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Andrology Lab Corner*

Contamination by Seminal Plasma Factors During Sperm Selection

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Contamination by semen constituents can hinder or decrease sperm hyperactivation and acrosome reaction (Andrews and Bavister, 1989; Han et al, 1990; Cross, 1993; Andrews et al, 1994; Cross, 1996; Cross and Mahasreshti, 1997; Mortimer et al, 1998), and zinc in particular hampers fertilization and early embryonic development (Tsunoda and Chang, 1977; Quinn et al, 1982; Van der Ven et al, 1983; Quinn and Begley, 1984; Blesbois and de Reviers, 1992; Vidal and Hidalgo, 1993; Stephenson and Brackett, 1999; Suzuki et al, 2002). In clinical practice, there are 2 main techniques to separate spermatozoa from seminal plasma: those based on sperm mobility ("swim-up") and gradient centrifugation. Both methods involve a considerable duration (20-60 minutes) of the preparation process. The extended time is partially necessary for the sperm to overcome the osmotic stress when spermatozoa migrate from the environment with high and variable osmolarity in seminal plasma to the lower osmolarity of the selection media (cf Björndahl et al, 2004; Cooper et al, 2004). One factor that could contribute to contamination of the insemination medium by seminal factors is simple diffusion during prolonged contact between seminal plasma and medium.

Although the main features of sperm preparation techniques are quite similar between different laboratories, there are no detailed golden standards for sperm preparation techniques. Technical studies in this field have been focused primarily on the yield of normal, motile sperm and secondarily on the exclusion of seminal factors promoting sperm damage by reactive oxygen species (cf Mortimer and Mortimer, 1992; Mortimer, 1994; Henkel and Schill, 2003). One way to reduce the risk for sperm damage caused by seminal plasma is to dilute and wash sperm with a synthetic medium before initiating swim-up. Furthermore, repeated washing after the selection step could reduce (by dilution) contamination from diffusion during selection, but such procedures are time consuming

(repeated washing and centrifugation) and might decrease the numeric yield of spermatozoa. Moreover, the stress induced by centrifugation affects sperm vitality (Mortimer, 1994). Therefore, methods based on direct swim-up from the liquefied semen and as few as possible timeconsuming steps are commonly used because of their relative simplicity.

In this study, we investigated whether zinc (seminal plasma marker for prostatic secretion) could diffuse from liquefied semen to the medium in which prepared spermatozoa are exposed to oocytes and compared swim-up to density gradient centrifugation.

Experiment 1

In the first series of experiments, zinc concentration was measured in media before and after sperm preparation (Table 1). Swim-up was performed in duplicate for each of 10 samples by laying 0.5 mL of semen (with a 1-mL sterile Falcon pipette; Becton Dickinson number 356521 [Becton Dickinson AB, Stockholm, Sweden]) under 2 mL of swim-up medium (Earles balanced salt solution supplemented with 10% human serum). Tubes (14-mL Falcon centrifuge tube, Becton Dickinson number 352001) were incubated at 37 °C and 5% CO_2 for 45 minutes, inclined at an angle of 45 °. After incubation, 1 mL of medium was carefully pipetted from the top meniscus of each tube with a glass Pasteur pipette (Labora number 010-1550-150 [Berman Labora AB, Stockholm, Sweden]). Duplicates were pooled and mixed with 2 mL of fresh medium (dilution 1:2). The tubes were then centrifuged (500 x *g*; 6 minutes), after which 3 mL of the supernatant was discarded, and to the remaining 1 mL, another 1 mL of fresh medium was added (total dilution 1:4, from swim-up suspension).

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Table 1. *Zinc concentration in supernatant of first wash and final sperm suspension with fresh medium, after swim-up, and gradient centrifugation compared with medium used; duplicate assays were performed for each of 10 semen samples*†

Gradient centrifugation was done with a 2-layer (90%-45%, 1 + 1 mL) discontinuous Pure Sperm gradient (Nidacon, Gothenburg, Sweden) on which 1 mL of semen was layered. After centrifugation (300 x *g*; 20 minutes), pellets were washed twice with 5 mL of the same medium as used for swim-up preparations and finally resuspended in 1 mL of fresh medium.

Zinc was determined in the media of the different sperm suspensions with a colorimetric technique for seminal zinc (WHO, 1999) and on the supernatant after centrifugation of the sperm suspension (20 minutes at 2500 x *g*).

Statistical analyses were performed with GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, Calif, www.graphpad.com).

The final sperm preparations after swim-up had zinc levels that were significantly higher (mean 28 µmol/L, range 14-72 µmol/L) than the concentration in the medium used (11 µmol/L; 1-sample *t* test, mean significant different from 11 µmol/L [medium concentration], $P < .05$).

Preparation by gradient centrifugation also resulted in zinc concentrations in the preparations statistically different from the level in the medium (12.5 vs 11.1 µmol/L; 1-sample *t* test, mean significant different from 11.1 µmol/L [medium level], $P < .001$), but actual concentration of zinc was only marginally increased (on average, 1.4 µmol/L), whereas in corresponding preparations after

swim-up, zinc concentrations were more than double (on average, increased by 17 µmol/L).

Experiment 2

In the second series of experiments, we measured zinc concentrations at different distances from the semen-medium interface (Table 2). "Swim-up" was done in duplicate from each of 5 different sperm-free seminal plasma pools, obtained as the supernatant after centrifugation (20 minutes at 2500 x *g*). After 45 minutes of incubation, the supernatants were removed in 0.5-mL portions starting from the top meniscus of each tube, representing different diffusion distances from the bottom layer of seminal plasma. The concentration of zinc increased toward the bottom of the swim-up tubes (Table 2: repeated measures ANOVA test, $P < .0001$, $F = 18.48$, $R^2 = .8221$, n = 5; with a linear trend: $R^2 = .6544$, $P < .0001$).

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Table 2. *Zinc concentration in swim-up medium at different diffusion distances (0.5-mL portions) with the use of 5 sperm-free seminal plasma pools void of spermatozoa after centrifugation*

Experiment 3

To investigate whether there was a time dependence in the concentration of zinc in the swim-up medium, we measured the zinc concentration in the top portion of medium after different durations of incubation in a third series of experiments (Table 3). We used triplicate swim-up preparations from 7 different sperm-free seminal plasma samples prepared as above. The top portions (1 mL) were taken off at 15, 45, and 120 minutes after start of incubation. There was as a significant increase in zinc concentration with longer incubation time (repeated measures ANOVA test, $P < .0001$, $F = 14.92$, $R^2 = .7132$, n = 7; with a linear trend: $R^2 = .5468$, $P < .0001$).

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Table 3. *Concentration of zinc in top 1 mL of swim-up medium after 15, 45, and 120 minutes incubation performed in triplicate from each of 7 sperm-free seminal plasma pools*

Discussion

Some 300 000 sperm preparations are performed every year for Assisted Reproduction Technique (ART) procedures in Europe alone (Nyboe Andersen et al, 2004), which emphasizes the concern for proper validation and quality control of these procedures. Swim-up and density gradient centrifugation are the most common techniques. In these experiments, we have measured zinc concentrations in the final medium and showed the proof-of-principle that swim-up preparation of sperm can result in significant amounts of seminal plasma constituents in the final medium used for sperm interaction with oocytes. In contrast to the swim-up procedure, density gradient centrifugation did not result in similarly high concentrations of zinc in the sperm-oocyte incubation medium.

The remaining concentrations of zinc we found in the media after swim-up preparation could be a significant negative factor for oocyte maturation (bovine: Stephenson and Brackett, 1999), fertilization (mouse: Aonuma et al, 1981), and early embryonic development (mouse: Vidal and

Hidalgo, 1993; bovine: Stephenson and Brackett, 1999; Table 4). Results also indicate that, among noncapacitated mammalian sperm, presence of zinc can inhibit the acrosome reaction (mouse: Aonuma et al, 1978, 1982; hamster: Andrews and Bavister, 1989; Andrews at al, 1994; human: Riffo et al, 1992) and that presence of seminal plasma constituents in the "insemination" medium can decrease fertilization during in vitro fertilization in mammals (hamster: Tsunoda and Chang, 1977; mouse: Quinn et al, 1982; Quinn and Begley, 1984; human spermatozoa and hamster oocytes: Van der Ven et al, 1983; boar: Suzuki et al, 2002) and fowls (Blesbois and de Reviers, 1992).

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Table 4. *Experimental data on effects of zinc in relation to sperm function, oocyte maturation, fertilization, and embryo development*

In clinical practice, the ART laboratory might be tempted to try and compensate for a low yield of motile sperm after swim-up preparation by using a larger proportion of the swim-up medium. If so, the present data indicate that the contamination of seminal compounds like zinc will increase in the droplet used for fertilization. Thus, the inhibitory effects of seminal components could contribute to an unsuccessful outcome of an ART treatment with initially low sperm yield at swim-up. Furthermore, in many ART laboratories, density gradient is the method of choice for sperm preparation under the assumption that a different and better population of sperm is selected. However, the lower contamination with seminal plasma constituents can be an ignored factor, making this technique preferable.

One way to reduce seminal contamination in a swim-up preparation would be to shorten the duration of the sperm selection procedure. This might be achieved by reducing the time required for adjustment to hypotonic shock; that is, start selection as soon as possible after ejaculation before the semen osmolarity has increased from 290 mOsm (Björndahl and Kvist, 2003; Cooper et al, 2004) and thus is more equivalent to the media used (cf Björndahl et al, 2004).

This study doesn't tell by which mechanism—and to what extent—seminal plasma contaminations like zinc contribute to the failure of ART procedures, but it indicates the importance of evaluating sperm selection techniques, not just with regard to the final concentration of motile and normal sperm. Also, possible contamination with seminal plasma constituents should be evaluated routinely when evaluating sperm preparation procedures to avoid negative influences on sperm function (capacitation) as well as oocyte maturation, fertilization, and early embryo development.

Footnotes

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