Nanowater enhances cryoprotective properties of glycerol-containing extenders used for ram semen freezing: A preliminary study spanning laboratory testing

DOI: 10.2478/aoas-2022-0008

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Received date: 3 May 2021
Accepted date: 9 December 2021

To cite this article: (2022). Szymanowicz J., Murawski M., Schwarz T., Bartlewski P.M. (2022). Nanowater enhances cryoprotective properties of glycerol-containing extenders used for ram semen freezing: A preliminary study spanning laboratory testing, Annals of Animal Science, DOI: 10.2478/aoas-2022-0008

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Nanowater enhances cryoprotective properties of glycerol-containing extenders used for ram semen freezing: A preliminary study spanning laboratory testing

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Abbreviated title: Cryopreservation of ram semen

Source of research funding: The present study was funded by the Polish National Center for Research and Development (grant no. 12011310/ZKO/PB-R3); “Badania Młodych” grant (no. BM-42604260 and BM 4247/2015); statutory funds of the Department of Animal Biology, University of Agriculture in Cracow, Poland and statutory funds of the Department of Swine and Small Ruminant Breeding (no. DS/KHTChIMP/3242/13), Cracow, Poland; Department of Biology and Animal Breeding, University of Life Sciences in Lublin; Department of Biomedical Sciences, University of Guelph, Guelph, ON, Canada; and Nantes Nanotechnology Systems in Bolesławiec, Poland (in-kind contribution).
Abstract

It has been suggested that nanowater (NW-water declustered in the cold plasma generator and characterized by a low freezing point and high diffusivity) could improve ram semen quality after freezing in glycerol-containing extenders. Eighteen ejaculates from six Olkuska rams were divided into six equal portions each, and then diluted (800×10⁶ spermatozoa/ml) and frozen in the fructose-skimmed milk-egg yolk Kareta extenders containing 3% or 7% of glycerol. The extenders were prepared with deionized water (DW-3% and DW-7%) or NW declustered for 15 min (NW15’) or 30 min (NW30’). Post-thaw sperm motility, proportions of sperm defects and percentages of apoptotic, necrotic, and live spermatozoa were determined. The proportion of spermatozoa with mid-piece defects was lower (P<0.05) in NW15’-3% compared with DW-3%. Sperm progressive motility was greater (P<0.05) for spermatozoa cryopreserved in both NW30’ (NW30’-3%/7%) extenders compared with their respective controls (DW30’-3%/7%). The proportion of necrotic spermatozoa 1 h after thawing was lower (P<0.05) in NW30’-7% compared with DW-7%, whereas the proportion of live cells detected immediately and 1 h after thawing was greater (P<0.05) in NW30’-7% than in DW-7%. In summary, NW enhanced cryoprotective effects of glycerol-containing extenders with an increase in sperm viability being greater with 7% than 3% of glycerol. Different declustering times appear to alter NW properties. These observations merit future studies of the utility of NW for semen cryopreservation in rams and other mammalian species. The specific mechanisms whereby NW ameliorates the quality of frozen-thawed ram spermatozoa remain to be elucidated.

**Key words:** ram, semen, cryopreservation, extender, glycerol, nanowater
Because spermatozoa lack the ability to adapt to subzero temperatures (Hezavehei et al., 2018), semen cryopreservation requires the use of cryoprotective agents (CPAs) to enable sperm survival and prevent structural damage under hypothermic conditions (Ciereszko and Strzeżek, 1989). Based on their ability to cross the cell membrane, CPAs are divided into two categories: permeating CPAs (capable of traversing plasma membrane; e.g., glycerol and dimethyl sulfoxide) and non-permeating CPAs (unable to diffuse into cytoplasm; e.g., raffinose, egg-yolk or skim milk). Permeating CPAs are non-ionic compounds (i.e., containing covalent bonds) that are highly soluble in water even at low temperatures; they can easily diffuse through cell membranes due mainly to their small molecular size (Debenedetti et al., 1989). Cryoprotective agents that permeate into cytoplasm replace the portion of intracellular water without excessively “dehydrating” the cell as they reach equilibrium (Trzcińska, 2006). Cryoprotective properties of permeating CPAs are associated with their ability to significantly reduce the concentration of electrolytes in the solvent (Anzar et al., 2002; FAO, 2012) and to decrease the degree of cell shrinkage caused by osmotic stress (Shinitzky and Barenholz, 1974). Moreover, permeating CPAs reduce intracellular ice formation because they freeze at lower temperatures than water (Shipley et al., 2007). Lastly, glycerol increases media viscosity (Takamura et al., 2012), which leads to further reduction in ice crystal formation and expansion.

A major disadvantage of using glycerol for sperm freezing is that it diffuses through the plasma membrane at a slower rate than water (Ball and Vo, 2001). Consequently, when glycerol is added to or removed from semen, spermatozoa still undergo rapid osmotic shrinking or swelling, respectively. Such an efflux or influx of fluid into mammalian cells may change their initial volume up to two-fold (Jiang and Sun, 2013). Ultimately, cell membrane damage and cell lysis due to osmotic shock may occur (Anzar et al., 2002), and ram spermatozoa have very low osmotic tolerance (Freshney, 2009). Therefore, in certain species, including rams, glycerol is added to semen extenders in a step-wise manner, beginning with low concentrations and then gradually increasing its content; each consecutive addition of glycerol is followed by sperm equilibration for several minutes (Morris, 2006; Davidson et al., 2015). Furthermore, glycerol at 37°C is cytotoxic and during thawing it can disturb normal cell metabolism, further decreasing semen viability.

A wide range of water sources can be used as diluents for the preparation of semen extenders, including mineralized drinking water and distilled or purified water (Ali, 2015; Murawski et al., 2015). Nanowater (NW) is produced through the low-frequency cold plasma
treatment of deionized water (DW), the process referred to as water declustering (Rusanov and Fridman, 1978; Muldrew and McGann, 1990; Białopiotrowicz et al., 2016). During this process, water molecules that under normal conditions form clusters or aggregates of up to 1,000 molecules are broken down into smaller clusters (Rusanov and Fridman, 1978; Bröll et al., 1999). Changes in the spatial configuration of NW are caused by breaking hydrogen bonds and result in various modifications of its physiochemical properties. Nanowater significantly increases solubility of many gases as well as inorganic and some non-polar (organic) substances (Białopiotrowicz et al., 2016). A difference in solubilizing ability between NW and DW arises from the relatively high dielectric constant ($\varepsilon$) of NW (Białopiotrowicz et al., 2016); the dielectric constant is an indicator of how well the solvent is able to separate ions. According to Bröll et al. (1999), NW molecules reduce the activation energy and hence effectively break the chemical bonds. Nanowater is also more efficient a carrier of solubilized substances compared with DW (Niemirowicz and Car, 2001; Mystowska and Dąbrowski, 2013) and hence may increase transmembrane transport of media constituents.

The major objective of the present study was to evaluate the effects of NW on post-thaw semen characteristics when it was used as a diluent for the 3% and 7% glycerol-containing Kareta extenders. We hypothesized that diluting the extenders with NW would improve the quality of frozen-thawed ram semen compared with DW-diluted extenders, because of the unique properties of NW.

**Material and methods**

**Animal management**

All experimental procedures complied with the European Community directives for animal experimentation and were conducted under the local animal care/bioethics committee authorization no. 165/2016. The present experiment utilized semen collected from six Olkuska breed rams aged 4–12 years and housed in a field research station of the Department on Animal Biotechnology (Agricultural University of Cracow) situated in Bielany, Poland (latitude: 50°2’55”N; longitude: 19°49’45”E). The rams remained indoors and received daily maintenance ratios of hay (0.3 kg/animal/day) and hay-silage (4 kg/animal/day); water and anti-parasitic, mineralized salt licks (Star Bloc Phyto Vers; Guyokrma Ltd., Prague, Czechia; Nosal et al., 2016) were available *ad libitum*. In addition, the animals received 150–300 g of concentrate per day (75% oats, 20% barley, and 5% rapeseed meal), for 1 week after shearing in spring (Elvidge and Coop, 1974).

**Semen collection and assessment**
Ejaculates were collected over 3 consecutive days in the mid-breeding season (November) into calibrated, pre-warmed (37°C) and insulated glass tubes attached to a pre-warmed (38°C) artificial vagina, in a presence of a teaser. Ejaculate volume, color and consistency were assessed immediately after collection. Semen concentration was determined using a Bürker-Turk counting chamber. A sample of ejaculate (25 μl) was diluted in 10 ml of 3% saline solution and then 10 μl of diluted semen was placed in a chamber and covered with a coverslip. Sperm count was completed using the phase-contrast microscope Nikon Eclipse 80i microscope (Nikon Corp., Tokyo, Japan) at 400× image magnification. An initial assessment of sperm motility was conducted in the Blom’s chamber on a warm plate (37°C), using the Nikon Eclipse 80i microscope at 200× magnification, as previously described by Murawski et al. (2015). All 18 ejaculates collected from the present Olkuska breed rams were classified as normal based on ejaculate volume, progressive sperm motility >70%, and lack of contamination with urine or other substances. The average ejaculate volume was 1.5±0.4 ml with a sperm concentration of 2.7±7.7×10⁹ spermatozoa/ml and progressive motility of 94.4±8.0%.

**Extender preparation and semen freezing**

Six glycerol-based extenders, modified from Kareta extender (Kareta et al., 1972), were prepared with: 1) deionized water (DW; Aqua Purificata®; Prolab, Gliwice, Poland) with 3% of glycerol (DW-3%); 2) DW with 7% of glycerol (DW-7%); 3) nanowater (NW; Nantes Nanotechnology Systems, Boleslawiec, Poland) declustered for 15 min with 3% of glycerol (NW15’-3%); 4) NW declustered for 15 min with 7% of glycerol (NW15’-7%); 5) NW declustered for 30 min with 3% of glycerol (NW30’-3%); or 6) NW declustered for 30 min with 7% of glycerol (NW30’-3%). The two desclustering times were chosen empirically based on previous laboratory testing and fertility trials using ram semen frozen in a commercial extender Triladyl® (MiniTub GmbH; Tiefenbach, Germany), and yielding the best results after artificial insemination (unpublished data). Ejaculates from each ram were divided into six equal parts and then frozen using the two-step freezing protocol as detailed below: 1) initial 30-min equilibration of diluted semen at 22°C (extender consisting of DW or NW and egg yolk (4:1) with addition of 1 g of fructose per 100 ml); 2) equilibration for 30 min to 4°C in a walk-in cooler; 3) further equilibration for 30 min at 4°C followed by 2-fold dilutions every 10 min to a final concentration of 3% or 7% vol/vol of glycerol (extender 6% or 14% vol/vol of glycerol, respectively); 4) further equilibration for 30 min at 4°C; 5) loading extended semen (final concentration of 800×10⁶ spermatozoa/ml) into 0.25-cc plastic straws (Rovers; Piaseczno, Poland); 6) equilibration in liquid nitrogen vapors
(−120°C) for 10 min; and 7) plunging the straws in liquid nitrogen (−196°C) before placing them in plastic goblets arranged in a liquid nitrogen container. All semen samples were thawed in a water bath at 37°C for 60 sec. After thorough dehumidification of the straws, semen samples were transferred into sterile analytical tubes for further analyses.

**Post-thaw semen evaluation**

*Semen motility analysis*

Determination of post-thaw semen motility used the same tools as those described above for the semen quality assessment. Briefly, semen motility was assessed with a computer-assisted Sperm Class Analyzer system (CASA; ver. 5.0; Microoptic® Automatic Diagnostic Systems, Barcelona, Spain) according to the manufacturer’s specifications, using a phase-contrast Nikon Eclipse 80i microscope and a 4-μl Leja® disposable counting chamber placed on a warming plate (37°C). The progressive changes in semen motility were recorded every 30 min for 1 to 1.5 h and then every 15 min until complete absence of motile spermatozoa. Throughout the duration of the test, semen samples were constantly kept in a water bath at 37°C. A field of analysis included all spermatozoa that were ≥5 mm away from the edge of the coverslip to avoid the confounding effects of peripheral sample drying on sperm motility.

*Sperm morphology assessment*

The proportions of normal and aberrant spermatozoa were estimated using a SpermBlue® kit (Microoptic SL Co., Barcelona, Spain) according to the manufacturer’s instructions. Histological smears were analyzed for sperm morphological defects including abnormal and detached heads, abnormal mid-pieces and tails, and proximal and distal cytoplasmic droplets. For each semen sample, two hundred spermatozoa were evaluated under oil immersion at 1000× magnification (Nikon Eclipse 80i microscope) in a bright view field. Sperm smears were prepared by dispensing 10 μl of semen on a glass slide and were left to air dry. Dried smears were placed vertically into a staining tray containing fixatives (i.e., SpermBlue® fixing solution) at 20°C for 2 min. All smears were then carefully removed from a staining tray and placed without washing for another 2 min in a tray containing SpermBlue® staining solutions. Slides were carefully removed from a staining tray and dipped slowly into a container filled with distilled water (two times for 3 sec). After washing, the slides were placed on a paper towel at a 60° angle for air drying. When slides were completely dry, they were mounted with Eukitt® (Sigma-Aldrich) and covered with a coverslip.

*Ultraviolet detection of alanine transferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP)*
Semen samples (20 μl per each enzyme analyzed) were placed in plastic, sterile reagent tubes and centrifuged for 6 min at 400×g in an Eppendorf 5415D centrifuge (Eppendorf AG, Hamburg, Germany). Seminal plasma was collected, transferred into a reagent tube and frozen at −20°C for later analyses. Measurements of ALT, AST and ALP were done using an Automated-Olympus-AU600 biochemical analyzer (Olympus Corporation; Tokyo, Japan) and optimized UV-test scoring system compliant with the guidelines of the International Federation of Clinical Chemistry. The sensitivity of the analytical methods used was 1U/l for all three enzymes measured in this experiment.

Detection of live, apoptotic and necrotic spermatozoa by flow cytometry

The Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen™, Becton Dickinson, Franklin Lakes, NJ, USA) and propidium iodide (PI) staining protocol were used for detection of viable, apoptotic and necrotic spermatozoa in frozen-thawed semen samples. Phospholipids are present in the outer and inner layers of the lipid bilayer in cell and plasma membranes. Freezing and thawing can disrupt the functioning of the intra-membrane transporters like flipases and flopases resulting in their translocation, which ultimately leads to the destabilization of the cell membrane and cell death (Bailey et al., 2005). In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V, conjugated to a fluorochrome FITC, is a 35–36 kDa Ca\(^{2+}\) dependent phospholipid-binding protein with a high affinity for PS, and it binds to cells with exposed PS. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI; PI is a marker of cell late apoptosis and necrosis as it migrates from the internal to the outer layer of the cell membrane as a result of advanced cell membrane destabilization (Parks, 1997; Duru et al., 2000).

Frozen semen samples were thawed as previously described and placed in sterile plastic tubes, washed twice with cold PBS and then re-suspended in 1× Binding Buffer to a final concentration of 1×10\(^6\) spermatozoa/ml. 100 μl of such solution (1×10\(^5\) cells) were transferred to a 5-ml plastic culture tube. After the addition of 5 μl of Annexin V FITC and 5 μl of PI, a sample was gently mixed and incubated for 15 min at room temperature in dark. Subsequently, 400 μl of 1× Binding Buffer was added to the test tube, sample was mixed gently by pipetting, and analyzed immediately in the flow cytometer BD Accuri™ C6 Plus (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were tested automatically using a sample loader with the acquisition criteria of 50,000 events (a number of particles detected
by the instrument) for each tube. Data acquired using a cytometric system were computed and a report was automatically generated by the BD Accuri™ C6 Plus software.

**Statistical analyses**

Statistical analyses were done with the SigmaPlot® statistical software (ver. 11.0 for Windows®; Systat Software Inc., Richmond, CA, USA). Each ejaculate (n=18) was divided 6 ways for each of the extenders studied. All single time-point observations were analyzed by one-way analysis of variance (ANOVA) to determine the effects of semen extenders and serial data were subjected to two-way repeated measures analysis of variance (RM-ANOVA) with the main effect of time, semen extender and the interactions of both terms. All results are expressed as mean±standard deviation (SD) unless otherwise stated and P values <0.05 were considered statistically significant.

**Results**

**Post-thaw sperm evaluation**

For greater clarity, only significant differences among treatment groups and their respective controls were included in this section of the paper. The mean duration of the period during which sperm motility *ex situ* could be detected was greater (P<0.05) in extenders dissolved in NW30’ compared with DW controls (Table 1). Sperm progressive motility in thawed semen samples was greater (P<0.05) for semen cryopreserved in NW30’-3% compared with its respective control (DW-3%) from 0 to 150 min after thawing (Fig. 1); it was greater (P<0.05) in NW15’-7% than in DW-7% group from 0 to 120 min; and it was greater (P<0.05) in NW30’-7% than in DW-7% from 165 to 195 min of incubation. Progressive motility was greater (P<0.05) in DW-7% compared with DW-3% Kareta extender up until 150 min after thawing. Within individual groups, significant declines in sperm progressive motility occurred at the following intervals: DW-3%: Times 0-105-150 min; DW-7%: Times 0-105-165-195 min; NW15’-3%: Times 0-90-135-195 min; NW15’-7%: Times 0-120-180 min; NW30’-3%: Times 0-105-150-195 min; and NW30-7%: Times 0-105-150-180-210 min. The proportion of spermatozoa with mid-piece defects was lower (P<0.05) in NW15’-3% compared with DW-3% (Table 2). The proportion of live spermatozoa was greater in NW30’-7% compared with DW-7% both immediately and 1 h after thawing, whereas the proportion of necrotic spermatozoa 1 h after thawing was greater (P<0.05) in DW-7% compared with NW30’-7% (Table 3). Alkaline phosphatase concentrations in semen samples frozen in extenders prepared with NW30’ were lower (P<0.05) compared with respective controls (Table 4).

**Discussion**
The freezability of ram semen is significantly lower compared with that in other mammalian species (Shipley et al., 2007; Murawski et al., 2015). Consequently, ejaculates from 5 to 10% of rams do not freeze well with the commonly used protocols and semen extenders (Salamon and Maxwell, 2000). Therefore, studies using ram semen provide a useful model for studying semen cryopreservation techniques in an array of animal species of veterinary interest and humans.

There is a paucity of information on the effects of declustering time on the physicochemical properties on NW. Results of the present experiment indicate that declustering times may impinge on the cryoprotective properties of NW used as Kareta extender diluent. The main beneficial effect of NW15’ was a reduction in midpiece defect rates of spermatozoa frozen in the Kareta extender containing 3% of glycerol, whereas an application of NW30’ mainly affected the proportions of live and necrotic spermatozoa after freezing in the 7%-glycerol Kareta extender and the motility, survival time and ALP release for both types of Kareta extenders (containing 3% or 7% of glycerol). The use of NW30’ with the 7%-glycerol extender ameliorated the cryoprotective properties of the Kareta extenders to a greater extent than NW15’.

Post-thaw structural changes in the sperm “motility apparatus”, the midpiece and flagella, are not always correlated with a decrease in sperm motility (Rocha et al., 2006; Chian and Quinn, 2010; Murawski et al., 2015). Our present results confirm this notion; a reduction in the proportion of midpiece defects in the NW15’-3% group was not accompanied by a significant difference in sperm progressive motility. Further, a significant improvement in sperm motility and survivability in NW30’ extenders was not due to reduction in sperm defect rates. One of the main causes of the adverse effects of cryopreservation on sperm motility is the phenomenon known as the cold shock (Pena et al., 2003). Changes taking place during the cold shock that lead to the weakening of sperm self-propelling ability include alterations in cell membrane structure and ionic transport (Grøndahl et al., 1994). Improved motility and survival time of Olkuska ram spermatozoa after freezing in the Kareta extender diluted in NW30’ may therefore be mediated, at least partly, by enhanced transmembrane transport and utilization of various extender components.

A rapid release of sperm cytoplasmic enzymes into semen extender usually takes place during the initial stages of the cold shock (Murawski et al., 2015), but it may also be caused by thawing (Almlid and Johnson, 1988). Alanine transferase and AST are intracellular enzymes permanently bound to sperm midpiece membranes, particularly to the
mitochondria, and so their abundance in semen extenders reflects the damage occurring in sperm mitochondria (Corteel, 1980; Ciereszko and Dąbrowski, 1994; Bronicka and Dembiński, 1999; Holt, 2000; Strzeżek and Kordan, 2003). Nikolopoulou et al. (1986) reported that ALP was a significant marker of acrosome membrane integrity. In the present experiment, ALP concentrations in control semen samples after thawing were consistently higher than those in extenders prepared with NW30’. A lack of differences in ALT and AST concentrations and a significant difference in ALP content between semen samples frozen in DW- or NW-containing extenders indicate that disruption of the acrosomal membrane was the main structural damage ameliorated by NW30’.

Sperm death may occur during all stages of semen cryopreservation process (Corteel, 1980; Samper and Morris, 1998; Neild et al., 2003). The three main reasons for sperm necrosis are the osmotic stress, intra- and extracellular ice crystal formation, and irreversible changes in cell membrane fluidity (Behera et al., 2015). Addition of cryoprotectants neutralizes adverse effects of exposure to low temperatures on lipid and protein conformation of cell membranes (Curry et al., 1994). However, based on the studies using equine semen incubated for up to 60 min in 37°C, glycerol (3.5 to 5%) may also induce damage to sperm membranes and mitochondria after thawing (Garcia et al., 2012). In the present study, there was no significant difference between Kareta extenders diluted in DW or NW in the proportion of spermatozoa positive for double fluorescence staining (ANN+/PI+) immediately after thawing. However, the cytometric analysis performed 1 h later revealed a significantly lower proportion of ANN+/PI+ spermatozoa in the extenders prepared with NW30’. This is intriguing and suggests that NW30’ may nullify cytotoxic effects of glycerol occurring after thawing of ram semen samples.

The assessment of post-thaw motility of Olkuska ram semen revealed that different combinations of glycerol concentrations and NW declustering times exerted effects on sperm kinematics and viability for different time periods after thawing. This creates a possibility for boosting the efficacy of artificial insemination (AI) in sheep. Specifically, NW30’-7% could be used for semen cryopreservation before intravaginal or transcervical AI (associated with longer sperm transport) whereas NW15’-7% and NW30’-3% could be employed for the cryoconservation of semen used for laparoscopic insemination (followed by shorter sperm transport). This supposition requires fertility trials.

Our results indicate that NW can enhance cryoprotective effects of glycerol-containing extenders on ram spermatozoa with the reduction in sperm necrosis/increase in survivability being greater with 7% than 3% glycerol. Different declustering times appear to alter
cytoprotective properties of NW, which warrants further studies of the utility of NW-based semen extenders for semen cryoconservation. The specific mechanisms whereby NW improves viability of ram spermatozoa remain to be elucidated.

Conflict of interests
The authors have no conflicts of interest to declare.

Acknowledgments
This work was presented, in a preliminary form, at the 46th Annual Conference of the International Embryo Transfer Society in New York (16–19 January 2020; Abstract #39).
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Table 1. Summary of Olkuska ram semen characteristics (determined in water bath at 37°C) following cryopreservation in modified Kareta extenders prepared with deionized water (DW, controls) or nanowater declustered for 15 min or 30 min (NW15’ or NW30’) and containing either 3% or 7% of glycerol. Values are expressed as mean±SD (n=18 ejaculates; 3 ejaculates/ram). Within a middle row, values denoted by the same number of asterisks are different (P<0.05)

| Variables/Extender          | DW-3% | DW-7% | NW15’-3 | NW15’-7 | NW30’-3 | NW30’-7 |
|-----------------------------|-------|-------|---------|---------|---------|---------|
| Maintenance of progressive motility (min) | 189.2±6     217.5±16.5 212.5±18 215.0±25 215.0±17 242.5±31.1 |
|                             |       | **    | .7      | .2      | 8*      | **      |
| Decline rate (%/min)        | 7.2±2.1 | 10.3±2.5 | 8.7±2.9 | 7.0±4.5 | 10.7±2.8 | 10.1±1.7 |
Table 2. Effect of freezing Olkuska ram semen in modified Kareta extenders on the proportion of spermatozoa with abnormal morphology. Values are expressed as mean±SD (n=18 ejaculates). DW-3%, DW-7%: control groups (extenders prepared in deionized water); NW15'/30'-3%/7%: extenders prepared in nanowater declustered for 15 min or 30 min; within columns, means denoted by different letter superscripts vary significantly: a, b – P<0.05

| Extender Type/region of defect | Type/region of defect | Extender Type/region of defect | Type/region of defect | Extender Type/region of defect | Type/region of defect | Extender Type/region of defect | Type/region of defect |
|-------------------------------|-----------------------|-------------------------------|-----------------------|-------------------------------|-----------------------|-------------------------------|-----------------------|
| Head                          | Mid-piece            | Tail                          | Detached head         | Proximal droplet              | Double tail           | DW-3%                        | 45.0±22.3             | 4.0±2.5 a               | 8.8±9.6               | 14.8±22.2              | -                      | -                      |
| Head                          | Mid-piece            | Tail                          | Detached head         | Proximal droplet              | Double tail           | DW-7%                        | 42.7±21.6             | 3.3±1.4               | 7.5±6.3               | 13.2±17.8              | 0.3±0.8               | -                      |
| Head                          | Mid-piece            | Tail                          | Detached head         | Proximal droplet              | Double tail           | NW15'-3%                     | 51.7±24.2             | 1.3±1.4 b              | 6.7±3.9               | 10.8±11.8              | 0.3±0.8               | -                      |
| Head                          | Mid-piece            | Tail                          | Detached head         | Proximal droplet              | Double tail           | NW15'-7%                     | 47.7±20.9             | 3.3±1.7               | 5.5±4.6               | 14.5±17.8              | -                      | -                      |
| Head                          | Mid-piece            | Tail                          | Detached head         | Proximal droplet              | Double tail           | NW30'-3%                     | 41.3±20.6             | 3.0±4.3               | 5.3±3.6               | 13.7±15.2              | 0.2±0.4               | -                      |
| Head                          | Mid-piece            | Tail                          | Detached head         | Proximal droplet              | Double tail           | NW30'-7%                     | 37.7±21.6             | 2.0±1.7               | 5.8±4.1               | 13.3±12.0              | 0.5±0.8               | 0.2±0.4               |
Table 3. The influence of freezing in modified Kareta extenders on cell survivability and the occurrence of apoptosis or necrosis (%) of Olkuska ram spermatozoa immediately after thawing and after 1-h incubation. Values are expressed as mean±SD (n=18 ejaculates).

DW-3%, DW-7%: control groups (extenders prepared in deionized water);
NW15'/30'-3%/7%: experimental groups including extenders prepared in nanowater declustered for 15 min or 30 min; within columns, pairs of means denoted by different letter superscripts vary significantly: a, b – P<0.05

| Cell type | Extender | Live cells | Necrotic cells | Apoptotic cells |
|-----------|----------|------------|----------------|----------------|
|           |          | Immediately after thawing |             |                |
|           | DW-3%    | 54.0±3.8   | 34.6±6.0       | 11.4±3.2       |
|           | DW-7%    | 50.4±5.4 a | 38.0±5.0       | 11.6±3.6       |
|           | NW15'-3% | 53.4±2.4   | 34.8±5.2       | 11.8±3.6       |
|           | NW15'-7% | 52.0±3.4   | 35.2±4.4       | 12.8±4.6       |
|           | NW30'-3% | 53.4±3.0   | 35.0±3.8       | 11.6±4.0       |
|           | NW30'-7% | 54.0±2.4 b | 34.4±5.0       | 11.6±4.4       |
|           |          | 1 h after thawing |             |                |
|           | DW-3%    | 50.0±4.0   | 39.7±6.0       | 10.3±2.8       |
|           | DW-7%    | 49.2±1.8 a | 31.4±3.8 a     | 9.4±2.2        |
|           | NW15'-3% | 47.8±4.2   | 40.0±5.4       | 12.2±4.6       |
|           | NW15'-7% | 49.4±4.4   | 39.6±4.8       | 11.0±2.6       |
|           | NW30'-3% | 51.0±3.0   | 40.0±3.8       | 9.0±1.4        |
|           | NW30'-7% | 54.0±5.4 b | 35.5±4.3 b     | 10.5±1.8       |
Table 4. Concentrations of ALT, AST and ALP in frozen-thawed Olkuska ram semen samples. Values are expressed as mean±SD (n=18 ejaculates). DW-3%, DW-7%: control groups (extenders prepared in deionized water); NW15'/30'-3%/7%: experimental groups including extenders prepared in nanowater declustered for 15 min or 30 min; within columns, pairs of means denoted by different letter superscripts vary significantly: a, b – P<0.05

| Extender  | ALT     | AST     | ALP       |
|-----------|---------|---------|-----------|
| DW-3%     | 17.7±5.0| 313.9±60.9| 4489.7±1930.7 a |
| DW-7%     | 16.8±5.0| 308.6±43.2| 3956.0±1116.4 a |
| NW15'-3%  | 19.3±3.7| 297.9±69.1| 3539.3±1469.6 |
| NW15'-7%  | 18.8±1.9| 290.6±70.6| 3327.9±1071.1 |
| NW30'-3%  | 15.6±5.2| 283.1±47.9| 3105.2±981.6 b |
| NW30'-7%  | 14.9±5.6| 261.7±68.6| 2683.0±707.8 b |
Figure 1. Changes in progressive motility of Olkuska rams’ spermatozoa *ex situ*. Standard deviation bars were omitted for greater clarity. Within each group (extender used), open white circles indicate consecutive significant (P<0.05) decreases in mean values (starting at Time 0 = thawing). DW-3%, DW-7%; control groups (Kareta extenders prepared with DW); NW15'/30'-3%/7%; experimental groups (Kareta extenders prepared with NW declustered for 15 min or 30 min). A table below the graph summarizes statistically significant differences between treatment groups and their respective controls (bold font) or between DW-3% and DW-7% (italics)

| Incubation time (min) | Pairs of significantly different mean values (P<0.05) |
|-----------------------|--------------------------------------------------------|
| 0                     | NW30'-3% vs. DW-3%; DW-3% vs. DW-7%; DW-7% vs.         |
|                       | NW15'-7%                                                |
| 30                    | NW30'-3% vs. DW-3%; DW-3% vs. DW-7%; DW-7% vs.          |
|                       | NW15'-7%                                                |
| 60                    | DW-3% vs. DW-7%; NW30'-3% vs. DW-C3%; DW-7% vs.         |
|                       | NW15'-7%                                                |
90  \( DW-3\% \text{ vs. } DW-7\% ; \text{ DW-7\% vs. NW15'-7}\% ; \)

\( DW-37\% \text{ vs. } DW-7\% ; \text{ NW30'-3\% vs. DW-3\% ; DW-7\% vs.} \)

105 \( NW15'-7\% \)

\( DW-3\% \text{ vs. } DW-7\% ; \text{ NW30'-3\% vs. DW-3\% ; DW-7\% vs.} \)

120 \( NW15'-7\% \)

135 \( NW30'-3\% \text{ vs. DW-3\% ; DW-3\% vs. DW-7\%} \)

150 \( DW-3\% \text{ vs. DW-7\% ; NW30'-3\% vs. DW-3\%} \)

165–195 \( NW30'-7\% \text{ vs. DW-7\%} \)