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Effect of glutamine and sugars after bull spermatozoa cryopreservation

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Abstract

The objective of this study was to evaluate the effects of the addition of different sugars (raffinose, sucrose, and trehalose) on bull spermatozoa cryopreserved in a commercial extender (Optidyl) supplemented with glutamine on semen parameters, fertilizing ability and superoxide dismutase (SOD) activity. Nine ejaculates for each bull were used in the study. Semen was frozen in five different extenders: raffinose 25 mM plus glutamine 3 mM (RGO), sucrose 25 mM plus glutamine 3 mM (SGO), trehalose 25 mM plus glutamine 3 mM (TGO), glutamine 3 mM (GO) and control (O). Insemination doses were processed so that each 0.25 mL straw contained 15 x 10^6sperm. Groups of GO and RGO resulted in the higher rates of subjective (54.0 ± 1.7% and 64.0 ± 1.1%; $P < 0.01$) and CASA motilities (53.0 ± 2.7% and 61.0 ± 4.4%; $P < 0.001$), respectively compared to the other groups. The supplementation of additives did not provide an effect on the level of post-thaw sperm CASA progressive motilities, the sperm motion characteristics and pregnancy rates. GO and RGO provided the better protective effect for sperm acrosome (4.0 ± 0.5% and 12.0 ± 0.6%) and total abnormalities (5.0 ± 0.3% and 13.0 ± 0.7%; $P < 0.001$), respectively. At the HOST values, the additives did not give to result the protective effect in comparison to Optydil extender without additives ($P > 0.05$). For pregnancy rates, there were no significant differences among the groups. The supplementation of additives did not provide any significant difference on the level of SOD activity ($P > 0.05$). It can be also thought that these sugars might have worked with glutamine in a synergy. Thereby, sugars such as raffinose and sucrose with glutamine in freezing extender may be recommended to facilitate bull semen freezability.

Keywords: Bovine; Frozen; Sugars; Glutamine; Fertility; Sperm

1. Introduction

Cryopreservation of bull semen is still challenging due to lower fertility when compared to fresh semen because the all processes of cryopreservation including cooling, freezing and thawing create oxidative stress on the sperm membrane [1–4]. Oxidative stress generally leads to loss of motility, swelling and the blebbing of the acrosomal membrane and disruption or increased permeability of the plasma membrane of spermatozoa [5,6]. It is well known that mammalian spermatozoa contain high concentrations of polyunsaturated fatty acids, and therefore are highly vulnerable to oxidative stress which is responsible for the generation of reactive oxygen species (ROS) [7,8]. Capacitation/acrosome reaction physiologically requires low

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concentrations of ROS. Although normally being a balance between the production of ROS and the scavenging capacity of the antioxidants, the excessive of ROS deteriorate sperm function and enzymatic activity [9,10]. The main objective of most experiments on successfull semen cryopreservation is to determine the appropriate extenders including cryoprotectant and antioxidants.

Egg yolk (EY) is a strong cryoprotectant agent that has been widely used in the extenders [11]. Since its strong cryoprotective effect, commercial extenders, such as Optidyl, have been producted by the incorporation of EY.

Sugars play a role in the protection of post-thaw sperm viability. Sugars such as raffinose, trehalose, when in a high concentration solution, promote the excretion of water out of the cell so as to decrease intracellular ice crystal formation, maintaining osmotic pressure of the extender and have a cryoprotective role. However, their cryoprotective effects are dependent on storage temperature, their molecular weight and the buffer used in the extender [12–14]. The beneficial antioxidative and cryoprotective effects of sugars on cold shock and freeze-thaw damage have been reported in some studies [15–18].

Aminoacids (AAs) ensure to protect the structure of biological membranes of sperm cells during the freeze-thaw process. Some plants and animals accumulate aminoacids in response to exposure to freezing temperatures. Amongst the aminoacids used in freezing extender, glutamine has been reported in several species such as Angora goat [19,20], ram [21], stallion [22] and bull [23] sperm cells against freeze-thawing damage.

This research is the first evaluation of the influence of different sugars in amino-acid supplemented extender on freezing/thawing process of bull semen. The objective was to determine the effects of sugars such as trehalose, sucrose, and raffinose in commercial Optydil® extender supplemented with glutamine on sperm parameters, fertilizing ability, and SOD activity after the freeze-thawing process.

2. Materials and methods

2.1. Chemicals

The Optydil® extender and additives were obtained from IMV Technologies (Şark Kemikal, Turkey) and Sigma-Aldrich (Interlab, Turkey), respectively.

2.2. Animals and semen collection

Ejaculates from three Brown-Swiss bulls (3 and 4 years of age) were used in the study. The bulls, belonging to the Lalahan Livestock Central Research Institute (Ankara, Turkey), were maintained under uniform feeding and housing conditions. A total number of 30 ejaculates (10 ejaculates for each bull) were collected from the bulls with the aid of an artificial vagina twice a week, according to AI standard procedures. Immediately after collection, the ejaculates were immersed in a warm water bath at 37 °C until their assessment in the laboratory. Only ejaculates containing spermatozoa with $> 80\%$ forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa/mL were used.

2.3. Semen extending, freezing and thawing

The groups established in the study were as follows:

RGO: Experiment group which was frozen in an Optydil extender with raffinose (25 mM) plus glutamine (3 mM).

SGO: Experiment group which was frozen in an Optydil extender with sucrose (25 mM) plus glutamine (3 mM).

TGO: Experiment group which was frozen in an Optydil extender trehalose (25 mM) plus glutamine (3 mM).

GO: Experiment group which was frozen in an Optydil extender with glutamine (3 mM).

O: Experiment group which was frozen in an Optydil extender without additives.

The volume of ejaculates and sperm concentrations were measured using a conical tube graduaded at 0.1 mL intervals and Accucell photometer (IMV, L'Aigle, France), respectively. The freezing extender used was a commercial extender (Optydil®). Each ejaculate was divided into five equal parts and diluted to a final concentration of 60×10^6/mL spermatozoa with the above mentioned extenders, respectively. Diluted semen samples were loaded into 0.25 mL French straws and cooled to 4 °C in 2 h, and then were frozen at a programmed rate of −3 °C/min from +4 to −10 °C; −40 °C/min from −10 to −100 °C; −20 °C/min from −100 to −140 °C using a digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen. At least after 24 h, frozen straws were thawed in a 37 °C water bath for 20 s.

2.4. Semen evaluation

2.4.1. Analysis of in vitro and in vivo semen parameters

Subjective motility was assessed using a phase-contrast microscope (100x magnification), with a warm stage maintained at 37 °C. A wet mount was made using a drop of semen placed directly on a microscope

slide and covered by a cover slip at same temperature. Sperm motility estimations were performed in three different microscopic fields for each semen sample. Besides estimating subjective sperm motility, a computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyse sperm motion characteristics. Thawed semen was diluted in a Tris-based extender (without egg yolk and glycerol). A semen sample (volume) was diluted and put onto a prewarmed chamber slide (Leja 4, Leja Products, Luzernestraat B.V., Holland) and sperm motility characteristics were determined with a 10x objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), total sperm motility (%), VAP (average path velocity, μm/s), VSL (straight linear velocity, μm/s), and VCL (curvilinear velocity, μm/s). For each evaluation, 10 microscopic fields were analysed to include at least 300 cells.

For the assessment of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 mL of Hancock solution [24]. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of sperm acrosome and total abnormalities were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (magnification 1000x, oil immersion).

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on coiled and swollen tails. A sample (volume) of each treatment was incubated with 300μL of a 100 mOsm hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37 °C for 60 min. After this time a sample (volume) was spread with a cover slip on a warm slide at 37 °C. A total of 200 sperm were evaluated with bright-field microscopy (100x magnification) and sperm with swollen or coiled tails were recorded [25,26].

The fertilizing ability of spermatozoa was calculated based on the pregnancy rates at 60 d by rectal palpation. A total number of 270 Brown-Swiss cows (between 2 and 5 y of age) were artificially inseminated with sperm treated with additives or not, each comprising the semen of one bull.

2.4.2. Biochemical assays

Biochemical assays were performed on sperm samples immediately after thawing following centrifugation and washing. An aliquot (500 μL) of semen from each sample was centrifuged at 800x g for 10 min, and the supernatant was removed and the pellet was washed with PBS. This procedure was repeated for three times. After the last centrifugation, Triton X-100 (0.1%) was added to the sperm pellets. The superoxide dismutase (SOD) activities, which were calculated for 2 x 10^8 sperm cells, were determined from supernatants [27,28].

2.4.3. Determination of SOD activity

The SOD activity was analysed with the technique of Sun and Zigman [29]. A volume of 0.4 mL sperm sample, 0.1 mL of 0.9% NaCl and 0.4 mL of chloroform was centrifuged for 10 min at 2000g. 0.5 mL of supernatant was used for the test. The reaction mixture contained 20 mL of 3 mmol/L xanthine, 10 mL of 0.6 mmol/L EDTA, 10 mL of 0.15 mmol/L nitroblue tetrazolium, 6 ml of 400 mmol/L sodium carbonate, and 3 ml of 1 g/L bovine serum albumin for 20 tests. 2.45 mL of this reaction mixture was added into 0.5 mL of supernatants in test tubes. An aliquot (50 μL) of xanthine oxidase (20 μmol/2 mL) was added into this mixture and was incubated at 25 °C for 20 min. Subsequently, the reaction was stopped by adding 1 mL of 0.8 mmol/L $CuCl_2$ to the test tubes. Then the absorbance was recorded at 560 nm using a UV/VIS spectrophotometer. SOD activity was expressed as U/g protein.

2.5. Statistical analysis

The results were expressed as mean ± SEM. Means were analyzed using a repeated measurement model of ANOVA, followed by Duncan's post hoc test to determine significant differences among the groups using the SPSS/PC computer program (version 12.0; SPSS, Chicago, IL). Differences with values of $P < 0.05$ were considered to be statistically significant. Pregnancy rates were evaluated using the chi-square test.

3. Results

As shown in Table 1, Group GO and RGO resulted in the higher rates of subjective (54.0 ± 1.7% and 64.0 ± 1.1%; $P < 0.01$) and CASA motilities (53.0 ± 2.7% and 61.0 ± 4.4%; $P < 0.001$), respectively compared to the other groups. The supplementation of additives did not provide an effect on the level of post-thaw sperm CASA progressive motilities, the sperm motion characteristics and pregnancy rates. GO and RGO provided the better protective effect for sperm acrosome (4.0 ± 0.5% and 12.0 ± 0.6%) and total abnormalities (5.0 ± 0.3% and 13.0 ± 0.7%; $P < 0.001$), respectively. At the HOST values, the additives did not give to result the protective effect in comparison to Optydil extender

Table 1
Mean ± SEM sperm parameters in frozen-thawed bull semen.

Groups	O	GO	RGO	SGO	TGO	P
Subjective motility (%)	39.0 ± 4.5[a]	54.0 ± 1.7[b]	53.0 ± 2.7[b]	38.0 ± 1.5[a]	39.0 ± 4.3[a]	**
Progressive motility (%)	18.0 ± 3.1	22.0 ± 1.3	23.0 ± 2.4	20.0 ± 2.0	16.0 ± 2.6	—
CASA motility (%)	51.0 ± 3.2[a]	64.0 ± 1.1[b]	61.0 ± 4.4[b]	47.0 ± 2.5[a]	49.0 ± 2.9[a]	***
VAP (μm/s)	116.1 ± 6.4	115.8 ± 5.5	112.6 ± 7.0	113.9 ± 5.6	101.3 ± 13.3	—
VSL (μm/s)	85.9 ± 2.0	85.9 ± 3.2	87.8 ± 3.9	87.6 ± 3.2	86.8 ± 2.6	—
VCL (μm/s)	219.6 ± 15.4	217.2 ± 13.5	201.3 ± 14.7	210.9 ± 13.8	213.0 ± 15.1	—
Acrosome abnormality (%)	10.0 ± 0.6[bc]	4.0 ± 0.5[a]	5.0 ± 0.3[a]	11.0 ± 0.6[c]	9.0 ± 0.6[b]	***
Total abnormalities (%)	21.0 ± 1.1[b]	12.0 ± 0.6[a]	13.0 ± 0.7[a]	21.0 ± 1.4[b]	19.0 ± 1.5[b]	***
HOST (%)	63.0 ± 1.9[b]	62.0 ± 2.3[b]	63.0 ± 2.0[b]	64.0 ± 1.3[b]	55.0 ± 1.1[a]	*
Pregnancy rate (%)	87.8 (65/74)	75.6 (34/45)	78.7 (37/47)	91.1 (41/45)	74.6 (44/59)	—
SOD (U/mg protein)	0.49 ± 0.03	0.5 ± 0.034	0.6 ± 0.04	0.5 ± 0.07	0.6 ± 0.03	—

[a–c] Different superscripts within the same row demonstrate significant differences among groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).
— No significant difference ($P > 0.05$).

without additives ($P > 0.05$). The supplementation of additives did not provide difference on the level of SOD activity ($P > 0.05$).

4. Discussion

Mammalian spermatozoa are susceptable to LPO which destroys the structure of the lipid matrix of spermatozoa membrane, due to the attacks of ROS formed from reduction of oxygen, during cryopreservation. The damage of lipid matrix finally causes loss of membrane integrity, membrane deterioration-due to membrane phase transitions, decreased sperm motility, loss in fertility and damage of the sperm DNA, through the oxidative stress and the production of cytotoxic aldehydes [5–10]. SOD is an enzyme, which scavenges ROS, such as superoxide anion and hydroxyl radicals, and thus protects mammalian sperm from oxidative damage [30]. In this study, additives did not increase SOD activity. Various factors complicate the cryopreservation of semen, like mechanical and biochemical stresses, oxygen-derived free radicals, high sensitivity to osmotic pressure changing, cooling, freezing and thawing processes.

Other important factors in sperm freezing protocols are cryoprotectants (e.g., glycerol) [12–14]. It was reported that glycerol alone is less effective than sugar/glycerol mixtures [27,28]. Adding sugars as cryoprotectant was suggested to ameliorate the complications. Non-permenant sugars can contribute to cellular dehydration before freezing where di- and tri- saccharides may be more influential than monosaccharides by increasing osmotic dehydration [31,29]. Raffinose plays a cryoprotective role, causing cellular osmotic dehydration during cryopreservation by interacting with membrane lipids and proteins and decreasing the risk of intracellular ice crystal formation [32,33]. The cryoprotective properties of various sugars against freezing–thawing induced damages have been well documented in various cell types [32,34,35]. The effect of raffinose related to oxidative stress has been considered as indirect effects of sugar signalling and triggering the production of specific ROS scavengers [36]. Raffinose also reported to be used in most procedures for blocking the extent of extracellular ice formation, enhancing the formation of a metastable glass or microcrystalline state and providing hypertonicity [31,33]. In this study, there were significant differences in sperm parameters of post-thawed bull semen in response to raffinose and glutamine supplemented to the extender (RGO). The results comprising subjective and CASA motilities and acrosome integrity represented clearly that using raffinose plus glutamine or alone glutamine in the extender might give better post-thawed sperm quality. Disaccharides were reported to provide superior protection for motility, plasma membrane integrity and fertilizing capability of post-thawed sperm against cryodamage in different species [13,16,28,29,33].

Disaccharides, which mammalian cells are impermeable to, prevent the irrevesible alteration in membranes and stabilise membrane bilayers during cooling-freezing states [37,38]. The concentration of sucrose is directly proportional to the amount of ice crystal and the temperature reduction occured during freezing [31]. In this study, SGO did not play an important role in maintaining sperm motility and acrosome integrity, in contrast with a study carried out in Iberian red deer epididymal spermatozoa [39]. But SGO gave a favorable effect on pregnancy rates, compared to that of other groups. However this difference was not statistically significant. Based on our results, we can suggest

that raffinose displayed cryoprotection on the sperm motility and morphology.

Trehalose showed better cryoprotection and post-thaw fertilizing ability in mouse, ram, and bull sperm in some reports, leading to diminished death and damage of sperm [33,40,41]. In this study, TGO did not provide a positive effect on maintaining semen quality parameters. This finding was in contrast with the results of the study of Aboagla and Terada performed in goat [42] who found significant improvement in goat sperm freezability with trehalose containing extender. Difference of the extender content and animal species may explain contradictory results.

Aminoacids may play a role in the maintanence of the sperm membrane lipids which are especially vulnerable to LPO damage [43]. The supplementation of various aminoacids to freezing media has been found to be beneficial for spermatological properties of many species [19–22,44]. Glutamine, a tripeptide, plays a regulatory role in several cell-specific processes, including the metabolism (e.g., oxidative fuel, lipogenic precursor), cell integrity (apoptosis, cell proliferation), protein synthesis and degradation, redox potential, respiratory burst, gene expression and extracellular matrix synthesis [43]. In regard to sperm functions, glutamine acts at the extra-cellular level, improving the motility of human and stallion sperm [45,46] and the fertilizing ability of human sperm [45] following the freeze-thawing process. In this study, the supplementation of the extender with glutamine led to higher motility rates, functional and acrosomal integrity of spern membrane. These results were similar to those previously reported for frozen–thawed sperm of the Angora goat, stallion and bull where glutamine addition also resulted in a significant increase in sperm motility and membrane integrity after thawing [20,22,23]. We may suggest that glutamine provide cryoprotection in mammalian sperm by decreasing cryodamage of sperm.

The HOST test assessed the resistance of the sperm plasma membrane to damage induced by the loss in permeability under the stress of swelling driven by the hypo-osmotic treatment. This, thus provided a form of a membrane stress-test, which is particularly useful for testing the membrane-stabilising action of antioxidants [34]. In regard to HOST values, the additives did not provide protective effect in comparison to Optydil extender without additives ($P < 0.05$) in this study.

The main aim of artificial insemination with frozen bull semen is to achieve succesfull pregnancy rate. In this study, fertility rate of SGO (91.1%) were slightly higher compared to other groups even though these differences were not statistically significant. Regarding our fertility results, it may be remarkable that sufficient pregnancy rates were obtained from all groups following cryopreservation/thawing of semen.

In conclusion, the percentages of post-thawed sperm motility, acrosome and functional plasma membrane integrity were found to be significantly higher in the groups of GO and RGO, compared to other groups. It can be suggested that these sugars might have worked with glutamine in a synergy. Sugars such as raffinose and sucrose with glutamine in freezing extender may be recommended to facilitate bull semen freezability. Further studies are required to evaluate the influence of sugar and aminoacid type on the sperm parameters and fertilizing ability in cryopreserved bull sperm.

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