Article

Nanowater Enhances Cryoprotective Effects of Glycerol-Containing Extenders Used for Ram Semen Freezing

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Simple Summary: Ram semen does not freeze well with most protocols and semen extenders currently used. Therefore, various cryoprotective substances such as glycerol are typically added to extenders prior to ram semen freezing. It was suggested that nanowater (NW-water obtained in the cold plasma generator and characterized by low freezing point and high diffusivity) could significantly improve ram semen quality after freezing. Our present results show that NW did enhance the protective effects of glycerol-containing semen extenders on ram spermatozoa, with the reduction in sperm mortality/overall increase in survivability being greater with 7% than 3% glycerol in freezing media. Different declusterization times (i.e., duration of cold plasma treatment to produce NW) appear to alter NW properties, which warrants future studies of the utility of NW-containing semen extenders for semen cryoconservation in rams and other mammalian species. While the specific mechanisms whereby NW ameliorates the quality of frozen-thawed ram spermatozoa remain to be fully understood, there is a great deal of evidence to suggest that its benefits are due to a combination of several factors including, but not limited to, thermoprotective effects, improved transport of soluble extender constituents and reduced ice crystal formation.

Abstract: Nanowater (NW-water declusterized in the cold plasma generator) can potentially ameliorate ram semen quality after freezing. Eighteen ejaculates from six Olkuska rams were divided into six equal portions each, and then diluted (800×10^6 spermatozoa/ml) and frozen in the fructose-skimmed milk-egg yolk Kareta extender containing 3% or 7% of glycerol (C3% and C7%) and diluted in deionized water (DW) or NW declusterized for 15 min (NW15)′ or 30 min (NW30′). All frozen-thawed semen samples were subjected to standard evaluation. In addition, *ex situ* survival time of spermatozoa was measured, and the proportions of apoptotic, necrotic and live sperm were determined by flow cytometry. The percentage of spermatozoa with mid-piece defects was lower ($p < 0.05$) in NW15′-3% compared with C3%. The mean survival time of spermatozoa was greater ($p < 0.05$) in NW30′ extenders compared with their respective controls. The proportion of necrotic spermatozoa 1 h after thawing was greater ($p < 0.05$) in C7% compared with NW30′-7%, whereas the proportion of live cells detected immediately and 1 h after thawing were greater ($p < 0.05$) in NW30′-7% than in C7%. NW enhanced cryoprotective effects of glycerol-containing extenders with an overall increase in sperm survivability being greater with 7% than 3% of glycerol.

Keywords: ram; semen; cryopreservation; extender; glycerol; nanowater

1. Introduction

Because spermatozoa lack the intrinsic ability to adapt to subzero temperatures [1], semen cryoconservation requires that semen extenders be supplemented with cryoprotective agents (CPAs) to enable sperm survival and prevent structural damage under hypothermic conditions [2]. Based on their ability to cross the cell membrane, CPAs are divided into two categories: permeating CPAs (capable of traversing plasmalemma; 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 that are highly soluble in water even at low temperatures; they can easily diffuse through cell membranes due mainly to their small molecular size [3]. CPAs that permeate into cytoplasm replace the proportion of intracellular water without excessively "dehydrating" the cell while they reach equilibrium [4]. Cryoprotective properties of permeating CPAs are associated with their ability to significantly reduce the concentration of electrolytes in the solvent [5] and to decrease the degree of cell shrinkage caused by osmotic stress [6]. Moreover, permeating CPAs reduce the intracellular ice formation by solidifying at lower temperatures than water [7]. Glycerol addition to semen extenders lowers the freezing point, and stabilizes sodium and chloride concentrations in spermatozoa [8]. Additionally, glycerol increases media viscosity [9], which leads to further reduction in ice crystal formation and expansion.

A major disadvantage of using glycerol for sperm freezing stems from the fact that it diffuses through the plasma membrane at a slower rate than water [10]. 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 even two-fold [11]. Ultimately, cell membrane damage and cell lysis may occur due to osmotic shock [8], and ram spermatozoa have very low osmotic tolerance [12]. Therefore, 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 prior to next dilution or the beginning of freezing protocol. Furthermore, glycerol at 37 °C (e.g., during thawing) shows significant toxicity and can disturb normal cell metabolism, which further decreases viable cell recovery rates after freezing [13,14].

Nanowater (NW) is obtained through the low-frequency cold plasma treatment of deionized water (DW), the process referred to as declusterization [15-17]. During this procedure, water molecules that under normal conditions form clusters or aggregates of up to 1,000 molecules are broken down into smaller clusters [15,18]. Changes in the spatial configuration of NW are caused by breaking hydrogen bonds and result in various modifications of its physiochemical properties. NW significantly increases solubility of many gases as well as inorganic and some non-polar (organic) substances within a cosolvent [17]. A difference in solubilizing ability between NW and DW arises from the relatively high dielectric constant (ε) of NW [17]; the dielectric constant is an indicator of how well the solvent is able to separate ions. According to Broll et al. [18], NW molecules reduce the activation energy and hence effectively stimulate the breaking of chemical bonds. Declusterized water is also more efficient a carrier of solubilized substances compared with DW [19,20] and hence may increase transmembrane transport of media constituents.

The major objective of the present study was to evaluate the effects of NW used as a diluent for the glycerol-containing semen extenders (3% and 7% Kareta extender; [21]) on post-thaw characteristics of ram semen. We hypothesized that NW, due mainly to its unique physicochemical properties, would significantly improve quality parameters of frozen-thawed ram spermatozoa as compared with DW. Laboratory testing utilized an array of morphological and functional evaluations to determine ram sperm quality.

2. Materials and Methods

2.1. Animals and Locality

All experimental procedures complied with the EC directives for animal experimentation and were conducted under the local animal care/bioethics committee authorization no. 165/2016. The present experiment utilized six clinically healthy Olkuska breed rams 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). During the summer months (anestrous period), all animals had unlimited access to pasture (grass and clover), and in the winter (breeding season), they 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.; http://www.guyokrma.cz; [22]) were available ad libitum. Additionally, the animals received 15-30 dag of concentrate (75% oats, 20% barley, and 5% rapeseed meal) per day for 1 week after shearing in spring [23].

2.2. Semen Collection and Initial Assessment

Major experimental procedures have been outlined in Figure 1. Ejaculates were collected into calibrated, pre-warmed (37 °C) and insulated glass tubes attached to a pre-warmed (38 °C) artificial vagina. Ejaculate volume, color and consistency were assessed immediately after collection. Semen concentration was determined in a Bürker-Turk chamber. A sample of ejaculate (25 µl) was diluted in 10 ml of 3% saline 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. Preliminary assessment of sperm motility was conducted in the Blom chamber on a warm plate (37 °C), using the Nikon Eclipse 80i microscope at 200× image magnification.

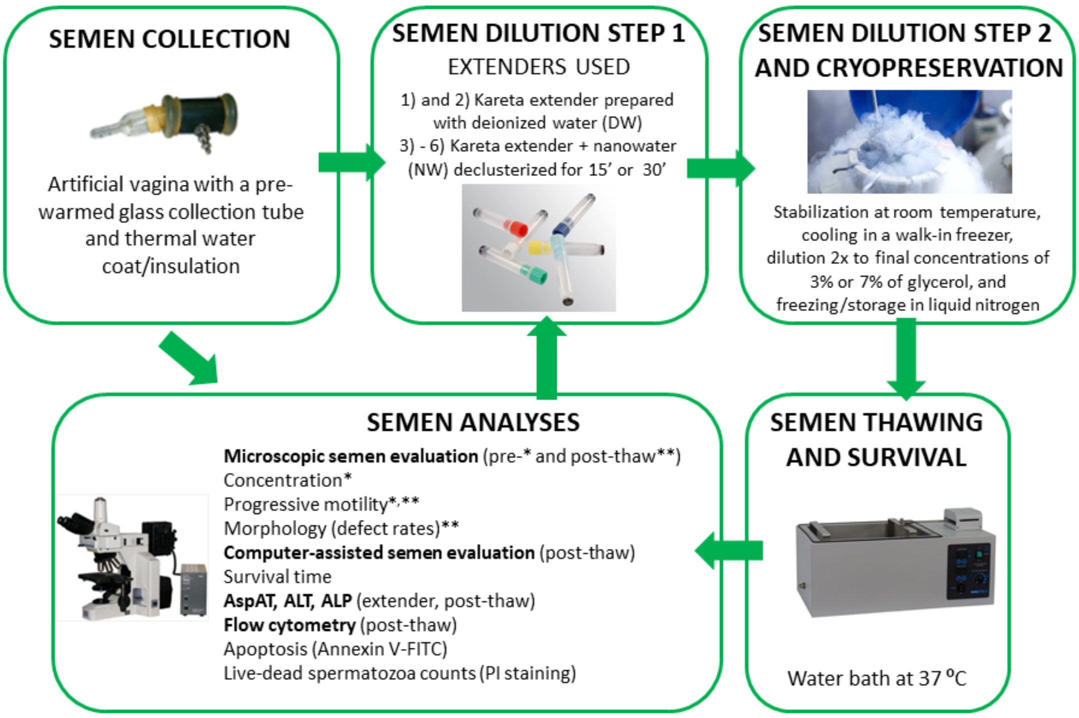


Figure 1. Experimental outline and main procedures.

2.3. Extender Preparation and Semen Freezing

Six types of extenders were prepared according to a modified Kareta protocol [21]; i. deionized water (DW; Aqua Purificata®; Prolab, Gliwice, Poland) with 3% of glycerol (DW3%); ii. DW with 7% of glycerol (DW7%); iii. nanowater (NW; Nantes Nanotechnology Systems, Boleslawiec, Poland) declusterized for 15 min with 3% of glycerol (NW3%-15′); iv. NW declusterized for 15 min with 7% of glycerol (NW7%-15′); v. NW declusterized for 30 min with 3% of glycerol (NW3%-30′); and vi. NW declusterized for 30 min with 7% of glycerol (NW3%-30′). The two desclusterization 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 in terms of post-thaw semen quality and pregnancy rates after artificial insemination [24]. Ejaculates from each ram were divided into six equal parts and then frozen using the two-step freezing protocol and as detailed below: i. initial 30-min equilibration of diluted semen at room temperature (extender consisting of DW or NW and egg yolk (4:1) with addition of 1 g of fructose per 100 mL); ii. equilibration for 30 min to 4 °C in a walk-in freezer; iii. further equilibration for 30 min at 4 °C and further dilution (2x) every 10 min to a final concentration of 3% or 7% of glycerol (extender 6% or 14% of glycerol, respectively); iv. further equilibration for 30 min at 4 °C; v. loading inseminates (final concentration of 800 × 10^6 spermatozoa/ml) into 0.25-cc plastic straws (Rovers; Piaseczno, Poland); vi. equilibration in liquid nitrogen vapors (–120 °C) for 10 min; and vii. 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.

2.4. Post-Thaw Microscopic Assessment of Semen Quality

After thorough dehumidification of straws, semen samples were transferred into sterile analytical tubes. Semen motility was assessed with a computer-assisted Sperm Class Analyzer system (ver. 5.0; Microoptic® Automatic Diagnostic Systems, Barcelona, Spain) using a phase-contrast Nikon Eclipse 80i microscope. All readings were obtained with a 4-µl Leja® disposable counting chamber placed on a warming plate (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.

Determination of spermatozoa survivability utilized the same tools as those described above for the motility assessment. A 250-µl sample of thawed semen was diluted in 1 ml of skimmed milk. Mortality rates of spermatozoa were recorded every 30 min for 1-1.5 h and then every 15 min until complete demise of all spermatozoa. During this test, semen samples were constantly kept in a water bath at 37 °C.

The proportions of normal and aberrant spermatozoa were estimated using a SpermBlue® kit (Microoptic SL Co., Barcelona, Spain) according to the producer's specifications. Histological smears were analyzed for sperm morphological defects including abnormal and detached heads, abnormal mid-pieces and tails, proximal and distal cytoplasmic droplets. For each semen sample, two hundred spermatozoa were evaluated under oil immersion at 1000× image 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.

2.5. Ultraviolet Detection of Alanine Transferase (ALT), Aspartate Aminotransferase (AspAT) and Alkaline Phosphatase (ALP)

Measurements of ALT, AspAT and ALP were done using an Automated-Olympus-AU600 biochemical analyzer (Olympus Corporation; Tokyo, Japan). It utilized an optimized UV-test scoring system compliant with the IFCC (International Federation of Clinical Chemistry) guidelines. Semen samples (20 µl) 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 –25 °C for later analyses.

ALT and AspAT are intracellular enzymes permanently bound to sperm midpiece membranes, particularly to the mitochondria, and so their abundance in semen extenders mainly reflects the damage occurring in sperm mitochondria [25-29]. With an analytical method sensitivity of 1U/l, ALT was detected based on the following chemical reactions:

$$\text{2-oxoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^{+} \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^{+}$$

ALT facilitates a transfer of amino groups from L-alanine to 2-oxoglutarate with concomitant L-glutamate and pyruvate formation. LDH catalyzes pyruvate reaction with NADH, which results in formation of L-lactate and NAD^{+}. NADH absorbance was detected at a 340-nm wavelength. Consumption of NADH lowers the absorbance and is in direct ratio to ALT activity in the probe. With an analytical method sensitivity of 1U/l, AspAT was detected based on the following reactions:

$$\text{2-oxoglutarate} + \text{L-aspartate} \xrightarrow{\text{AspAT}} \text{L-glutamate} + \text{oxaloacetate}$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^{+} \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^{+}$$

AspAT catalyzes transamination reaction of 2-oxoglutarate, which results in the formation of L-glutamate and oxaloacetate. A reduction of oxaloacetate to L-malate by NADH is catalyzed by malate dehydrogenase. Consumption of NADH lowers the absorbance and is directly relevant to AspAT activity in the sample.

Nikolopoulou et al. [30] reported that ALP was a significant marker for acrosome membrane integrity. With an analytical method sensitivity of 1U/l, ALP was detected based on the following reaction:

$$\text{pNPP} + \text{AMP} \xrightarrow[\text{Mg}^{2+}]{\text{FA}} \text{pNP} + \text{AMP}^{-}\ \text{PO}_4$$

ALP activity is determined by measuring the rate of conversion of p-nitrophenyl phosphate (pNPP) into p-nitrophenol (pNP) in the presence of magnesium and zinc ions and with 2-amino-2-methyl-1-propanol (AMP) as a phosphate acceptor, at pH = 10.4. The change in absorbance due to pNP formation, measured at the wave lenght of 410/480 nm, is directly proportional to the enzyme activity in the sample.

2.6. 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 layer 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 [31]. 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 [32,33].

Frozen semen samples were thawed immediately before analyses in a water bath (37 °C) 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 x 10^6 spermatozoa/ml. 100 µl of such solution (1x10^5 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 30,000 events for each tube; 50,000 cells were analyzed per each event. Data acquired using a BD Accuri™ C6 Plus system were computed and a report was automatically generated by the BD Accuri™ C6 Plus software.

2.7. Statistical Analyses

Statistical analyses were done with the SigmaPlot® for Windows® statistical software (ver. 11.0; Systat Software Inc., Richmond, CA, USA). All single time-point observations were analyzed by one-way analysis of variance (ANOVA) and serial data were subjected to two-way repeated measures analysis of variance (RM-ANOVA). All results are expressed as mean ± SD unless otherwise stated and p values < 0.05 were considered statistically significant.

3. Results

3.1. Ejaculate Characteristics

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 mean ejaculate volume was 1.5 ± 0.4 ml, with sperm concentration of 2.7 ± 7.7 x 10^9 spermatozoa/ml and mean progressive motility of 94.4 ± 8.0%.

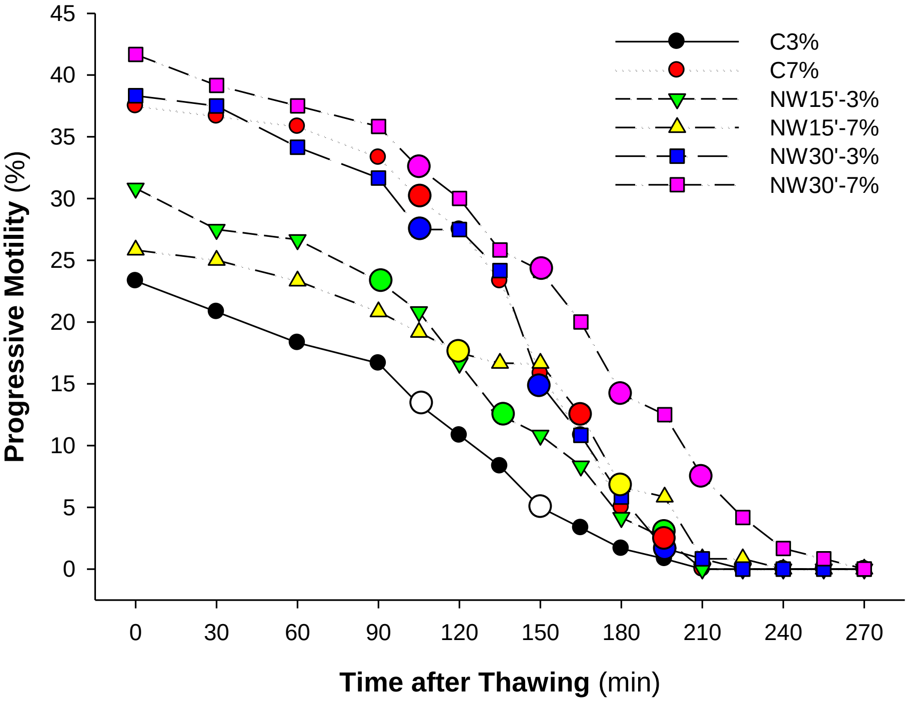
3.2. Post-Thaw Semen Evaluation

For greater clarity, only significant differences among treatment groups and their respective controls were included in this section of the paper. The mean survival time of spermatozoa 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 controls (C3%) from 0 to 150 min after thawing (Figure 2); it was greater (p < 0.05) in NW15′-C7% tan in C7% group from 0 to 120 min; and in was greater (p < 0.05) in NW30′-7% than in C7% from 165 to 195 min of incubation. Progressive motility was greater (p < 0.05) in Kareta extenders C7% compared with C3% up until 150 min post-thawing. Within individual groups, significant declines in sperm progressive motility occurred at the following intervals: C3%: Times 0-105-150 min; C7%: 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 midpiece defects was lower (p < 0.05) in NW15′-3% compared with C3% (Table 2). The proportion of live spermatozoa was greater and the proportion of necrotic spermatozoa was less in NW30′-7% compared with C7% immediately and 1 h after thawing, whereas the proportion of necrotic spermatozoa 1 h after thawing was greater (p < 0.05) in C7% compared with NW30′-7% (Table 3). ALP concentrations in extenders prepared with NW30′ were lower (p < 0.05) compared with the control groups (Table 4).

Table 1. Summary of Olkuska ram semen characteristics (determined in water bath at 37 °C; mean ± SD) following cryoconservation in modified Kareta extenders prepared with deionized water (Control: C) or nanowater declusterized for 15 min or 30 min (NW15′ or NW30′) and containing either 3% or 7% of glycerol.

Variables/Extender	C3%	C7%	NW15′-3%	NW15′-7%	NW30′-3%	NW30′-7%
Survival Time (min)	189.2 ± 6.0*	217.5 ± 16.5**	212.5 ± 18.7	215.0 ± 25.2	215.0 ± 17.8*	242.5 ± 31.1**
Mortality 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

Values are means of 18 ejaculates (3 ejaculates/ram). Within rows, values denoted by the same number of asterisks (* or **) are different ($p < 0.05$).



Incubation Time (min)	Pairs of Significantly Different Mean Values ($p < 0.05$)
0	**NW30′-3% vs. C3%**; *C7% vs. C3%*; **C7% vs. NW15′-7%**
30	**NW30′-3% vs. C3%**; *C7% vs. C3%*; **C7% vs. NW15′-7%**
60	*C7% vs. C3%*; **NW30′-3% vs. C3%; C7% vs. NW15′-7%**
90	*C7% vs. C3%*; **C7% vs. NW15′-7%**;
105	*C7% vs. C3%*; **NW30′-3% vs. C3%; C7% vs. NW15′-7%**
120	*C7% vs. C3%*; **NW30′-3% vs. C3%; C7% vs. NW15′-7%**
135	**NW30′-3% vs. C3%**; *C7% vs. C3%*
150	*C7% vs. C3%*; **NW30′-3% vs. C3%**
165-195	**NW30′-7% vs. C7%**

Figure 2. Changes in Olkuska ram sperm progressive motility monitored ex situ until the complete demise of spermatozoa. Standard deviation bars were omitted for better visibility. Within each group (extender used), larger circles represent consecutive significant ($p < 0.05$) decreases in mean values (starting at Time 0 = thawing). C3%, C7%: control groups (Kareta extenders prepared with DW); NW15'/30′-3%/7%: experimental groups including extenders prepared in NW declusterized 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 C3% and C7% (italics).

Table 2. The influence of cryoconservation in modified Kareta extenders on the percentages of segmental defect of frozen-thawed Olkuska ram spermatozoa.

Extender	Type/Region of Segmental Defect					
	Head	Midpiece	Tail	Detached head	Proximal droplet	Double tail
C3%	45.0 ± 22.3	4.0 ± 2.5^{a}	8.8 ± 9.6	14.8 ± 22.2	ND	ND
C7%	42.7 ± 21.6	3.3 ± 1.4	7.5 ± 6.3	13.2 ± 17.8	0.3 ± 0.8	ND
NW15'-3%	51.7 ± 24.2	1.3 ± 1.4^{b}	6.7 ± 3.9	10.8 ± 11.8	0.3 ± 0.8	ND
NW15'-7%	47.7 ± 20.9	3.3 ± 1.7	5.5 ± 4.6	14.5 ± 17.8	ND	ND
NW30'-3%	41.3 ± 20.6	3.0 ± 4.3	5.3 ± 3.6	13.7 ± 15.2	0.2 ± 0.4	ND
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

Values are means of 18 ejaculates. C3%, C7%: control groups; NW15'/30'-3%/7%: extenders prepared in NW declusterized for 15 min or 30 min; within columns, means denoted by different letter superscripts vary significantly: ^{ab} $p < 0.05$. ND-not detected.

Table 3. The influence of cryoconservation in modified Kareta extenders on cell survivability and the occurrence of apoptosis or necrosis (%) of Olkuska ram spermatozoa immediately after thawing and following a 1-h incubation period.

Extender	Cell Type		
	Live Cells	Necrotic Cells	Apoptotic Cells
	Immediately after Thawing		
C3%	27.0±1.9	17.3±3.0	5.7±1.6
C7%	25.2±2.6^{a}	19.0±2.5	5.9±1.8
NW15'-3%	26.7±1.2	17.4±2.6	5.9±1.8
NW15'-7%	26.0±1.7	17.6±2.2	6.4±2.3
NW30'-3%	26.7±1.5	17.5±1.9	5.8±2.0
NW30'-7%	27.0±1.2^{b}	17.1±2.5	5.8±2.2
	1 h after Thawing		
C3%	25.0±2.0	19.7±3.0	5.2±1.4
C7%	24.6±0.9^{a}	20.7±1.9^{a}	4.7±1.1
NW15'-3%	23.9±2.1	20.0±2.7	6.1±2.3
NW15'-7%	24.7±2.2	19.8±2.4	5.5±1.3
NW30'-3%	26.0±1.5	19.5±1.9	4.5±0.7
NW30'-7%	27.2±2.7^{b}	17.6±4.3^{b}	5.1±1.8

Values are means of 18 ejaculates. C3%, C7%: control groups; NW15'/30'-3%/7%: experimental groups including extenders prepared in NW declusterized for 15 min or 30 min; within columns, pairs of means denoted by different letter superscripts vary significantly: ^{ab} $p < 0.05$.

4. Discussion

Freezability of ram semen is significantly lower compared with that in other mammalian species. Consequently, ejaculates from 5-10% of rams do not freeze well with the commonly used protocols and semen extenders [34]. In fact, boar semen is the most sensitive to low temperatures followed by the ram, stallion, and cat [35]. Therefore, studies using ram semen provide a useful model for studying and amelioration of semen cryopreservation techniques for an array of animal species of veterinary interest and humans.

Table 4. Concentrations of enzymes ALT, ALP and AspAT measured in semen extender samples after thawing.

Extender	Enzyme Concentrations (U/l)		
	ALT	AspAT	ALP
C3%	17.7 ± 5.0	313.9 ± 60.9	4489.7 ± 1930.7[a]
C7%	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 ± 168.6	2683.0 ± 707.8[b]

Values are means of 18 Olkuska ram ejaculates. C3%, C7%: control groups; NW15'/30'-3%/7%: experimental groups including extenders prepared in NW declusterized for 15 min or 30 min; within columns, pairs of means denoted by different letter superscripts vary significantly: [ab] $p < 0.05$.

There is a paucity of information on the effect of declusterization time on the physicochemical properties on NW. Results of the present experiment revealed that declusterization time might impinge on the cryoprotective properties of NW used as a 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′ affected the percentages of live and necrotic spermatozoa cryopreserved in the 7%-glycerol Kareta extenders and the motility, survival time and ALP release for both types of Kareta extenders (containing 3% or 7% of glycerol). Clearly, more positive effects of NW30′ were associated with the use of the 7%-glycerol extender and it ameliorated the cryoprotective properties of the Kareta extenders to a greater extent than NW15′. More research is needed on the mechanisms whereby NW obtained using different declusterization periods can potentiate the effects of semen extenders containing permeable cryoprotectants.

Freezing and thawing procedures reduce sperm motility to a lesser degree than they do affect sperm structural integrity, suggesting that post-thaw structural changes in the sperm “motility apparatus”, the midpiece and flagella, are not always correlated with a decrease in sperm motility [36,37]. Our present results confirm this notion; a reduction in the percentage of midpiece defects in NW15′extender containing 3% of glycerol 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 associated with any difference in sperm defect rates. One of the main causes of the adverse effects of cryopreservation on gamete motility is the phenomenon known as the cold shock [38]. Changes taking place during the cold shock that lead to the weakening of sperm self-propelling ability include alterations in the cell membrane structure and consequently the disturbances in ionic transport across the membrane [39]. Improved motility and survival time of Olkuska ram spermatozoa after freezing in the Kareta extender diluted in NW30′ may therefore be mediated, at least in part, by enhanced intracellular transport and utilization of various extender components during the cold shock phase. In general, the cryoprotective properties of semen extenders are a result of their chemical composition and interactions among the various extender components and glycerol [34]. Moreover, the hyperoxidation and formation of reactive oxygen species (ROS) during the freezing and thawing of semen samples can damage the mitochondrial sheath and tail axoneme, further reducing sperm motility [40]. Interestingly, based on a recent study of boar semen storage in a liquid phase, it was proposed that the main mechanism of the protective actions of NW could include both the improved membrane transport and utilization of seminal plasma/semen extender constituents as well as neutralization of accumulating ROS and direct thermoprotective effects [41]. A specific mechanism whereby NW exerts its beneficial effects during semen freezing and thawing remain to be elucidated.

A rapid release of sperm cytoplasmic enzymes into semen extender usually takes place during the initial stages of the cold shock, but it may also be associated with the damage that occurs in plasma membranes during semen thawing [42]. A leakage of intracellular enzymes from spermatozoa detected after thawing is invariably associated with a decline in their viability and fertilizing potential [25,43-45]. In the present experiment, ALP concentrations in control extenders were consistently higher than those in the extenders prepared with NW declusterized for 30 min. A lack of differences in ALT and AspAT concentrations and a significant difference in ALP content between semen extender samples prepared with DW or NW indicate that disruption of the acrosomal membrane is the main structural damage that occurs in ram spermatozoa frozen in glycerol-containing extenders that can be ameliorated by NW30′.

Sperm mortality after cryopreservation is a well-documented consequence of an exposure to subzero temperatures in several mammalian species [37,46,47]. Sperm death may occur during all consecutive stages of the cryopreservation process [48]. The three main reasons for sperm necrosis are the osmotic stress, intra- and extracellular ice crystals formation, and irreversible changes in the cell membrane fluidity [49]. Exposure to low temperatures and addition of cryoprotective agents have the opposing effects on lipid and protein conformation of cell membranes [50]. However, based on the studies using equine semen incubated for up to 60 min in 37 °C, glycerol (3.5-5%) may induce rapid depolymerization of flagellar actin (sperm motility), and damage to sperm membrane and mitochondria (structural defects and necrosis); evidently, cytotoxic effects of glycerol are related to both osmotic and non-osmotic events during semen freezing and thawing [51]. In the present study, the annexin/phosphatidylserine (ANN/PI) staining showed no significant differences between both Kareta extenders (3% and 7% glycerol) prepared with DW or NW in the percentage of spermatozoa positive for double fluorescence staining (ANN^+/ PI^+) immediately after thawing. This contrasts with the results of earlier studies in bucks documenting an increase in the percentage of apoptotic spermatozoa after thawing [52]. However, the cytometric analysis performed 1 h later showed 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 residual glycerol manifest after thawing of ram semen samples. In the present experiment, a decline in sperm motility/viability at 37ºC was effectively slowed down in NW30′-containing extenders up to 150 min and 195 min after thawing, for NW30′-3% and NW30′-7%, respectively. Therefore, beneficial effects of NW used for ram semen freezing could potentially extend into the period after deposition of frozen-thawed semen in the female reproductive tract, resulting in an improvement of insemination efficiency. This supposition warrants further studies and fertility trials.

The assessment of frozen-thawed sperm motility and survivability at 37 °C revealed that progressive motility of Olkuska ram spermatozoa: i. was greater in NW30′-3% compared with its respective control and it was greater in C7% than in C3% up to 150 min after thawing; ii. was greater for NW15′-7% than for C7% for 2 h after thawing; and iii. was greater in NW30′-7% compared with C7% from 165 to 195 min after thawing. Collectively, these observations can be interpreted to suggest that different combinations of glycerol concentrations and declusterization times of NW change the properties of semen extenders to such a degree that their effects of sperm kinematics and viability are exerted at different stages of the freezing process and after thawing. Adequate concertation on glycerol appears critical for ensuring improved semen motility after thawing. The addition of NW declusterized for 30 min to the 3% Kareta extender and of NW declusterized for 15 min to the 7% Kareta extender can further improve semen motility for 2-2.5 h after thawing. Interestingly, NW declusterized for 30 min and added to the 7% Kareta extender improved progressive motility of spermatozoa between 165 and 195 min after thawing. This is intriguing and opens a possibility of boosting the efficacy of AI in sheep. Specifically, NW30′-7% could potentially be used for semen preservation before intravaginal or transcervical AI whereas NW15′-7% and NW30′-3% could be employed for the cryoconservation of semen subsequently used for laparoscopic insemination. More studies are needed to confirm this utility of various NW preparations.

5. Conclusions

In closing, our results indicate that NW can enhance cryoprotective effects of glycerol-containing extenders on ram spermatozoa with the reduction in sperm necrosis/overall increase in survivability being greater with 7% than 3% glycerol. Different declusterization times appear to alter cytoprotective properties of NW, which warrants further studies of the utility of NW-based semen extenders for semen cryoconservation. While the specific mechanisms whereby NW improves viability of frozen spermatozoa remain to be fully elucidated, indirect evidence accumulates that its beneficial effects are a combination of several influences including, but very likely not restricted to, thermoprotective functions, improved bioavailibity and cellular transport of extender components, as well as reduced ice crystal formation and hyperoxidation [24,41].

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