Cryobiology 65 (2012) 93–97

Contents lists available at SciVerse ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

The effects of different sugars on motility, morphology and DNA damage during the liquid storage of rat epididymal sperm at 4 °C

Serpil Sarıözkan [a,*], Mustafa Numan Bucak [b,*], Fazile Canturk [c], Saim Özdamar [c], Arzu Yay [c], Pürhan Barbaros Tuncer [d], Servet Özcan [e], Neslihan Sorgucu [c], Yusuf Caner [c]

[a] Erciyes University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Kayseri, Turkey
[b] Selcuk University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Konya, Turkey
[c] Erciyes University, Faculty of Medicine, Department of Basic Sciences, Kayseri, Turkey
[d] Lalahan Livestock Central Research Institute, Lalahan, Ankara, Turkey
[e] Erciyes University, Faculty of Physical Sciences, Department of Biology, Kayseri, Turkey

ARTICLE INFO

Article history:
Received 21 March 2012
Accepted 14 May 2012
Available online 23 May 2012

Keywords:
Chilling
Rat sperm
Fructose
Raffinose
Trehalose

ABSTRACT

This study evaluated the protective effects of supplementation with three different sugars on the motility, morphology and DNA integrity of rat epididymal sperm chilled and stored at 4 °C Epididymides were obtained from each donor. Rat epididymal sperm was diluted in Ham's F10 plus raffinose, Ham's F10 plus trehalose, Ham's F10 plus fructose, and Ham's F10 medium for control purposes. Thereafter, the extended sperm were chilled and stored in liquid form at 4 °C. Sperm motility, morphological abnormalities and DNA damage were determined at 0 and 12 h after chilling. No significant difference was observed in any of the parameters evaluated at 0 h, before storage ($P > 0.05$). After 12 h of storage, all sugar additives led to statistically higher motility, normal sperm morphology and DNA integrity in comparison to the control group. Raffinose gave the best motility percentages (32.86 ± 1.84%) after 12 h of storage at 4 °C, compared to the other groups ($P < 0.001$). In conclusion, Raffinose, trehalose and fructose provided a better protection of sperm functional parameters against chilling injury, in comparison to the control group.

Introduction

Rats are among animal models widely used in biotechnological research. Although rats are naturally easy to breed, the breeding of transgenic or genetically modified strains remains a challenge [7]. Liquid storage and cryopreservation are reliable techniques for the preservation of semen, and live offspring can be generated by artificial insemination or in vitro fertilization using the preserved sperm of rats [18]. The chilling and freezing of semen results in the deterioration of sperm motility, viability and DNA integrity. Actually, appropriate chilling and cryopreservation protocols have already been developed for many species, including the bull [5,31], ram [9,32], goat [37], and mouse [24]. However, limited data is available on the successful chilling, liquid storage and cryopreservation of rat sperm since cryopreservation ability of rat sperm and testicular tissue ranges from poor to fair in terms of functional survival and reproducibility [7]. Besides, rat sperm is extremely vulnerable to physical stress and handling processes such as chilling, freezing and thawing, due to their size and low water permeability [15,25].

Sugars such as raffinose (trisaccharide), trehalose (disaccharide) and fructose (monosaccharide) play a role in the protection of post-thaw sperm viability by providing an energy source, promoting the excretion of water out of the cell so as to decrease intracellular ice crystal formation, and maintaining the osmotic pressure of the extender. However, their protective effects are dependent on storage temperature, molecular weight, and the particular buffer used in the extender [1,13,14]. The beneficial antioxidative and protective effects of sugars against the cold shock and freeze–thaw damage of sperm have been reported in many studies [21,43].

We designed the current study aiming to examine the effect of sugars such as trehalose, fructose and raffinose on motility, morphology and DNA integrity of rat epididymal sperm during liquid storage at 4 °C.

Materials and methods

Chemicals

The additives (fructose F2543, trehalose T 0167 and raffinose R7630) and other chemicals used in this study were obtained from Local representative of Sigma–Aldrich Chemicals.

* Corresponding authors.
E-mail addresses: sariozkan75@yahoo.com (S. Sarıözkan), mustafanbucak@yahoo.com (M.N. Bucak).

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http://dx.doi.org/10.1016/j.cryobiol.2012.05.007

Animals

Twenty male Wistar albino rats (weighing 200–250 g) were used as semen donors. They were obtained from the Experimental and Clinical Research Centre of Erciyes University, Kayseri, Turkey. Rats were housed under standard laboratory conditions (in polycarbonate cages and were exposed to a 12 h/12 h light/dark cycle at a room temperature of 22–24 °C and relative humidity of 55–60%). A commercial pellet diet (2600 kcal/kgME, 7% crude cellulose, and 23% crude protein) and fresh drinking water were given *ad libitum*. The protocol for the use of experimental animals was approved by the Institutional Review Board of the National Institute of Health and Local Committee on Animal Research.

Experimental design and sample collection

Mature male rats were killed under anesthesia (ketamine and xylazine, i.p.), and their epididymides were excised. The epididymides collected from each donor were placed in a plastic dish containing 3 ml of Ham's F10 medium without Hepes and BSA (Biological Industries, Kibbutz Beit Haemek Israel, Lot: 930747). The epididymides were dissected with anatomical scissors, to obtain epididymal sperm, in 1 ml of Ham's F10 medium in a Petri dish to allow viable spermatozoa to swim out for 10–15 min at 37 °C. Tissue debris was removed from the medium by filtering through a strainer. The sperm suspension was then gently placed in eppendorf tubes. The number of sperm in the samples was determined by direct examination under the microscope using a haemocytometer. The base semen extender supplemented with raffinose, trehalose and fructose at 5 mM dose was prepared. Supplementation with different sugars (Ham's F10 plus raffinose – HR, Ham's F10 plus trehalose – HT, Ham's F10 plus fructose – HF, Ham's F10 – control) was used to dilute rat sperm. Only semen with a sperm motility >50% and a sperm concentration of 1–3 $\times$ 10^8 spermatozoa/mL was processed in the present study.

Semen dilutions and chilling

In the experiment, 20 mature male rats were used as semen donors, and were allocated to four extender groups (five rats/per experimental group). The epididymides of five rats belonged to one group. Each group consisted of five rats and had five replicates. Each rat with their epididymides was accepted as a replicate. Epididymal sperm derived from both epididymides of each twenty rats was diluted with one of the four different semen extenders containing raffinose (5 mM-HR), trehalose (5 mM-HT), fructose (5 mM-HF) or no supplementation, to a final concentration of 1–3 $\times$ 10^7/mL spermatozoa, respectively. Thereafter, diluted semen samples (10 ml) were kept in glass tubes and cooled from 37 to 5 °C, at a rate of 0.2–0.3 °C/min in a cold cabinet, and were maintained at 5 °C during 12 h.

Evaluation of sperm parameters

Evaluations of sperm motility, morphological abnormalities and DNA damage were performed prior to chilling (moment 0) and at 12 h after chilling. All the evaluations were made after dilution.

Evaluation of sperm motility

Sperm motility was evaluated by placing a 5-μL drop of diluted semen on a slide at 37 °C, and then examining it under a light microscope at $\times$100. Motility estimations were performed from three different fields in each sample and mean used as the final motility.

Assessment of sperm morphological abnormalities

To determine the percentages of morphological abnormalities, sperm samples were stained with eosin–nigrosin (1.67% eosin, 10% nigrosin and 0.1 M sodium citrate) were prepared. The percentages of sperm head, tail and total abnormalities were determined by counting a total of 200 sperm per slide under a light microscope at 400$\times$ magnification [28,38].

Assessment of sperm DNA damage using the comet assay

Diluted semen samples were centrifuged at 300*g* for 10 min at 4 °C. Supernatant was removed and the remaining sperm cells were washed with (Ca2+ and Mg2+ free) PBS [4]. Sperm DNA damage was investigated using the single-cell gel electrophoresis (comet) assay that was generally performed at high alkaline conditions. Firstly, sperm was embedding in agarose gel. In brief, each microscope slide was pre-coated with a layer of 1% normal melting point agarose in PBS and dried thoroughly at room temperature. Next, 100 μl of 0.7% low melting point agarose at 37 °C was mixed with 10 μl of the cell suspension and dripped onto the first layer. Slides were allowed to solidify for 5 min at 4 °C in a moist box. The coverslips were removed and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM dithiothereitol (pH 10) for 1 h at 4 °C. Then the slides were incubated overnight at 37 °C in 100 μg/ml proteinase K (Sigma) added to the lysis buffer. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at room temperature at 25 V and was adjusted to 300 mA. Subsequently, the slides were washed with a neutralizing solution of 0.4 M Tris, pH 7.5, in order to remove the alkali ions and detergents. After neutralization, the slides were stained with 50 μl of ethidium bromide (1 μg/ml) and covered with a coverslip. All steps were performed under dim light to prevent further DNA damage [16,34].

The images of 100 randomly chosen nuclei were analyzed visually. Observations were made at a magnification of 400x using a fluorescent microscope (Olympus, Japan). Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a 'comet' pattern (Fig. 1), whereas whole sperm heads, without a comet (Fig. 2), were not considered to be damaged [41].

Statistical analysis

The study was replicated five times. Results were expressed as mean ± SEM. Means were analyzed by one-way analysis of variance, followed by Tukey's post hoc test to determine significant differences among the groups for all sperm parameters using the SPSS/PC computer programme (version 12.0, SPSS, Chicago, IL). Differences with values of $P < 0.001$ were considered to be statistically significant.

Results

The effects of raffinose, trehalose and fructose on rat sperm motility, morphological abnormalities and DNA damage, prior to chilling (moment 0) and at 12 h of chilling, are presented in Tables 1 and 2, respectively. No significant difference was observed in any of the parameters evaluated at 0 h of storage. Conversely, after a 12 h-storage, all sugar additives tested led to statistically higher motilities in comparison to the control group ($P < 0.001$), and raffinose gave the best motility values. Similarly, sugar supplementations provided a better protection against chilling injury since

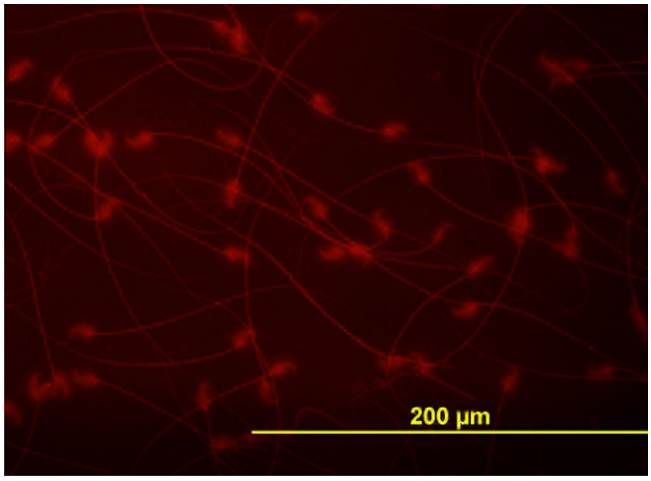

Fig. 1. This appearance with a comet pattern shows the spermatozoa with damaged DNA.

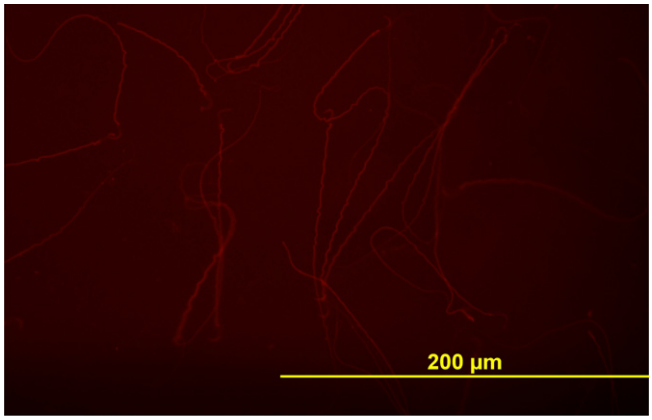

Fig. 2. This appearance without a comet pattern shows the spermatozoa with undamaged DNA.

results obtained for sperm morphological abnormalities, including head, tail and total percentages, and DNA damage percentage were higher when compared to control ($P < 0.001$).

Discussion

The transmission of genetic information to future generations depends on the DNA integrity of sperm. DNA integrity has become an important indicator of fertile sperm. However, sperm cells are susceptible to a number of endogenous and exogenous factors. As sperm possess limited defensive cytoplasm [33], significant DNA damage may occur as a result of oxidative damage arising from the absence of a defense system [40]. Therefore, DNA damage can result in infertility [17].

This study investigated the effects of the sugars trehalose, raffinose and fructose on the motility, morphological abnormalities and DNA damage of rat epididymal sperm chilled and stored at 4 °C. Normally, as cryopreservation and freezing induce the formation of intracellular ice crystals, and membrane alterations due to cold shock, phase changes in membrane lipids and osmotic pressure alterations, there are important differences in terms of fertilizing ability between fresh and frozen-thawed sperm [7,42].

Sperm cells are highly vulnerable to in vitro manipulations, which lead to the loss of sperm functions [25]. Additionally, cold shock not only deteriorates the functional parameters of sperm, but also triggers DNA damages [12]. Cryopreservation of rat sperm is more difficult than that of some other mammals [25,26]. DNA alterations and chromatin abnormalities arising from cold shock also affect early embryonic development [10].

The integrity of sperm DNA has a vital importance to the sperm cell. Some authors suggest that sperm DNA integrity is a more objective marker of sperm function as opposed to the sperm parameters such as motility [20]. DNA damage could be result of free radical induced damage [28,39]. In this study, although sperm functional parameters declined with time of storage, all sugar additives led to statistically higher sperm parameters and DNA integrities, in comparison to the control group. Similarly, all semen extenders with additives provided a better protection against cold shock for the sperm head and tail, total abnormalities, and DNA damage, when compared to the controls. These results show that the sugars used are apparently beneficial additives, which affect sperm survival after the chilling of sperm. Although sugars cannot diffuse across the plasma membrane, they are able to create an osmotic pressure, inducing cell dehydration. Besides, these sugars interact with phospholipids in the plasma membrane, increasing sperm survival during chilling period [8,19]. The current results are similar to those reported in studies carried out in ram [3], goat [2], boar [21] and mouse [36] sperm.

Table 1
Mean ± SEM sperm parameters in fresh epididymal rat spermatozoa prior to chilling (moment 0).

Groups	Motility (%)	Head abnormality (%)	Tail abnormality (%)	Total abnormalities (%)	DNA damage (%)
HR	69.00 ± 1.53	2.50 ± 0.43	2.83 ± 0.70	5.33 ± 0.61	2.53 ± 0.45
HT	66.67 ± 2.47	2.50 ± 0.62	2.33 ± 0.49	4.83 ± 0.75	1.38 ± 0.49
HF	69.17 ± 2.39	2.00 ± 0.52	2.50 ± 0.43	4.50 ± 0.56	1.70 ± 0.30
Control	70.00 ± 1.29	2.33 ± 0.61	2.83 ± 0.65	5.17 ± 0.48	1.90 ± 0.53
P	–	–	–	–	–

–: No significant difference.

Table 2
Mean ± SEM sperm parameters in chilled rat spermatozoa at 4 °C for 12 h.

Groups	Motility (%)	Head abnormality (%)	Tail abnormality (%)	Total abnormalities (%)	DNA Damage (%)
HR	32.86 ± 1.84^{a}	6.43 ± 0.87^{b}	9.00 ± 0.72^{b}	15.43 ± 1.11^{b}	7.21 ± 1.78^{b}
HT	24.29 ± 2.54^{b}	6.14 ± 0.67^{b}	8.14 ± 0.46^{b}	14.29 ± 1.04^{b}	2.80 ± 1.01^{b}
HF	17.14 ± 1.84^{c}	8.29 ± 1.08^{b}	9.57 ± 0.75^{b}	17.86 ± 0.96^{b}	3.32 ± 0.84^{b}
Control	10.00 ± 1.54^{d}	27.88 ± 2.19^{a}	15.75 ± 1.11^{a}	43.63 ± 2.41^{a}	18.85 ± 2.49^{a}
P	***	***	***	***	***

$^{a-d}$: Different superscripts within the same column demonstrate significant differences among groups ($^{***}P < 0.001$).

Normally, we obtained a lower cryoprotective effect for monosaccharides (fructose), when compared to di- (trehalose) and tri- (raffinose) saccharides. Some papers report that di- and tri-saccharides may be more influential than monosaccharides in that they increase osmotic dehydration [19,29]. Raffinose has been used in many procedures to block extracellular ice formation, provide hypertonicity and enhance the formation of a metastable glass or microcrystalline state [35]. As mitochondria produce ATP, they contribute greatly to the maintenance of motility [23]. In this study, it was determined that raffinose gave the best motility percentage. The current finding is similar to the results obtained by Tuncer et al. [37], who demonstrated a marked reduction in acrosome abnormalities and DNA damage in goat sperm when raffinose was added to the extender. We found that supplementation of all three sugars played a significantly beneficial role in the maintenance of sperm motility, intact morphology and DNA integrity in rat sperm against chilling injury. Besides, raffinose particularly induced a stronger protective effect than trehalose and fructose in preserving sperm motility. Although rat sperm has problematic process in terms of handling and chilling, these challenges can be overcome by the supplementation of the semen extender with cryoprotective additives and by suitable handling procedures prior to storage period.

Additionally, if alternative methods for the handling, chilling and freezing of rat spermatozoa are developed, these methods might prove beneficial for different animal models including the boar, stallion and small ruminants, which still face challenges in term of cryopreservation. For example, the widespread dissemination of semen from a small number of ram with superior genetic merit to remote areas in order to inseminate a large number of ewes requires preservation of semen under formed artificial environment. Two major systems of sperm storage (liquid and frozen) have been achieved for preservation. Preservation of sperm generally requires a reduction or arrest of the metabolism of sperm cells, thereby, prolonging their fertile life [22,44]. The freezing–thawing of spermatozoa results in more reduced rates of sperm motility and fertilizing capacity when compared to liquid storage at 5 °C [11]. On the other hand, cooled semen suffers from a decrease in motility and membrane integrity, accompanied by a reduction in fertility for extending days of liquid storage (e.g. 12 h of storage) [6,22,30]. Successful liquid storage of spermatozoa is dependent on the reversible reduction of survival and metabolic activity of spermatozoa. This is achieved by providing effective environmental conditions for the cooled semen, which to present have focused on the development of beneficial extenders that maintain motility, DNA integrity and fertilizing ability of spermatozoa during liquid storage [27,30]. In the present study, it was concluded that raffinose, trehalose and fructose provided a protective effect, which improves motility and prevents DNA damage and morphological injury of rat sperm for 12 h of storage). Supplementation with these sugars prior to the liquid storage may be recommended to facilitate the enhancement of sperm storage techniques for the livestock industry and laboratory animals, and human in vitro fertilization studies.

Statement of funding

The authors would like to thank the staff of Hakan Çetinsaya Experimental and Clinical Research Centre of Erciyes University, Faculty of Medicine for their valuable assistance.

References

[1] A.A. Abdelhakeam, E.F. Graham, I.A. Vazquez, K.M. Chaloner, Studies on the absence of glycerol in unfrozen and frozen ram semen: development of an extender for freezing: effects of osmotic pressure, egg yolk levels, type of sugars, and the method of dilution, Cryobiology 28 (1991) 43–49.
[2] E.M. Aboagla, T. Terada, Trehalose enhanced fluidity of the goat sperm membrane and its protection during freezing, Biol. Reprod. 69 (2003) 1245–1250.
[3] E. Aisen, V. Medina, A. Venturino, Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations, Theriogenology 57 (2002) 1801–1808.
[4] M. Arabi, Bull spermatozoa under mercury stress, Reprod. Dom. Anim. 40 (2005) 454–459.
[5] J.F. Bilodeau, S. Blanchette, I.C. Gagnon, M.A. Sirard, Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen, Theriogenology 56 (2001) 275–286.
[6] M.N. Bucak, N. Tekin, Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen, Small Rumin. Res. 73 (2007) 103–108.
[7] B. Charreau, L. Tesson, J.P. Soulillou, C. Pourcel, I. Anegon, Transgenesis in rats: technical aspects and models, Transgenic Res. 5 (1996) 223–234.
[8] Y. Chen, R.H. Foote, C.C. Brockett, Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm, Cryobiology 30 (1993) 423–431.
[9] K. Coyan, N. Baspinar, M.N. Bucak, P.P. Akalın, M.B. Ataman, A.D. Omur, S. Gungor, Influence of methionine and dithioerythritol on sperm motility, lipid peroxidation and antioxidant capacities during liquid storage of ram semen, Res. Vet. Sci. 89 (2010) 426–431.
[10] M.J. D'Occhio, K.J. Hengsberger, S.D. Johnston, Biology of sperm chromatin structure and relationship to male fertility and embryonic survival, Anim. Reprod. Sci. 101 (2007) 1–7.
[11] G. Evans, W.M.C. Maxwell, Handling and examination semen, in: W.M.C. Maxwell (Ed.), Salamon's Artificial Insemination of Sheep and Goat, Butterworths, Sydney, 1987, pp. 93–106.
[12] L. Gandini, F. Lombardo, A. Lenzi, M. Spano, F. Dondero, Cryopreservation and sperm DNA integrity, Cell Tissue Bank 7 (2006) 91–98.
[13] M.A. Garcia, E.F. Graham, Development of a buffer system for dialysis of bovine spermatozoa before freezing. II. Effect of sugars and sugar alcohols on post-thaw motility, Theriogenology 31 (1989) 1029–1037.
[14] J.J. Garde, A. Olmo, A.J. Soler, G. Espeso, M. Gomendio, E.R. Roldan, Effect of egg yolk, cryoprotectant, and various sugars on semen cryopreservation in the endangered Cuvier's gazelle (Gazella cuvieri), Anim. Reprod. Sci. 108 (2008) 384–401.
[15] M. Hagiwara, J.H. Choi, R.V. Devireddy, K.P. Roberts, W.F. Wolkers, A. Makhlouf, Cellular biophysics during freezing of rat and mouse sperm predicts post-thaw motility, Biol. Reprod. 81 (2009) 700–706.
[16] G. Haines, B. Marples, P. Daniel, I. Morris, DNA damage in human and mouse spermatozoa after in vitro-irradiation assessed by the comet assay, Adv. Exp. Med. Biol. 444 (1998) 789–791.
[17] C.M. Hughes, V.J. McKelvey-Martin, S.E.M. Lewis, Human sperm DNA integrity assessed by the comet and ELISA assays, Mutagenesis 14 (1999) 71–75.
[18] N. Kashiwazaki, Y. Seita, A. Takizawa, N. Maedomari, J. Ito, T. Serikawa, Techniques for in vitro and in vivo fertilization in the rat, Methods Mol. Biol. 597 (2010) 311–322.
[19] C. Koshimoto, P. Mazur, The effect of the osmolality of sugar-containing media, the type of sugar, and the mass and molar concentration of sugar on the survival of frozen-thawed mouse sperm, Cryobiology 45 (2002) 80–90.
[20] C.C. Love, S.P. Brinsko, S.L. Rigby, J.A. Thompson, T.L. Blanchard, D.D. Varner, Relationship of seminal plasma level and extender type to sperm motility and DNA integrity, Theriogenology 63 (2005) 1584–1591.
[21] C. Malo, L. Gil, N. Gonzalez, R. Cano, De.I. Blas, E. Espinosa, Comparing sugar type supplementation for cryopreservation of boar semen in egg yolk based extender, Cryobiology 61 (2010) 17–21.
[22] W.M.C. Maxwell, S. Salamon, Liquid storage of ram semen: a review, Reprod. Fertil. Dev. 5 (1993) 613–638.
[23] C.M. Medeiros, F. Forell, A.T. Oliveira, J.L. Rodrigues, Current status of sperm cryopreservation: why isn't it better, Theriogenology 57 (2002) 327–344.
[24] N. Nakagata, M. Okamoto, O. Ueda, H. Suzuki, Positive effect of partial zona-pellucida dissection on the in vitro fertilizing capacity of cryopreserved C57BL/6J transgenic mouse spermatozoa of low motility, Biol. Reprod. 57 (1997) 1050–1055.
[25] E. Nakatsukasa, T. Inomata, T. Ikeda, M. Shino, N. Kashiwazaki, Generation of live rat offspring by intrauterine insemination with epididymal spermatozoa cryopreserved at–196 °C, Reproduction 122 (2001) 463–467.
[26] K. Niwa, M.C. Chang, Various conditions for the fertilization of rat eggs in vitro, Biol. Reprod. 11 (1974) 463–469.
[27] H. Paulenz, L. Söderquist, T. Adnøy, O.H. Fossen, K.A. Berg, Effect of milk-and TRIS-based extenders on the fertility of sheep inseminated vaginally once or twice with liquid semen, Theriogenology 60 (2003) 759–766.
[28] K.T. Rajesh, K. Doreswamy, B. Shrilatha, M. Muralidhara, Oxidative stress associated DNA damage in testis of mice. induction of abnormal sperms and effects on fertility, Mutat. Res. 513 (2002) 103–111.
[29] S. Salamon, Deep freezing of ram semen: recovery of spermatozoa after pelleting and comparison with other methods, Aust. J. Biol. Sci. 21 (1968) 355–360.
[30] I. Salvador, M.P. Yaniz, E.A. Gomez, M.A. Silvestre, Effect of solid storage on caprine semen conservation at 5 °C, Theriogenology 66 (2006) 974–981.
[31] S. Sariozkan, M.N. Bucak, P.B. Tuncer, P.A. Ulutas, A. Bilgen, The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation, Cryobiology 58 (2009) 134–138.

[32] S. Sariozkan, M.N. Bucak, P.B. Tuncer, U. Tasdemir, H. Kinet, P.A. Ulutas, Effects of different extenders and centrifugation/washing on postthaw microscopic-oxidative stress parameters and fertilizing ability of Angora buck sperm, Theriogenology 73 (2010) 316–323.
[33] G.A. Sega, E. Generoso, Measurement of DNA breakage in specific germ cell stages of male mice exposed to acrylamide, using an alkaline elution procedure, Mutat. Res. 242 (1990) 79–87.
[34] N.P. Singh, R.E. Stephens, X-ray induced DNA double-strand breaks in human sperm, Mutagenesis 13 (1998) 75–79.
[35] B.T. Storey, E.E. Noiles, K.A. Thompson, Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation, Cryobiology 37 (1998) 46–58.
[36] N. Tada, M. Sato, J. Yamonoi, T. Mizorgi, K. Kasai, S. Ogawa, Cryopreservation of mouse spermatozoa in the presence of raffinose and glycerol, J. Reprod. Fertil. 89 (1990) 511–516.
[37] P.B. Tuncer, M.N. Bucak, S. Sariozkan, F. Sakin, D. Yeni, I.H. Cigerci, M. Gundogan, The effect of raffinose and methionine on frozen/thawed Angora buck (Capra hircus ancryrensis) semen quality, lipid peroxidation and antioxidant enzyme activities, Cryobiology 61 (2010) 89–93.
[38] G. Türk, A. Ateşşahin, M. Sönmez, A. Yüce, A.O. Ceribaşi, Lycopene protects against cyclosporine A-induced testicular toxicity in rats, Theriogenology 67 (2007) 778–785.
[39] J. Twigg, N. Fulton, E. Gomez, D.S. Irvine, R.J. Aitken, Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants, Hum. Reprod. 13 (1998) 1429–1436.
[40] A.A. Van Loon, E. Sonneveld, J. Hoogerbrugge, G.P. Van der Schans, J.A. Grootegoed, P.H.M. Lohman, R.A. Baan, Induction and repair of single strand breaks and DNA base damage at different cellular stages of spermatogenesis of the hamster upon in vitro exposure to ionising radiation, Mutat. Res. 294 (1993) 139–148.
[41] F.F. Verit, A. Verit, A. Kocyigit, H. Ciftci, H. Celik, M. Koksal, No increase in sperm DNA damage and seminal oxidative stress in patients with idiopathic infertility, Arch. Gynecol. Obstet. 274 (2006) 339–344.
[42] P.F. Watson, Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function, Reprod. Fertil. Dev. 7 (1995) 871–891.
[43] H. Woelders, A. Matthijs, B. Engel, Effects of trehalose and sucrose, osmolality of the freezing medium, and chilling rate on viability and intactness of bull sperm after freezing and thawing, Cryobiology 35 (1997) 93–105.
[44] M. Yoshida, Conservation of sperm: current status and new trends, Anim. Reprod. Sci. 60 (2000) 349–355.