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*Original Research*

# The utility of nanowater for ram semen cryopreservation

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## Abstract

Nanowater (NW; water declusterized in the low-temperature plasma reactor) has specific physicochemical properties that could increase semen viability after freezing and hence fertility after artificial insemination (AI) procedures. The main goal of this study was to evaluate ram semen quality after freezing in the media containing NW. Ejaculates from 10 rams were divided into two equal parts, diluted in a commercially available semen extender (Triladyl®; MiniTüb GmbH, Tiefenbach, Germany) prepared with deionized water (DW) or NW, and then frozen in liquid nitrogen. Semen samples were examined for sperm motility and morphology using the sperm class analyzer system and light microscopy. Cryo-scanning electron microscopy (cryo-SEM) was employed to determine the size of extracellular water crystals in frozen semen samples. Survival time at room temperature, aspartate aminotransferase (AspAT) and alkaline phosphatase (ALP) concentrations post-thawing as well as conception/lambing rates after laparoscopic intrauterine AI of 120 ewes were also determined. There were no significant differences between DW and NW groups in sperm progressive motility ($26.4 \pm 12.2$ and $30.8 \pm 12.4\%$) or survival time ($266.6 \pm 61.3$ and $270.9 \pm 76.7$ min) after thawing and no differences in the percentages of spermatozoa with various morphological defects before or after freezing. There were, however, differences ($P < 0.05$) in AspAT (DW: $187.1 \pm 160.4$ vs. NW: $152.7 \pm 118.3$ U/l) and ALP concentrations (DW: $2198.3 \pm 1810.5$ vs. NW: $1612.1 \pm 1144.8$ U/l) in semen samples post-thawing. Extracellular water crystals were larger ($P < 0.05$) in ejaculates frozen in NW-containing media. Ultrasonographic examinations on day 40 post-AI revealed higher ($P < 0.05$) conception rates in ewes inseminated with NW (78.3%) compared with DW semen (58.3%), and the percentages of ewes that carried lambs to term were 73.3% and 45.0% in NW and DW groups, respectively ($P < 0.01$). In summary, the use of a semen extender prepared with NW was associated with a substantial improvement in the fertilizing ability of frozen-thawed ram semen and lamb productivity of inseminated ewes.

**Keywords:** Cryopreservation, freezing, nanowater, semen, sheep, ram

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## Introduction

Cryopreservation is a common and cost-effective method of mammalian gamete storage.[1,2] In a vast majority of human andrological cases, the primary indications for semen freezing and banking revolve around various medical conditions and environmental hazards (Figure 1). Alternatively, the propagation of and/or salvaging valuable genetics are the main grounds for semen cryopreservation in animal husbandry (for subsequent use in female-only commercial operations) and conservation programs (Figure 1). To mention but a few specific considerations for semen freezing: (i) oncologists estimate that nearly 30% of their male patients choose to freeze their sperm;[3] (ii) in dairy cattle, approximately a fifth of the breedable female population in the world is now bred by artificial insemination (AI) using frozen semen;[4] (iii) although semen banking is only effective in exotic species whose reproductive biology has been well-characterized, already at the end of the last decade, European cryo-spermbanks stored semen samples from more than 40 endangered mammalian species including elephants, giant pandas, wild felids, and primates.[5]

In spite of the differences between humans and animal species in the application of semen cryopreservation, maintaining high sperm viability after deep freezing invariably remains a major goal in assisted reproductive technologies (ARTs). Semen freezing procedures cause adverse changes in sperm structure and function. The percentage of motile sperm amount and velocity are significantly reduced after thawing,[6] and overall sperm viability can be decreased by even 50% of initial (pre-freezing) values.[7] Irreversible damage to the sperm occurs during both the freezing and thawing processes.[8] Cooling appears to be the major stressor inflicted on spermatozoon cytoplasmic membranes and causes phospholipid re-orientation into a different configuration;[7] this is the main reason for the addition of cryoprotectants to semen extenders. However, cytotoxic properties of some cryoprotectants that become manifest immediately after thawing may cause further damage to the sperm.

Because of the low success rate of AI using frozen-thawed semen, it had been recommended in the past that only semen samples with high sperm counts and progressive motility be stored and used. Although the current efficiency of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) make cryopreservation of all samples containing any amount of live sperm appropriate, the effectiveness of AI still relies on satisfactory sperm parameters in whole inseminate doses after freezing. There has been intensive research aimed to ameliorate cryopreservation techniques currently used by manipulating the cooling/freezing rates[9] and chemical composition of semen extenders.[10] Andrabi[11] concluded that even under the most optimal conditions, it was inevitable that some damage would occur to semen during the freezing procedures. Clearly, the need to improve cryopreservation protocols still exists. Ram semen freezability is lower compared with that of other mammalian species, and so a considerably higher number of sperm are required for a dose of inseminate.[2] As ram semen has very low cryotolerance/cryosurvival rates, it lends itself as an ideal experimental model for studying various aspects of semen freezing.

Water subjected to cold plasma treatment (a.k.a. declusterization) is termed nanowater (NW).[12–15] During this procedure, water molecules that under normal conditions form aggregates (clusters) of up to 1000 are broken down into single molecules measuring 1 nM each.[15,16] NW is characterized by low viscosity, high diffusivity, and very low

| Main reasons for semen cryopreservation | Relative significance | |
|---|---|---|
| | Human beings | Animal species of agricultural and veterinary interest |
| Life-threatening/chronic diseases, environmental hazards, medical treatments or surgical procedueres that may/will cause a permanent sterilization or genetic demage | ● | • |
| A serious risk of testicular injury | ● | • |
| Semen preservation and storage for long-distance transport and/or deferred insemination (incl. post-humous conception) | • | ● |
| To secure good quality semen/spermatozoa for assisted reproductive technologies (incl. cryopresevation of epididymal and testicular samples) | ● | ● |
| Oligospermia that requires multiple semen collections over time and pooling for use in a single insemination | ● | • |
| Semen donation by a directed/designated donor(s) preceded by a qurantine during which the donor is screened for infectious diseases or genetic aberrations | • | ● |
| Sperm banks for conservation programs, sales and/or trade | · | ● |
| R&D of cryopreservation techniques, semen extenders, etc. | • | ● |

**Figure 1** General summary of major reasons for semen cryopreservation in medical and agricultural/veterinary practices. A size of the circles in the last two columns denotes a relative significance of various factors, with the largest circle signifying: ''highly important and frequently used'', medium-sized: ''confined to specific situations with less widespread application(s)''; and a small one: ''marginal/infrequent use.'' This synopsis was based largely on references Walters *et al.*[1] and FAO[2]

density.[12,15] It also has a low dielectric constant allowing it to dissolve even non-polar compounds (i.e., lipids) into solution; in fact, NW dissolves 35–40% more substances than the same volume of normal water.[12,15] It also raises the solubility of gases and salts by ~50%, making it possible to obtain highly concentrated solutions.[12,15] NW is therefore more efficient a carrier of nutrients and inorganic constituents than ordinary water.[12,15] In addition, it possesses antimicrobial properties.[12,17,18] Consequently, NW is suitable as a substitute for organic solvents in a broad range of industrial and laboratory applications. Lastly, the water treated in low-temperature plasma reactors freezes at −67°C and has a zero coefficient of thermal expansion at freezing;[12,15] these properties of NW may potentially reduce the incidence of structural damage occurring during cryopreservation of gametes.

In light of these considerations, the main objective of the present study was to evaluate ram semen quality after freezing in the medium diluted in NW. We hypothesized that specific physicochemical properties of NW would significantly increase the viability of frozen-thawed ram semen leading to improved conception and lambing rates in artificially inseminated ewes.

## Materials and methods

### Animals

All experimental procedures performed on live animals were approved by the local animal care committees. The present study utilized three PON (Polska Owca Nizinna or Polish Lowland Sheep) and four BCP (synthetic breed: Berrichon × Charolaise × PON) rams that had been trained to ejaculate into an artificial vagina and 120 multiparous BCP ewes housed in the field research station of the Department of Sheep and Goat Breeding (University of Life Sciences in Lublin) situated in Bezek, Poland (latitude: 51°12′48"N; longitude: 23°16′46"E). Animals were aged 4 to 12 years, clinically healthy and in a good overall condition (body condition score of 2–3 on a scale from 1 to 5).[19] The lifetime parity of ewes averaged six lambs. During the summer, all animals had unlimited access to pasture, and in the winter, they remained indoors and received daily maintenance ratios of hay (0.3 kg/animal/day) and hay-silage (4 kg/animal/day), and water *ad libitum*. Additionally, after shearing (ewes and rams), eight weeks before the expected lambing and during pregnancy and lactation (ewes), the animals received 15–30 dag of concentrate/day (75% oats, 20% barley, and 5% rapeseed meal).

### Semen collection, evaluation and processing

Semen was collected into a pre-warmed (~30°C) artificial vagina. Two types of extenders were prepared for semen freezing by mixing commercially available Triladyl® (MiniTüb GmbH; Tiefenbach, Germany) with deionized water (DW; Aqua Purificata®; Prolab, Gliwice, Poland) or NW (obtained through the treatment of DW in a cold plasma generator; Nantes Nanotechnology Systems, Bolesławiec, Poland) and *Gallus domesticus* egg yolk in 1:3:1 (vol/vol/vol) proportion, respectively. Ejaculates from all rams were divided into two equal parts and, after being diluted in the extender prepared in either DW or NW to a final concentration of $400 \times 10^6$ spermatozoa/mL, placed in 0.25-cc plastic straws (Rovers, Piaseczno, Poland) and then frozen using the following protocol: an initial 30-min cooling to 20°C followed by equilibration for 120 min to 4°C, both performed in a walk-in freezer; further equilibration in liquid nitrogen fumes (−120°C) for 10 min; and plunging in liquid nitrogen (−196°C) before placing the straws into plastic goblets arranged in a liquid nitrogen container.

Before freezing, semen samples were evaluated for sperm concentration, progressive motility and morphology. The percentage of motile spermatozoa was determined using a Nikon Eclipse 80i microscope (Nikon Corp., Tokyo, Japan) at 200 × image magnification on a warm glass slide (~37°C). The amounts of normal and aberrant spermatozoa were estimated according to the classification developed by Blom[20] at 1000 × magnification using an eosin-nigrosine staining and a bright view field (Table 1). Briefly, histological smears were analyzed for sperm morphological defects including abnormal or detached heads, abnormal mid-pieces and tails, and distal and proximal cytoplasmic droplets. For each semen sample, 200 spermatozoa were evaluated under oil immersion.

Approximately six months later, the samples were thawed and examined for sperm motility and morphology using the Sperm Class Analyzer (SCA) ver. 5.0 (Microoptic® Automatic Diagnostic Systems, Barcelona, Spain) and a Nikon Eclipse 80i microscope. Survival time of spermatozoa at room temperature as well as aspartate aminotransferase (AspAT) and alkaline phosphatase (ALP) concentrations in semen plasma were also determined. For microscopic examination, slides with semen smears were stained using the Sperm Blue kit (Microoptic SL Co., Barcelona, Spain). All samples were processed for staining within 1 h after thawing. The specimens were photographed and images captured digitally using the SCA® CASA System.

**Table 1** Percentages (±SD) of spermatozoa with different morphological defects detected microscopically before freezing (regular font) and after thawing (italics) of ram semen samples ($n = 10$) diluted in the extender prepared with deionized water (DW) or nanowater (NW)

| Group/semen abnormalities | Abnormal heads | Abnormal mid-pieces | Abnormal tails | Loose heads | Proximal droplets | Distal droplets | Total defects |
|---|---|---|---|---|---|---|---|
| DW | 0.20 ± 0.48 | 2.30 ± 1.55 | 13.70 ± 13.29 | 2.17 ± 4.39 | 0.10 ± 0.32 | 0.05 ± 0.16 | 20.90 ± 11.31 |
| | – | *3.15 ± 2.65* | *16.20 ± 6.82* | *6.80 ± 5.31* | – | – | *23.65 ± 14.64* |
| NW | – | 1.05 ± 0.90 | 13.65 ± 5.40 | 0.80 ± 1.06 | – | – | 15.90 ± 7.14 |
| | – | *1.75 ± 0.34* | *13.75 ± 14.60* | *3.35 ± 3.37* | – | – | *18.95 ± 14.32* |

AspAT and ALP concentrations in thawed ejaculates were determined by a method based on the recommendations of the International Federation of Clinical Chemistry. The laboratory analyzes utilized a biochemical analyzer Vitros (Ortho Clinical Diagnostics, High Wycombe, UK) and were performed at 37°C. The analytical method used was based on the ''dry slide technology'' employing the thin film diagnostic laminae (ALP: Abbott Laboratories Poland Ltd., Warsaw, Poland; and AspAT: DiaLab Laboratoria Medyczne, Wroclaw, Poland). Enzyme quantities were determined by the kinetic UV method using direct potentiometry, which utilizes ion-selective electrode modules for electrolyte analysis without sample dilution.[21] A specific chemical reaction in ALP concentration measurement is given below.

*2-amino-2-methylo-1-propnol + p-pnitrophenyl phosphate + $H_2O$*

$$\downarrow ALP$$

*4-nitrophenol + 2-amino-2-methylo-1-propanol phosphate*

AspAT assays were based on keto acids transamination from 2,4-double nitrophenylohydrazine; AspAT catalyzes the reversible reaction of L-aspartate as detailed below:

*L-aspartate + 2-oxygtlutate→L-glutamate + oxaloacetate*

### Scanning electron microscopy and image analyses

Scanning electron microscopy (SEM) examinations in the present experiment were performed to indirectly assess the size of extracellular ice crystals forming in the frozen semen samples. Cryo-scanning electron microscopy (cryo-SEM) is a technique that permits the assessment of the fully hydrated ultrastructure of sperm samples in the frozen state without the introduction of artefacts caused by thawing, chemical fixation or other types of sample processing. Therefore, this method also allows for visualization of the topography of the sample, including the distribution and size of the water crystals forming during the initial freezing process.

Plastic straws with semen were broken manually inside the liquid nitrogen tank, following which the plastic sheath was removed and a cylindrical frozen semen sample mounted on a mechanical grip holder of the LEO SEM 1430 VP microscope (Zeiss Inc., Jena, Germany) equipped with the energy dispersive X-ray (EDX) detector system. The settings of an electron microprobe were as follows: 20-kV, 80-μA beam current, and vacuum ($10^{-5}$ Pa × μF) maintained throughout the entire observation period. The approximate depth of the electron beam penetration in the samples studied was ~1 μm. Selected images ($n = 24$) at 2500 × to 12000 × magnification were photographed (Figure 2) and saved as digital graphic files. Finally, the average size (area) of the micro-compartments corresponding to the water crystals within the frozen semen sample was computed using the ImageProPlus® analytical software (Media Cybernetics, San Diego, CA, USA).

### Laparoscopic insemination

Fertility after laparoscopic AI using semen samples collected from BCP rams was determined in a commercial flock of 120 BCP ewes. Estrous cycle was synchronized in August with intravaginal sponges containing 30 mg of fluorogestone acetate (Chronogest®; MSD Animal Health, Boxmeer, Holland) that were left in place for 14 days. All ewes were injected with 500 IU of equine chorionic gonadotropin i.m. (Folligon®; Intervet Int., Warsaw, Poland) at the time of sponge removal. Two days later, the ewes underwent laparoscopic AI. Animals were food-deprived and had restricted access to water for 16 h before the procedure, which was performed under general anesthesia induced 15 min before insemination (Sedazin: 0.4 mL i.m., containing 20 mg of xylazine/mL; Biowet, Puławy, Poland). Ewes were then placed in a dorsal recumbency in a custom-made laparoscopy rack. The abdominal region was prepared by shearing the wool and disinfecting the skin with 70% ethanol. The ewes' hindquarters were lifted up to an approximate 45° angle. An experienced operator used the trocar to make two punctures in the abdominal wall for insertion of a laparoscope and a fiberoptic light source (Karl Storz-Endoskope GmbH & Co. KG, Tuttlingen, Germany). This was preceded by a locally applied analgesia (1.5 mL of 5% polocaine + 0.05% adrenaline s.c.; Biowet, Drwalew, Poland). The abdominal cavity was distended with carbon dioxide to help visualize the uterus and separate it from the abdominal wall. Subsequently, an inseminator placed a pipette containing a semen dose through a laparoscope puncture site and deposited semen into the lumen of both uterine horns.

### Ultrasonographic pregnancy detection and lambing

The pregnancy rate was confirmed 40 days after AI by transabdominal ultrasonography using an Aloka ProSound 2 scanner (Hitachi Aloka Medical Co., Tokyo, Japan) connected to a hand-held 5-MHz linear-array probe. Sheep were examined in a standing position, restrained by a member of the station personnel. The number, sex, and birth weight of lambs were recorded at natural (non-synchronized) lambing that took place in the month of January.

### Statistical analyses

All single time point observations were compared between the two groups of animals by Student *t*-test using the SigmaPlot® for Windows® statistical software (ver. 11.0; Systat Software Inc., Richmond, CA, USA). Chi-squared test (Brendt and Snedecor formula) was used for the analysis of proportions. All results are given as mean ± SD. $P$ value $< 0.05$ was considered statistically significant.

## Results

### Semen evaluation and cryo-SEM

The mean volume of ejaculate measured directly in a calibrated collection flask was 2.3 ± 1.1 mL with $4.7 \pm 1.4 \times 10^9$/mL spermatozoa. The progressive motility of freshly obtained semen was 87.1 ± 4.7%. There were no significant differences between the two types of extenders used in the proportion of spermatozoa with various morphological defects detected before freezing (Table 1).

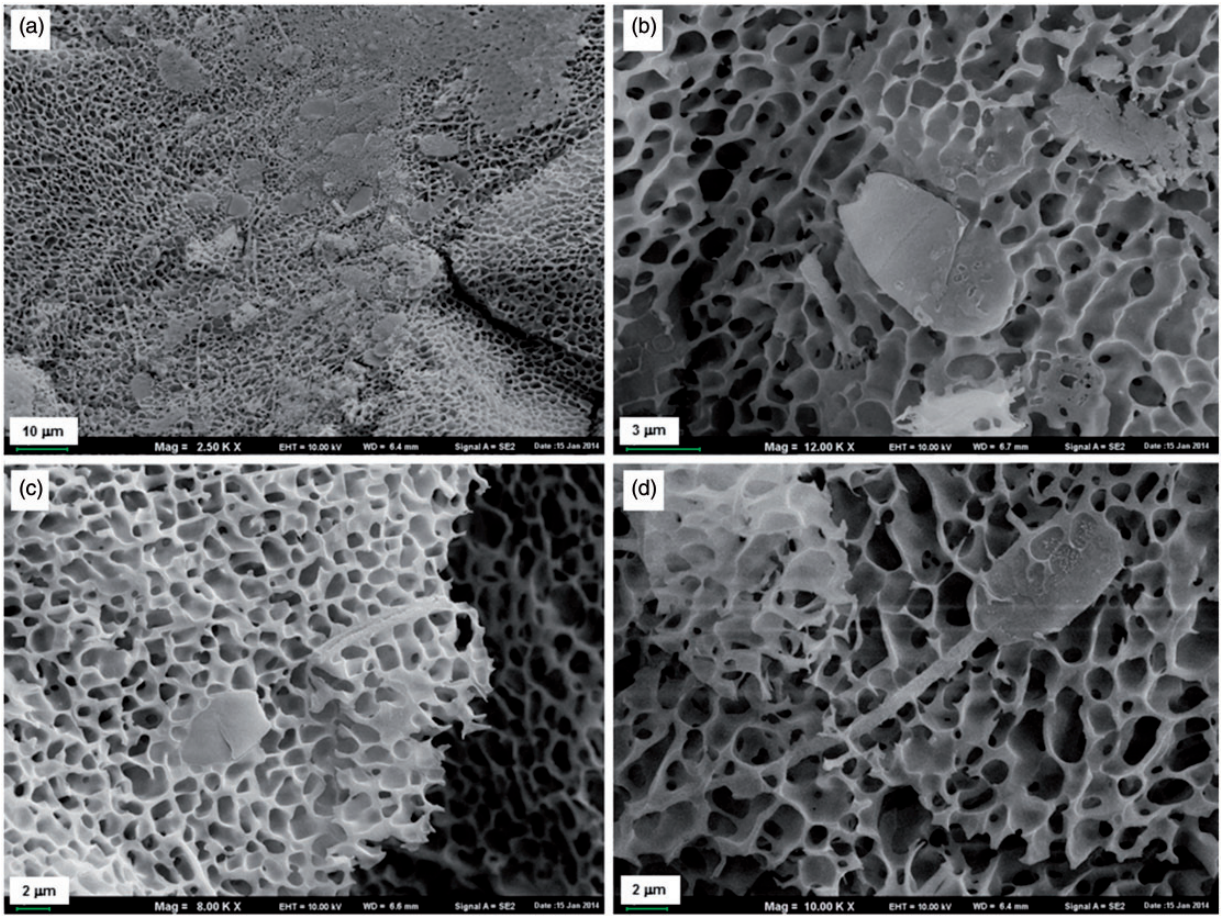


**Figure 2** Cryo-scanning electron microscopy (cryo-SEM) images of frozen ram semen samples at different magnifications (A: 2500×, B: 12000×, C: 8000× and D: 10000×) and diluted in the extender prepared with deionized water (A and C) or nanowater (B and D). A honeycomb-like structure represents extracellular water crystals forming during the freezing procedure. (a) Multiple spermatozoa visualized in a frozen specimen; (b) loose heads; (c) a loose head and a detached tail; and (d) a normal, intact spermatozoon

There were no differences ($P > 0.05$) between DW and NW groups in terms of sperm progressive motility ($26.4 \pm 12.2$ vs. $30.8 \pm 12.4\%$, respectively) and survival time ($266.6 \pm 61.3$ vs. $270.9 \pm 76.7$ min, respectively) after thawing. No signs of swelling were observed, and the acrosome stained dark blue, whereas the postacrosomal region stained pale blue. Only numerical differences in the percentage of spermatozoa with various structural abnormalities were observed ($P > 0.05$; Table 1). There were, however, significant differences between the two types of extenders tested in AspAT (DW: $187.1 \pm 160.4$ vs. NW: $152.7 \pm 118.3$ U/l) and ALP concentrations (DW: $2198.3 \pm 1810.5$ vs. NW: $1612.1 \pm 1144.8$ U/l) post-thawing. The mean cross-sectional area of micro-compartments determined in cryo-SEM electronograms was less in DW than in NW group ($0.33 \pm 0.29$ vs. $0.94 \pm 0.19\,\mu m^2$, respectively).

### Conception and lambing rates, lamb characteristics

Ultrasonographic examinations conducted on day 40 post-AI revealed higher ($P < 0.05$) conception rates in ewes inseminated with NW ($n = 60$) compared to DW group ($n = 60$) semen (78.3 and 58.3%, respectively). The percentages of ewes that carried lambs to term were 73.3 and 45.0% in NW and DW groups, respectively ($P < 0.01$). There were no significant differences between the two groups in the mean number of lambs per ewe ($1.9 \pm 0.7$ vs. $1.9 \pm 0.9$), sex ratio ($58.6 \pm 39.9$ vs. $60.6 \pm 37.7\%$; expressed as a proportion of ram lambs), and weight of lambs ($4.0 \pm 0.9$ vs. $4.0 \pm 1.0$ kg; DW vs. NW groups, respectively) recorded at lambing.

## Discussion

Ram semen is considerably more difficult to freeze than semen of other mammalian species and ejaculates from 5–10% of rams simply do not freeze successfully with currently available techniques and extenders.[22] Although a relatively high proportion of ram spermatozoa retain their motility after freezing, only about 20–30% remain biologically functional.[8] Salamon and Maxwell[8] observed that the freezing-thawing processes reduced motility to a lesser degree than structural integrity of ram spermatozoa, suggesting that the plasma and acrosome membranes are more vulnerable than the parts of the spermatozoon involved in locomotion (i.e., mid-piece and tail). Our present results are in contrast with those observations; differences in the proportions of spermatozoa with various morphological

defects were only numerical, but there was a 60% reduction in sperm progressive motility post-thawing. This would suggest that undesirable changes in ram sperm motility after freezing are caused mainly by ultrastructural (i.e., undetectable using light microscopy) and biochemical alterations.

In the present study, the Triladyl® (Tris-based) semen extender diluted in NW provided beneficial effects on sperm fertilizing ability but not motility or survival time of the spermatozoa subjected to the freezing procedure. The cryoprotective properties of semen extenders are due chiefly to their chemical composition and interactions among the various components.[23] NW possesses specific physicochemical properties that may result in improved delivery of biologically active substances across biological membranes. Moreover, the present cryo-SEM results revealed that the amount of extracellular water crystals was decreased in frozen semen samples diluted in an NW-containing extender. When tissues or cell suspensions are cooled slowly, water migrates out of cells and forms ice crystals in the extracellular space; too much extracellular ice can cause mechanical damage to cell membranes due to crushing.[2] It is attractive to speculate that a combination of a greater diffusivity and lower crystallization rate of NW as compared with DW ameliorated ram semen viability in this study.

AspAT is an intracellular enzyme[24] whose abundance and activity primarily reflect the damage occurring in the mid-piece area of the spermatozoon;[25] this is because AspAT is permanently bound to the sperm mid-piece membranes and particularly to the mitochondrial membranes.[26] Alkalaine phosphatase (ALP) in turn is a dephosphorylating enzyme involved in glycolytic reactions.[27] Semen requires metabolic energy generated by mitochondria from mid-piece ATP to sustain motility.[28] Approximately 20% lower concentrations of ALP and AspAT in the plasma of semen diluted in NW may indicate significantly enhanced stability of the plasma and mitochondrial membranes.[29]

Most mammalian species show a reduction in fertility after insemination with frozen-thawed semen, which is thought to be associated mainly with the reduced sperm motility, thereby requiring an increase in the number of sperm per inseminate necessary to achieve reasonable conception rates.[30] Sperm progressive motility is directly correlated with fertility, whereas the proportion of abnormal spermatozoa is inversely related to fertility of animals inseminated with the frozen-thawed semen.[31] However, a spermatozoon may be motile, but damaged, in which case it is unlikely that such a cell will fuse with the oocyte and fertilize it; damage can be ultrastructural, biochemical, and/or functional.[8] Frozen ram semen can be expected to result in conception rates of 65% when using laparoscopic intrauterine AI,[22] but the outcomes are highly variable depending on the animals' health status, inseminator's skills, and several other intrinsic and extrinsic factors.[32] In the present fertility trial, the ewes inseminated with ram semen prepared with NW exceeded their counterparts inseminated with semen diluted in DW in conception rates by 20%. This difference appears to reflect the results of AspAT and ALP analyses rather than the percentages of abnormal spermatozoa and sperm motility post-thawing. Therefore, the fertility outcome after laparoscopic AI using frozen-thawed ram semen, within the concentrations used in this study, may be predominantly dependent on, and potentially predicted from, semen biochemical indices and functional viability rather than the percentage of abnormal spermatozoa detected by microscopy and their motility.

Another intriguing observation in the present study was the fact that the difference in the lambing rate between the two subsets of ewes studied approached to 30%, which was a consequence of increased pregnancy losses in DW group after day 40 of gestation (5 vs. 13.3% for the NW and DW groups, respectively; $P < 0.05$). In healthy flocks of sheep, fetal losses after pregnancy diagnosis is performed are usually <2%, but some losses may occur even in healthy ewes, with a higher level observed in more prolific genotypes.[33] Causes are most often infectious but may also be nutritional deficiencies or toxins.[33] Among the most commonly diagnosed causes of abortion are iodine deficiency, selenium deficiency and some plant toxins (e.g., locoweed).[33] All of these factors can be ruled out in the present situation. Stress and/or trauma due to restraint during pregnancy detection may have potentially caused fetal losses in the ewes of the present study,[29] but it is rather unlikely as the percentage of losses was significantly greater in the DW than NW group even though all animals were handled in the same manner. Therefore, genetic causes that in humans account for 5–15% of abortions[34] cannot be ruled out. Although in naturally breeding sheep, the dam's genetic ability to sustain pregnancy has 5.5 times the effect of the embryo's ability to survive (and the sire of the embryo only affects the embryo quality),[35] these influences may change after an application of ARTs. It is possible that the exposure of ram semen to NW improved overall viability of frozen-thawed ram semen resulting in fewer genetic abnormalities (e.g., aneuploidies) and greater rates of embryonic/fetal survival in inseminated ewes. As this is only a speculation, that particular aspect warrants further studies.

We concluded that the use of a semen extender prepared with NW was associated with a substantial improvement in the fertilizing ability of frozen-thawed ram semen and reduction in fetal losses following laparoscopic AI of ewes. NW appears to exert beneficial cyto- and cryoprotective effects and, albeit the specific mechanisms of these effects remain to be elucidated, it can be recommended for semen freezing. It is anticipated that the use of semen diluents prepared with NW may facilitate the reduction in the number of sperm per inseminate dose without any deleterious effects on fertility and embryonic/fetal development after AI in mammals.

**Author contributions:** MM, TSc and JG conceived the present study and were primarily responsible for whole animal experiments, data analyses and the preparation of the final version of this manuscript; JG and KP co-organized and conducted the fertility trial; JG and PMB were responsible for all aspects of data analyses and manuscript preparation; ZO and IJ provided nanowater for the present experiments;

TMG and DAZ secured funding and adequate animal access for the duration of the project; KP, AS and AK coordinated consecutive parts of the present study and data acquisition; TS carried out cryo-scanning electron microscopy (cryo-SEM) of ram semen samples.


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## REFERENCES


1. Walters EM, Benson JD, Woods EJ, Crister JK. The history of sperm cryopreservation. In: Pacey AA, Tomlinson MJ (eds). *Sperm banking: Theory and practice*. Cambridge, UK: ©Cambridge University Press, 2009
2. FAO. Cryoprservation of animal genetic reasources. FAO Animal production and Health Guidelines No. 12, Rome, 2012
3. Zapzalka DM, Redmon JB, Pryor JL. A survey of oncologists regarding sperm cryopreservation and assisted reproductive techniques for male cancer patients. *Cancer* 1999;**86**:1812–7
4. Thibier M, Wagner HG. World statistics for artificial insemination in cattle. *Livest Prod Sci* 2002;**74**:203–12
5. Fickel J, Wagener A, Ludwig A. Semen cryopreservation and the conservation of endangered species. *Eur J Wildl Res* 2007;**53**:81–9
6. Hallak J, Kolettis PN, Sekhon VS, Thomas AJ, Agarwal A. Cryopreservation of sperm from patients with leukemia. *Cancer* 1999;**85**:1973–8
7. Lemma A. Effect of cryopreservation on sperm quality and fertility. In: Milad Manafi (ed.) *Artificial insemination in farm animals*, 2011, ISBN 978-953-307-312-5, InTech, http://www.intechopen.com/books/artificial-insemination-in-farm-animals/effect-of-cryopreservation-on-sperm-quality-and-fertility (accessed 9 October 2014)
8. Salamon S, Maxwell WMC. Frozen storage of ram semen: II. Causes of low fertility after cervical insemination and methods of improvement. *Anim Reprod Sci* 1995;**38**:1–36
9. Woelders H. Fundamentals and recent development in cryopreservation of bull and boar semen. *Vet Quart* 1997;**19**:135–8
10. Christanelli MJ, Squires EL, Amann EL, Pickett BW. Fertility of stallion semen processed, frozen and thawed by a new procedure. *Theriogenology* 1984;**22**:39–45
11. Andrabi SMH. Fundamental principles of cryopreservation of *Bos taurus* and *Bos indicus* bull spermatozoa. Mini review. *Int J Agri Biol* 2007;**9**:367–9
12. Dereń E. Living water. *Polish Sci Voice* 2011;**34**:20–1
13. Niemirowicz K, Krętkowski R, Mystkowska J, Cechowska-Paszko M, Jelkin I, Oszczęda Z, Car H. NANOwoda-woda o zmienionych właściwościach i nowych możliwościach aplikacji. 55 Zjazd PTCHEM i SITPCHM-''Chemia dla środowiska i cywilizacji'', 16–20 September 2012 in Białystok, Poland [conference abstract, in Polish]
14. Niemirowicz K, Car H. Nanonośniki jako nowoczesne transportery w kontrolowanym dostarczaniu leków. *Chemik Nauka-Technika-Rynek* 2012;**66**:868–81. ([in Polish])
15. Mystkowska J, Dabrowski JR. Physical and chemical properties of deionized water and saline treated with low-pressure and low-temperature plasma. *Chemik* 2013;**67**:722–4
16. Rusanov VD, Fridman AA. Possible destruction of large molecules in water by cold-plasma flow. *Zh Fiz Khim+* 1978;**52**:92–6
17. Bai N, Sun P, Zhou H. Inactivation of Staphylococcus aureus in water by a cold He/O-2 pressure plasma microjet. *Plasma Process Polym* 2011;**5**:424–31
18. Julak J, Scholtz V, Kotucova S, Janouskova O. The persistent microbicidal effect in water exposed to the corona discharge. *Phys Medica* 2012;**28**:230–9
19. Russel AJF, Doney JM, Gunn RG. Subjective assessment of body fat in live sheep. *J Agric Sci* 1969;**72**:451–4
20. Blom E. The ultrastructure of some characteristic sperm defects and a proposal for a new classification of the bull spermiogram. *Nord Vet Med* 1973;**25**:383–91. ([in Danish, author's translation of the title])
21. Katz SA. Direct potentiometric determination of urease activity. *Anal Chem* 1964;**36**:2500–1
22. Shipley CFB, Buckrell BC, Mylne MJA, Pollard J, Hunton JR. Artificial insemination and embryo transfer in ewes. In: Youngquist RS, Threlfall WR (eds). *Current therapy in large animal theriogenology 2*. St. Louis: Saunders Elsevier, 2007
23. Garde JJ, Soler AJ, Cassinello J, Crespo C, Malo AF, Espeso G. Sperm cryopreservation in three species of endangered gazelles (*Gazella cuvieri*, *G. dama mhorr*, and *G. dorcas neglecta*). *Biol Reprod* 2003;**69**:602–11
24. Bronicka A, Dembiński Z. Current criteria and conditions influencing the quality of boar semen. *Medycyna Wet* 1999;**55**:436–9. ([in Polish with English abstract])
25. Strzezek J, Kordan W. Effects of decondensing agents on chromation stability of boar spermatozoa-radioisotopic study. *Anim Sci Pap Rep* 2003;**21**:167–81
26. Ciereszko A, Dabrowski K. Relationship between biochemical constituents of fish semen and fertiliy: the effect of short-term storage. *Fish Physiol Biochem* 1994;**12**:357–67
27. Mann T. *The biochemistry of semen and of the male reproductive tract*. London: Methuen, Wiley, New York, 1964
28. Windsor DP. Mitochondrial function and ram sperm fertility. *Reprod Fertil Dev* 1997;**9**:279–84
29. Silva PF, Gadella BM. Detection of damage in mammalian sperm cells. *Theriogenology* 2006;**65**:958–78
30. Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci* 2000;**60**:481–92
31. Apu AS. Fertility of fresh and frozen-thawed Black Bengal goat spermatozoa. In: Auvijit Saha Apu (ed.). *Fertility of fresh and frozen-thawed goat semen*. LAP Lambert Academic Publishing – ISBN: 978-3-659-18666-0, 2012, https://www.lap-publishing.com/site/lap-the-publisher/11
32. Candappa IBR, Bartlewski PM. A review of advances in artificial insemination (AI) and embryo transfer (ET) in sheep, with the special reference to hormonal induction of cervical dilation and its implications for controlled animal reproduction and surgical techniques. *Open Reprod Sci J* 2011;**3**:162–75
33. Menzies PI. Abortion in sheep: diagnosis and control. In: Youngquist RS, Threlfall WR (eds). *Current therapy in large animal theriogenology 2*. St. Louis: Saunders Elsevier, 2007
34. Simpson JL, Carson SA. Genetic and nongenetic causes of pregnancy loss. *Glob Libr Women's Med (ISSN: 1756-2228)* 2013. DOI: 10.3843/GLOWM.10319, http://www.glowm.com/section_view/heading/Genetic%20and%20Nongenetic%20Causes%20of%20Pregnancy%20Loss/item/318
35. Shorten PR, O'Connel AR, Demmers KJ, Edwards SJ, Cullen NG, Juengel JL. Effect of age, weight, and sire on fetal survival in sheep. *J Anim Sci* 2013;**91**:4641–53


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