa.

Materials and Methods

Sperm specimen handling

Frozen-thawed specimens from 3 bulls were used in this study (ABS Global, Inc., Madison, WI, USA). The medium used for bovine semen cryopreservation consisted of 2.9% (w/v) sodium citrate containing 20% (v/v) chicken egg yolk (SC-EY) and 7% (v/v) glycerol (Sullivan and Elliot 1971; Pace et al. 1981). The medium used for isolation of swim-up and ZSC™ specimens was Ham's F-10 containing 3% (w/v) BSA (SpermPrep™ medium; ZBL, Inc., Lexington, KY, USA). The frozen specimens were thawed at 37°C for 10 sec and transferred to a water bath at 21°C for 1 min to complete thawing (Correa et al. 1996). Specimens were slowly diluted, to prevent abrupt changes in osmotic pressure, 1 : 1 (v/v) with SC-EY media without glycerol (Correa and Zavos 1995). Following dilution, the specimens were centrifuged (15 ml conical centrifuge tube) for 8 min at $400 \times g$ and reconstituted to their original volume with SC-EY media. The reconstituted specimens were split into 3 (0.5 ml) aliquots. Aliquot 1, 2 and 3 corresponded to control, swim-up, and ZSC™ specimens, respectively. Specimens were assessed (at 0 and 1 hr postincubation) for sperm concentration ($\times 10^6$/ml), the percentage and grade of motility (0 to 4; Zavos et al. 1995), the occurrence of osmotic shock (Correa and Zavos 1995) and the percentage of spermatozoa reactive to the hypoosmotic swelling (HOS) test (Jeyendran et al. 1984; Correa and Zavos 1994). Sperm concentration and motility characteristics were determined via the use of the Makler counting chamber (Makler 1980). The quality of sperm progressive motility was graded as follows: Grade 0, no movement; Grade 1, oscillating movement but stationary; Grade 2, slow movement with no fixed direction; Grade 3, slow progressive movement; and Grade 4, fast progressive movement. The proportion of spermatozoa with coiled tails represents spermatozoa with biochemically active and intact membranes that were injured by osmotic shock damage, resulting in the loss of sperm viability (Correa and Zavos 1995 Correa et al. 1996, 1997). The HOS test was employed to measure the proportion of spermatozoa with biochemically active and intact plasma membranes (Jeyendran et al. 1984). The HOS test was performed by adding 0.1 ml of the sperm specimen to 1.0 ml of a 100 mOsm/l HOS diluent, followed by incubation at 37°C for 1 hr (Correa and Zavos 1994). The swelling patterns of spermatozoa exposed to the HOS test were assessed at the end of incubation (Jeyendran et al. 1984). The total functional sperm fraction (TFSF; Zavos and Centola

1990b), an inclusive term that takes into consideration the total sperm number, the percentage of motility and the percentage of spermatozoa reactive to the HOS test, was calculated to assess the overall quality of the spermatozoa.

Swim-up procedure

The swim-up procedure was performed by gently overlayering 0.7 to 1.0 ml of Ham's F-10 medium over Aliquot 2. The conical centrifuge tube was held at a 90° angle for 1 hr (37°C) to allow the maximal number of spermatozoa to migrate into the ovarlayered medium. Following incubation, the maximum volume (80%) of the overlayered medium was carefully removed without disturbing the sperm specimen-overlayered medium interface and the recovered spermatozoa were assessed as previously described.

ZSC™ procedure

The ZSC™ procedure was performed by placing Aliquot 3 (0.5 ml) into the conical cylinder of the ZSC™ device (ZBL, Inc.) and bringing the surface of the sperm specimen to the same level as the upper boundaries of the conical cylinder (Fig. 1). Following this step, 0.7 to 1.0 ml of Ham's F-10 medium was placed into the ZSC™ column completely saturating the periconical area and a certain level of the epiconical area of the glass column (Fig. 2). The ZSC™ device was capped and allowed to set in a controlled environment at 37°C for 1 hr to allow high quality, motile spermatozoa to rise or migrate from the conical cylinder into the epiconical and periconical areas of the overlayered medium (Fig. 2). The harvesting or removal of the overlayered medium from the ZSC™ at the end of incubation was done by gently placing a 22G needle into the periconical area, followed by aspiration of the medium until all of the overlayered medium was removed (Fig. 3). The recovered spermatozoa were assessed as previously described.

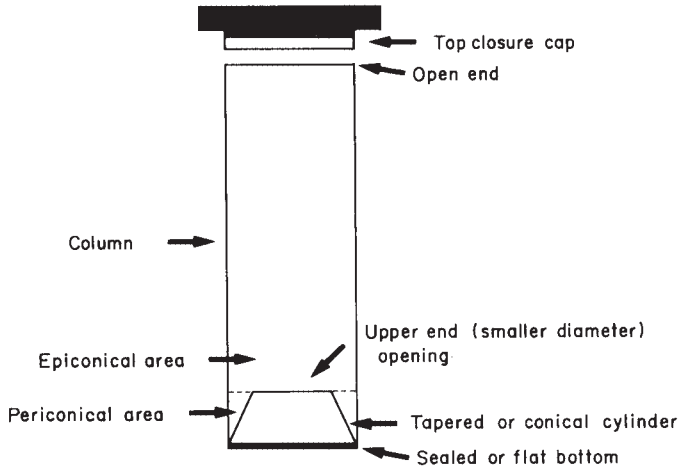

Fig. 1. The ZSC™ device and its various compartments used for harvesting high quality spermatozoa.

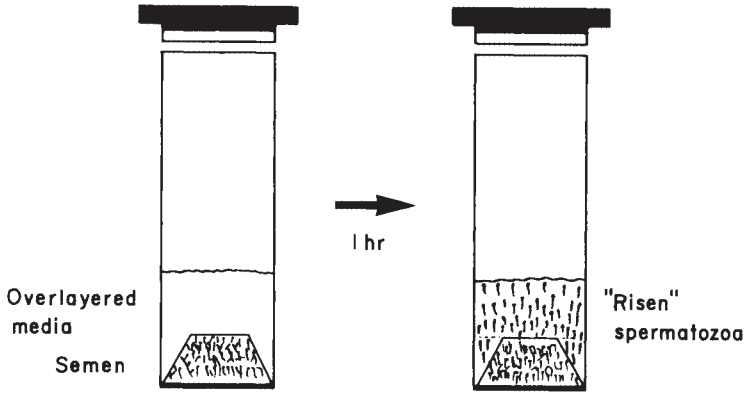

Fig. 2. Diagrammatic illustration of the ZSC™ device depicting the mode of use on a step by step basis during sperm manipulation and rise of the spermatozoa.

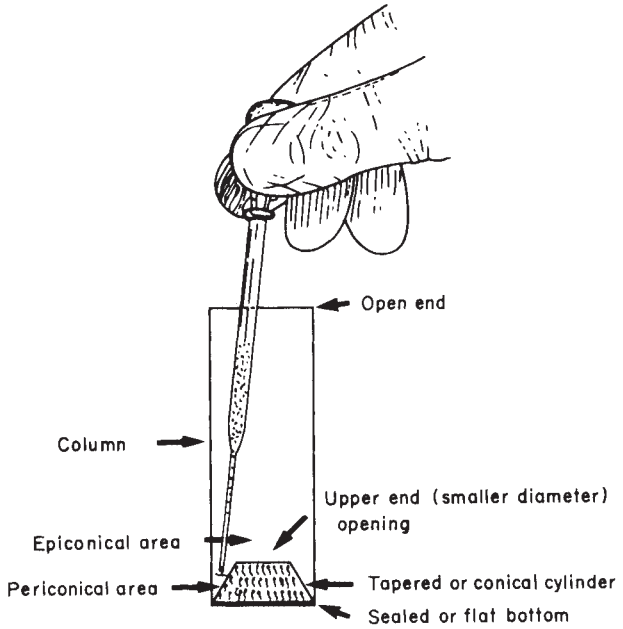

Fig. 3. Diagrammatic illustration of the ZSC™ device and harvest of the overlayered media containing the spermatozoa at the end of the incubation period.

Statistics

The results were reported as means ± S.D. The data was analyzed by ANOVA procedures using the SAS Statistical Package (SAS Institute Inc. 1989). The experiment was replicated 5 times.

Results

The results obtained are summarized in Table 1. Higher numbers of spermatozoa were recovered when sperm specimens were processed via the ZSC™ device (1.6 fold increase) than with the coventional swim-up ($p < 0.05$). When compared to control specimens, the sperm recovery for specimens processed via conventional swim-up and the ZSC™ method were 17 and 27%, respectively.

TABLE 1. *Quantitative and qualitative characteristics of frozen-thawed bovine spermatozoa selected via a conventional or a standardized (ZSC™ method) swim-up technique (means ± S.D.*)*

Sperm specimen	Sperm characteristics assessed					
	Total sperm ($\times 10^6$)	Motility (%)	Grade (0 to 4)	Osmotic shock[a] (%)	HOS[b] (%)	TFSF[c] ($\times 10^6$)
Control (0 hr)	11.4 ± 2.3^d	68.4 ± 4.1^d	3.4 ± 0.1^d	12.8 ± 1.7^d	39.2 ± 2.8^d	3.1 ± 0.7^d
Control (1 hr)	11.4 ± 2.3^d	57.5 ± 5.3^e	3.2 ± 0.4^e	16.7 ± 2.6^e	26.5 ± 3.2^e	1.8 ± 0.5^e
ZSC™ (1 hr)	3.1 ± 0.7^e	87.1 ± 2.6^f	3.7 ± 0.1^f	6.9 ± 2.2^f	45.3 ± 3.4^f	1.2 ± 0.3^f
Swim-up (1 hr)	1.9 ± 0.5^f	86.5 ± 3.4^f	3.7 ± 0.1^f	7.1 ± 2.5^f	44.0 ± 3.1^f	0.7 ± 0.3^g

*The experiment was replicated 5 times.

[a]Osmotic shock, % spermatozoa with coiled tails; [b]HOS, hypoosmotic swelling test (% swollen spermatozoa); [c]TFSF, total functional sperm fraction (total sperm × % motility × delete; % HOS test response)

[d,e,f,g]Values with different superscripts within columns are significantly different ($p < 0.05$).

More importantly, when the ZSC™ method was applied, up to 38% of the TFSF was recovered, where only 22% of the TFSF was recovered when the conventional swim-up method was used as compared to the control (0 hr) values. Swim-up and ZSC™ selected specimens were qualitatively similar to each other ($p > 0.05$) and all of the qualitative characteristics of these specimens were significantly improved over those of control specimens ($p < 0.05$). The qualitative characteristics of control specimens, and subsequently the TFSF, decreased significantly during 1 hr of incubation ($p < 0.05$). Furthermore, the occurrence of osmotic shock in spermatozoa from control specimens increased during incubation ($p < 0.05$).

DISCUSSION

The results obtained in this study indicate that the ZSC™ method yielded spermatozoa of similar qualitative characteristics as those recovered with the conventional swim-up method. However, the ZSC™ method yielded a significantly higher number of total sperm and than did the conventional swim-up method. The ZSC™ technique enabled the harvesting of the medium closest to the underlayered sperm specimen, which maximized the number of sperm recovered (Zavos 1992). When the TFSF value was calculated for both techniques (Zavos and Centola 1990b), then the TFSF value for the ZSC™ method was 1.7 fold higher ($p < 0.05$) than the TFSF value obtained with the conventional swim-up method. The ZSC™ procedure was also less cumbersome and easier to use than the swim-up procedure. Because of its unique design the ZSC™ enables the end user to layer the semen specimen and the isolation medium with much greater ease and a lower margin of error (Zavos 1992). The same seems to be true during the recovery of the overlayered medium that contains the motile, healthy

population of spermatozoa (Zavos 1992).

In the various swim-up techniques, motile spermatozoa migrate from the underlayered medium (sperm specimen) and migrate across the sperm specimen-isolation medium interface to isolate themselves from the original sperm population (Fulgham and Alexander 1990; Zavos 1992). The ZSC™ device consists of a column with a conical cavity located on the bottom were the semen specimen is placed, and from where the sperm rise or migrate into the overlayered medium then swim-down into the peripherally situated medium and are subsequently recovered at the end of the procedure. The sperm migration is continuous, with no backward flow of spermatozoa at the sperm specimen-isolation medium interface (Zavos 1992). Because the ZSC™ method enabled the recovery of up to 100% of the isolation media volume (compared to 80% with the swim-up method), it also enabled the recovery of most of the spermatozoa that migrated from the sperm specimen into the isolation media with no possibility of mixing the two, which is the case with the swim-up method most of the time, and which can contaminate the recovered specimen with dead and immotile spermatozoa (Zavos 1992). Also, the sperm specimen recovered via the ZSC™ method can be used directly (without further processing) for intrauterine insemination (IUI), in vitro fertilization (IVF) or other forms of assisted reproductive technologies.

The ZSC™ method is a standardized technique which would enable the clinician and the researcher to draw various inferences and conclusions during comparisons between different specimens from the same or different subjects. When all assessed parameters were noted and all clinical improvements and efficiency of the method were compared to the swim-up technique, the sperm manipulation procedure of choice was clearly the ZSC™ method.

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