Theriogenology 125 (2019) 203–209

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Pomegranate seed in diet, affects sperm parameters of cloned goats following freezing-thawing

Hamid Zarepourfard [a], Ahmad Riasi [a, **], Mohsen Frouzanfar [b], Mehdi Hajian [c], Mohammad Hossein Nasr Esfahani [c, *]

[a] Department of Animal Science, College of Agriculture, Isfahan University of Technology, Isfahan, Iran
[b] Department of Biochemistry, Fars Science and Research Branch, Islamic Azad University, Fars, Iran
[c] Department of Reproductive Biotechnology at Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran

ARTICLE INFO

Article history:
Received 9 June 2018
Received in revised form
28 October 2018
Accepted 29 October 2018
Available online 12 November 2018

Keywords:
Semen cryopreservation
Pomegranate seed
Sperm motility
Cloned goats
Essential fatty acids

ABSTRACT

Semen cryopreservation is affected by individual differences and use of cloned animal from the same source is the main tool to eliminate genetic variation. Pomegranate seed (PS) contains fatty acids and phenolic compounds which have antioxidant properties. Essential fatty acids and antioxidants are vital for production of healthy sperm by improving sperm membrane integrity and protecting sperm from oxidative stress.

In this study, the effect of dietary supplementation of PS on some blood metabolites and sperm parameters following freezing-thawing of semen of cloned goats were investigated. 5–6 years cloned male goats (N = 9, 50 ± 2 kg) were randomly assigned to three different isocaloric and isonitogenous diet groups: supplemented with 0 (control), 30 and 60% (g/100 g of barley) replaced with PS for a total of 9 weeks. Sperm collection was carried out within 10–14 weeks. Semen samples were diluted whit cryoprotectant and frozen in liquid nitrogen. Sperm parameters, reactive oxygen species (ROS) as well as, ability to induce fertilization were evaluated following freezing/thawing.

According to the results of our study, treatment with PS induce higher plasma cholesterol production compared to control group at 8th week. However, testosterone and MDA (malondialdehyde) level of blood plasma were not significantly affected by PS treatment. In comparison to control group, PS supplementation significantly improved total sperm motility and viability in both 30 and 60% PS groups and reduced ROS production. Cleavage rate and developmental competency to blastocyst stage were similar to fresh sperm. In conclusion, dietary supplementation with PS can improve sperm motility and viability following freezing-thawing and maintain developmental competency.

1. Introduction

It is well-accepted that the results of any experiment on animals are inevitably influenced by the genetic and uncontrolled environmental factors [1]. For evaluation of independent factor(s) in animal science, especially in science-based medicine experiments, attempts have been made to control the environmental influences, nevertheless genetic variation remains as a major nuisance factor [2]. One approach that may partly solve this problem is providing a homogenous large sample size. The other approach is the employment of identical twins or use cloned animals from the same donor cell source [3].

One of the most important issues in animal breeding, which has yet to be addressed is investigating the effects of dietary supplements on reproductive outcomes. Artificial insemination (AI) in farm animals offers many benefits in terms of decreasing the risk of sexually transmitted diseases through semen quality control, using superior sires to improve rate of genetic gain, and managing kidding time [4,5]. AI benefits from both fresh/liquid and frozen semen, but its successful conception and economic profit mainly depend on sperm cryopreservation technique [6]. Many efforts have been developed for optimization of semen cryopreservation output aiming to improve fertilization rate [7,8]. However, semen cryopreservation technology hasn't been

* Corresponding author.
** Corresponding author.
E-mail addresses: mh.nasresfahani@royaninstitute.org (A. Riasi), ariasi@cc.iut.ac (M.H. Nasr Esfahani).

https://doi.org/10.1016/j.theriogenology.2018.10.030
0093-691X/

optimized for majority of domestic animals and a variety of cellular injuries may lead to decreasing sperm viability and motility [9,10]. In addition, excessive production of ROS plays a central role in induction of cryo-injury [11]. Evidences suggest that high ROS level can result in infertility through sperm membrane damage and DNA fragmentation [12–14]. Naturally, semen has a high innate antioxidant capacity [15]. However, loss of these antioxidants during sperm processing or their dilution or removal of semen plasma will inevitably increase ROS production. On the other hand, mature spermatozoa have a negligible cytoplasm to store any type of anti-oxidant. Therefore, spermatozoa is highly prone to ROS induce injuries [16].

Spermatozoa are sensitive to lipid peroxidation due to their high content of polyunsaturated fatty acids (PUFAs) [17–19]. On the other hand, the lipid composition of sperm plasma membrane is essential for providing membrane fluidity as well as allowing sperm to undergo capacitation, acrosomal reaction, and sperm-oocyte membrane fusion [20,21]. In vitro supplementation of antioxidants and/or PUFA into semen extenders improves cryo-survival rate of spermatozoa [22]. Recent evidence suggested dietary supplementation with flax-seed oil can significantly improve sperm cryo-survival parameters [23]. Thus, using diets containing both antioxidant and appropriate PUFA may be able to increase sperm fertility attributes and a higher fertilization rate. In this study, we showed that supplementation of PS containing a combination of fatty acids such as punicic acid and antioxidants including flavonoids, polyphenols and vitamin such as tocopherols [24,25] which can improve semen quality and reduce sperm cryo-injury. Using nine cloned Lori-Bakhtyari goats were a noteworthy privilege in this study.

2. Material and methods

2.1. Chemicals

All chemical reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise indicated.

2.2. Pomegranate seed's composition

Pomegranate seed was analyzed according to Kornsteiner et al., 2006 [26]. Fatty acid composition was determined using gas chromatography (Agilent 6890, UK) with a FID (Flame Ionization Detector) and a capillary column (Table 1). The chemical compounds of PS was measured using spectrophotometry based on Capannesi et al., 2000 [27] supplementary table 1.

Table 1
The type and percentage of fatty acids in pomegranate seed.

Fatty acids	%
Myristic acid (C14:0)	0.1
Palmitic acid (C16:0)	6.3
Palmitoleic acid (C16:1 9c)	0.3
Stearic acid (C18 (0))	5.1
Oleic acid (C18:1c c9)	10.8
Conjugated linoleic acid (C18:2 11t,15c)	0.3
Linoleic acid (C18:2 n6)	9.8
Arachidic acid (C20:0)	0.1
Alpha-linolenic (omega 3) (C18:3)	1.1
Gamma-Linolenic acid (C18:3,9c.12c,15c)	64.8
Punicic acid (C18:3 n3 9c,11t,13c)	0.1
Eicosadienoic acid (C20:2)	0.2
Behenic acid (C22:0)	0.8
Erucic acid (C22:1c)	0.3

2.3. Animals and location

This experiment was carried out at the animal farm of Reproductive Biotechnology research center, Royan Institute (Isfahan, Iran latitude 32°39′N). Nine fertile cloned male goats from the same donor cell source [28] with 5–6 years old and mean live weight of 50 ± 2 kg were randomly allotted to three dietary groups, each group with three replicates, and housed in individual pens. The diets were formulated according to the small ruminant nutrition system) SRNS), Cornell University (Version:1,9,4468). The experimental groups were contained 30 and 60% PS. In addition, the diet with no supplements was considered as the control group. For balancing the diet energy, PS were replaced with barley in the treatment groups. The dietary ingredients are shown in supplementary Table 2. The goats were fed with their corresponding diets for a total period of 9 weeks, and the sperm collection was carried out at weeks 10–14 [29–31].

2.4. Blood samples

In all three dietary groups, 15 ml of jugular blood samples were collected in the morning before the starting date of the project week 0, and continued to 4th and 8th weeks following treatment. For serum separation, the collected blood samples were stored for 2 h at room temperature followed by a centrifugation at 1200 rpm for 30 min. The sera were stored at −70 °C for further analysis.

2.5. Biochemical analysis of blood serum

Commercial colorimetric diagnostic kits were used to measure cholesterol (Pars Azmoon Kits; Pars Azmoon, Tehran, Iran) by an automated analyzer (Technicon-RA 1000 Auto analyzer; DRG Instruments GmbH, Marburg, Germany). Testosterone concentration was determined using a commercial RIA kit (Demeditec Diagnostic, GmbH, Germany) by a fully automated ELISA reader (Stat Fax® 3200 Microplate Reader; MIDSCI Co., Palm City, USA). Plasma malondialdehyde (MDA) level was detected using commercial biochemical assay kits produced by Boehringer Mannheim, Germany or Randox Private Ltd., UK. The data was analyzed using an automated biochemical analyzer (ABX Mira, Montpellier, France).

2.6. Extender preparation

The basic extender comprised of 2.7 g Tris, 1.4 g Citric Acid, 10 g fructose; 15% (w/v) egg yolk and 7% (v/v) glycerol was added to a basic extender [32]. The osmotic pressure and pH of the extender were 420 mOsm/kg and 6.8, respectively.

2.7. Semen collection and processing

Semen collection and processing were carried out according to Forouzanfar et al. [33]. In brief, ejaculates were obtained by artificial vagina from the goats twice a week, started from 10 to 14 week which was corresponded to the breeding season (October–November 2016). The collected samples of raw semen from each group were transported to the laboratory at 35 °C. A total of six ejaculates with at least 70% motility, 80% morphologically normal appearance, and >1 ml volume were used for the freezing step. The diluted semen with the extender (1:20 v/v) was cooled to 4 °C for two hours, drawn into the 0.5 ml straws (Biovet, L'Agile, France), hematocrit sealed, and stored at 4 °C for 1 h for more equilibration. The straws were exposed to liquid nitrogen (LN) vapor for 12 min, plunged into LN, and stored in LN until the thawing process for evaluation of sperm parameters. Thawing step was carried out by plunging the straws into a 37 °C water bath for 30 s followed by sperm analysis by a well-trained staff [34].

2.8. Assessment of sperm motility, viability, and ROS production

Evaluation of sperm motility was carried out as previously described by Shafiei [34]. After the thawing, 4–5 straws from each replicate were diluted with fertilization medium (Tyrode's albumin lactate pyruvate medium- Fert-TALP) to final concentration of 1×10^6 spermatozoa/ml. The percentage of sperm with rapid progressive that passed fast in a straight line (class A), slow progressive that move forward but tend to travel in a curved line (class B), and non-progressive that do not move forward despite that they move their tails (class C), as well as the total motility, which refers to the population of sperm that display any type of movements were measured using a computer-assisted sperm analysis (CASA) system (Video Test, ltd: version Sperm 2.1© 1990–2004, Russia) The control of the system is carried out by a calibration test by a neubauer chamber and microscopic ruler. For each sperm sample, 10 μl was placed on a sperm counter (Sperm Processor, Aurangabad, India) and sperm concentration and motility for each sample were analyzed and reported [35].

A hypo-osmotic swelling test (HOST) was used for detecting sperm membrane functionality [36]. Sperm viability was evaluated using a live-dead staining, eosin-nigrosin staining as described previously [37]. All experiments were repeated more than three times.

We used flow cytometer (FAC Scan; Becton Dickinson, San Jose, CA) to determine the ROS content in diluted-thawed semen as described previously [38]. In brief, the samples from each group were centrifuged at 700 rpm for 10 min, the pellet was resuspended in 1 ml of phosphate buffer saline (PBS). The percentage of ROS-positive spermatozoa was measured following incubation of one million sperm/ml with 5 μM of 2′, 7′-Dichlorofluorescin diacetate (DCF-DA) for 30 min at room temperature. Regarding mechanism of ROS reaction with DCF, it is note-worthy that once DCF-DA enters the cell, it loses its ester group and upon interaction with ROS, it produces a fluorescence compound. Live sperm produce a detectable amount of physiological ROS and therefore, the sperm may become DCF positive. In order to differentiate between the physiological amount of ROS and the pathological ROS production, we reduced the concentration of DCF-DA (5 μM) so that only sperm cells which produced extra-physiological concentration of DCF-DA were detectable.

2.9. In vitro maturation (IVM) and fertilization (IVF)

IVM, IVF and embryo culture were performed as described by Forouzanfar et al. [39]. Goat ovaries were recovered at the local slaughterhouse, placed in normal saline (0.9% sodium chloride) at a temperature between 25°Cand 35 °C, and then transported to the laboratory within 2 h. The cumulus–oocyte complexes (COCs) comprised at least 3–4 and above layers of cumulus cells, and oocytes with a uniform cytoplasm and homogenous distribution of lipid droplets in the cytoplasm were recovered by aspiration from follicles of more than 2 mm diameter on the surface of the ovaries and selected for the IVM. The selected COCs were washed three times in the aspiration medium (HEPES-tissue culture media +10% fetal calf serum + 100 IU/ml heparin) and then cultured in maturation medium (tissue culture medium 199 + 10%fetal calf serum + 5 mg/ml FSH + 5 mg/ml LH + 0.1-mMcysteamine) in 5% CO_2 at 39 °C and maximum humidity for 20–22 h.

2.10. Sperm preparation

1. Three straws representing one freezing operation in each replicate were thawed, pooled, and washed through two gradients (40%–80% solutions) of Pure Sperm (Nidacon; Gothenburg, Sweden) to separate motile sperm with normal morphology by centrifugation (700 rpm for 15 min at room temperature). It is important to note that rate of sperm motility after sperm processing was higher than 90% in all samples.
2. Fresh sperm was washed by capacitation solution, then it was put in humidified air condition by 5% of CO_2 at 39 °C for 45 min. In order to separate motile sperm with normal morphology, centrifugation were performed (700 rpm for 15 min at room temperature).

Matured COCs were partially stripped of the cumulus cells, transferred into 100 ml drops of fertilization medium, and fertilized *in vitro* at 39 °C and 5% CO_2, 5% O_2, and 90% N_2 atmosphere. For IVF, sperm at a final concentration of 2×10^6 sperm were incubated with 20–25 matured oocytes/100-ml droplet for 22 h under the same gas atmosphere condition as for IVM. Cumulus cells were removed from presumptive zygotes by vortexing in 2 ml PBS for 1 min. Embryos were transported to modified synthetic oviduct fluid (SOF) medium containing fetal calf serum (FCS) (10% v/v) under mineral oil in a humidified atmosphere of 5% CO_2 and 90% N_2 at 39 °C. Subsequently cleavage and blastocyst rate in each group were assessed on Days 3 and 7 after insemination, respectively and differential staining of blastocysts was assessed on Day 7 after insemination.

2.11. Statistical analysis

The results are reported as the mean ± standard error (SE) for each experiment. The data of each attribute was analyzed using one-way analysis of variance (ANOVA) following the Tukey post-hoc for mean comparisons. P-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Fatty acids content of pomegranate seed (PS)

The results of GC-Mass analysis for PS showed that 87.7% of fatty acid contents in PS were unsaturated fatty acids (Table 1). Among them, gamma-linolenic acid, oleic acid, and linoleic acid constituted 64.8, 10.8, and 9.8% of total fatty acids respectively, while punicic, eicosadienoic, palmitoleic, conjugated linoleic, erucic, and alpha-linolenic acids comprised 2.3% of the total fatty acids.

3.2. Blood serum parameters

Fig. 1 presents the mean value for testosterone, cholesterol, and MDA concentrations in blood plasma of cloned goats at 0, 4th and 8th week of experiment. The results for cholesterol concentration in plasma showed that the treatment groups induce higher cholesterol production compared to the control group at 4th and 8th week (P-value<0.05). However, the testosterone and MDA levels of blood plasma were not significantly affected by the treatment with PS (P > values 0.05).

3.3. Sperm parameters

As shown in Table 2, a significant effect was observed following treatment with PS on sperm motility of cloned goats following freezing-thawing process. The PS supplementation significantly improved total motility in both PS30 and PS60 groups compared to the control group. Using 30% PS significantly improved, the class B motility compared to both control and PS60 groups. This resulted to a higher class A + B motility in this group compared to the other groups. Furthermore, class A + B motility was significantly

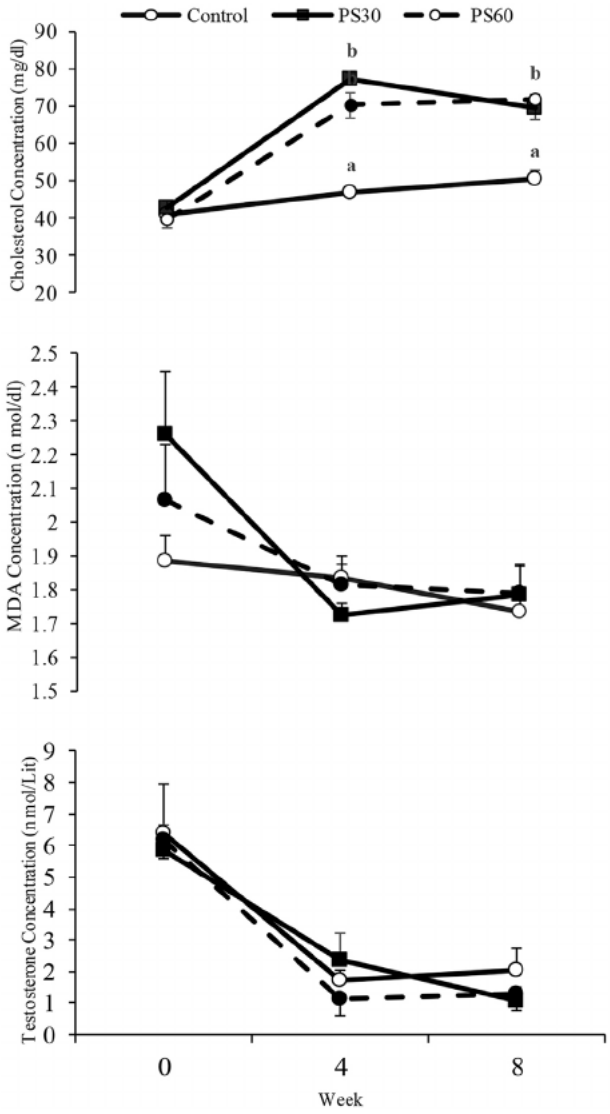

Fig. 1. Testosterone, Cholesterol and MDA of goat's blood fed non supplemented PS (CON), PS30 and PS60.

improved for PS30 and insignificantly for PS60 over control. In addition, supplementation with PS60 increased the class C motility compared to the PS30 and control groups.

Table 3 presents the mean percentage of sperm viability, sperm membrane integrity and DCF-positive sperm for ROS content in semen samples of Lori-Bakhtyari cloned goats following freezing-thawing procedure. Percentages of sperm viability and membrane integrity were significantly higher in both PS supplemented groups compared to the control group, while PS60 group showed the highest means for the aforementioned parameters compared to the other groups. Results for ROS production showed that diet supplemented with PS in both treatment groups significantly reduced the rate of DCF-positive sperm compared to the control group.

The analysis of sperm fertilizing ability and developmental competence showed that there was no significant ($P < 0.05$) difference between the cleavage rate and the developmental competence to reach the blastocyst between the fresh sperm group and PS60 (Fig. 2). The inner cell mass (ICM), trophoblast and total cell number were not significantly different between the two groups (Fig. 3).

4. Discussion

Mammalian semen quality depends on season, breed, genetic background, sire health, age, and most importantly on diet [40–43]. Investigating the general roles of dietary supplements on reproduction performance is of particular interests for artificial reproductive technique (ART) researchers [44,45]. However, genetic and environment cross-talk has been remained as a conflicting factor for food supply experiments. Using cloned animals from the same cell source can remove the breed, genetic value, health, and age as unwanted factors and subsequently provide a more accurate evaluation of dietary treatments [23]. Several *in vitro* experiments have been done to improve sperm quality via addition of antioxidants [22,46], vitamins [47] and fatty acids [48] into semen extenders. Therefore, our experiment was also designed to improve sperm cryopreservation potential/resistance via supplementation with PS.

In this study, initial analysis revealed presence of a high content of unsaturated fatty acids in PS. Therefore, based on background literature [41], we hypothesized that supplementation with PS can increase the PUFA content of sperm membrane or antioxidants content of seminal plasma, it can improve sperm quality following freezing-thawing process. Fig. 1 shows a testosterone decrement from week 0 to week 4 and remained low by week 8 but no significant difference was observed between groups. Unlike testosterone, the values for cholesterol in blood plasma showed a significant increase at weeks 4 and 8 in both treatment groups compared to the control group. Cholesterol can stabilize sperm membranes at low temperatures [49,50]. Sperm cells from species having higher ratio of cholesterol: phospholipid are more resistant to cold shock during freezing-thawing process [51]. Therefore, improving sperm parameters following freezing-thawing may be attributed to higher cholesterol content of sperm membranes. However, further assessment of antioxidant levels and cholesterol level of both sperm and seminal plasma are required to improve this conclusion.

A positive correlation has been reported between total blood level of cholesterol concentration and testes volume [52]. In addition, cholesterol concentration in seminal plasma positively correlated with sperm motility and count [52].

Table 2
Effect of dietary supplementation with 0 (control), 30 and 60% PS, on the sperm motility of Bakhtyari cloned bucks following freezing-thawing.

Group	Post thawing motility (%)± SE				
	Class A	Class B	Class C	Class A + B	total
Control	2.86 ± 0.37	11.33 ± 0.90^{a}	22.70 ± 1.72^{a}	13.97 ± 0.80^{a}	35.32 ± 2.01^{a}
PS30	2.98 ± 0.37	17.13 ± 1.29^{b}	22.61 ± 1.41^{a}	20.21 ± 1.43^{b}	42.82 ± 1.47^{b}
PS60	3.12 ± 0.53	12.17 ± 1.30^{a}	34.31 ± 2.05^{b}	15.3 ± 1.46^{a}	49.60 ± 2.41^{b}

Different superscripts within the same column demonstrate significant differences at $P < 0.05$. Groups contain: Control; without PS, PS30; supplemented with 30% PS and PS60; supplemented with 60% PS.
Sperm motility characteristics in computer - assisted semen analysis (CASA): class A, represents goats sperm with progressive and fast motility, class B, represents goat sperm with progressive but slow motility and class C, represents goat sperm with non-progressive motility.

Table 3
Percentage of sperm viability, intact membrane integrity (HOST +) and DCF-positive (ROS contents) in semen samples of Lori-Bakhtyari cloned goats following freezing-thawing processes.

Group	viability (%) ± SE	Membrane integrity −HOST^{+} (%) ± SE	ROS content(%) ± SE
Control	32.3 ± 0.77^{a}	34.2 ± 0.38^{a}	62.0 ± 1.61^{a}
PS30	40.1 ± 1.28^{b}	40.4 ± 0.85^{b}	38.7 ± 3.76^{b}
PS60	49.4 ± 2.98^{c}	47.6 ± 0.60^{c}	42.8 ± 4.72^{b}

Different superscripts within the same column demonstrate significant differences at $P < 0.05$. Groups contain: Control; without PS, PS30; supplemented with 30% PS and PS60; supplemented with 60% PS.

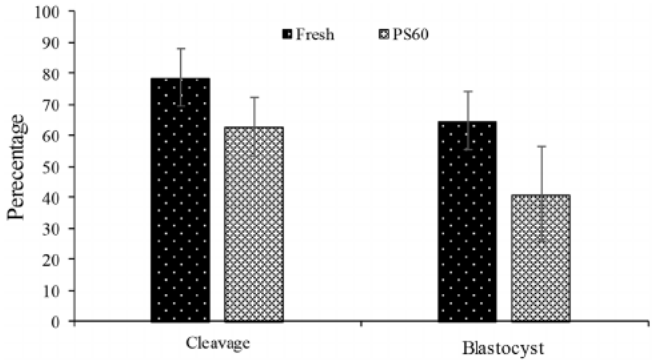

Fig. 2. In vitro development of goat cumulus–oocyte complexes fertilized by frozen-thawed goat semen with supplemented PS in diet. Results are expressed as mean ± standard error of the mean (SEM). Groups contain: Fresh sperm; PS60: sperm freeze-thawed diet supplemented with 60% PS.

Our results showed percentages of sperm total motility, viability and membrane integrity were significantly higher in both PS supplemented groups compared to the control following freezing-thawing process. Furthermore, diet supplemented with PS in both treatment groups significantly reduced the rates of ROS production. ROS which produced *in vivo*, as results of normal cellular respiration or *in vitro* by exogenous sources during cryopreservation process, makes sperm cells susceptible to ROS injuries. It has been reported that, phenolic compound contents of PS have antioxidant properties [53,54], as we expect, another positive effect of PS in goats diet may be attributed to its antioxidant properties. It has been reported, dietary supplementation using different fatty acid sources, water/oil-soluble vitamins and probably minerals, improved the semen quality in bovine [55], horse [56], ram [57] and goat [58]. These improvements are likely to be related to increasing the antioxidant contents of seminal plasma or/and sperm.

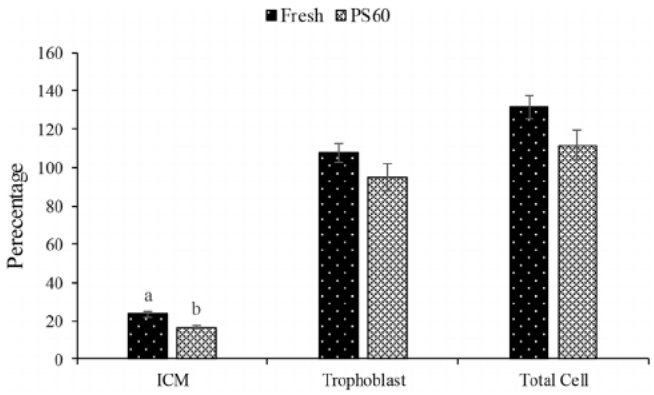

Fig. 3. Result of differential staining Blastocysts Day 7. Results are expressed as mean ± standard error of the mean (SEM). Different superscripts within the same column demonstrate significant differences at $P < 0.05$. Groups contain: Fresh sperm and PS60: sperm freeze-thawed diet supplemented with 60% PS.

Results of chemical analysis showed that linoleic acid and gamma-linolenic acid are the most prevalent constitute of fatty acids in PS. Therefore, improvement of sperm parameters in this study could be attributed to the existence of different fatty acids, although this finding needs further investigation, as we did not measure and compared the constitute of these amino acids before and after treatment and between groups. In accordance with our result, Esmaeili et al. showed dietary supplements including fish oil (n-3 source) improved the quality of ram's spermatozoa compared with dietary n-6 and saturated sources [59].

The ultimate goal of semen cryopreservation is to achieve high fertilization and developmental competency rate. Therefore, based on our previous experience and others knowing that fertilization and developmental competency rate are lower when semen samples were compared to samples that were not treated [60–62], thus we compared the fertilization and developmental competency between fresh semen samples from untreated group as golden standards with PS supplemented cryopreserved groups. The result revealed, despite a small reduction, no significant difference was detected for cleavage rate and ability of cleaved embryos to reach the blastocyst stage. Assessment of quality of blastocyst between the two groups revealed no significant difference in terms of total cell number and trophoblast but the number of cells in ICM were slightly but significantly reduced. Taken together these results suggest PS supplementation improves the quality of semen sample that can well tolerate cryo-stress during freezing and thawing.

5. Conclusion

The combination of reproductive technique along with dietary supply has opened a new avenue for improving *in vivo* sperm capacity for semen cryopreservation, especially for those sires which candidate for. According to the results of our study, dietary supplementation with PS can improve sperm motility, vitality and number of sperm with intact plasma membrane following freezing -thawing. This improvement is likely related to both antioxidant properties and high contents of fatty acids in PS. To solidify this conclusion, the concentrations of both antioxidants and fatty acids need to be determined both in PS and in seminal plasma before and after treatment.

Conflicts of interest

None.

Acknowledgments

This study was supported by the Royan Institute and Isfahan University of Technology. Also the authors would like to express their gratitude to staff of Royan Institute for their full support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at

https://doi.org/10.1016/j.theriogenology.2018.10.030.

References

[1] Liu Y, Webber S, Bowgen K, Schmaltz L, Bradley K, Halvarsson P, et al. Environmental factors influence both abundance and genetic diversity in a widespread bird species. Ecology and evolution 2013;3:4683–95.

[2] Kafkafi N, Benjamini Y, Sakov A, Elmer GI, Golani I. Genotype–environment interactions in mouse behavior: a way out of the problem. Proc Natl Acad Sci U S A 2005;102:4619–24.

[3] Coulon M, Baudoin C, Abdi H, Heyman Y, Deputte B. Social behavior and kin discrimination in a mixed group of cloned and non cloned heifers (Bos taurus). Theriogenology 2010;74:1596–603.

[4] Ax R, Dally M, Didion B, Lenz R, Love C, Varner D, et al. Artificial insemination. Reproduction in farm animals 2000:376–89.

[5] Eaglesome M, Garcia M. Disease risks to animal health from artificial insemination with bovine semen. Revue scientifique et technique-Office international des épizooties 1997;16:215–25.

[6] Wang W, Luo J, Sun S, Xi L, Gao Q, Haile A, et al. The effect of season on spermatozoa motility, plasma membrane and acrosome integrity in fresh and frozen–thawed semen from xinong saanen bucks. Reprod Domest Anim 2015;50:23–8.

[7] Dorado J, Rodriguez I, Hidalgo M. Cryopreservation of goat spermatozoa: comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology 2007;68:168–77.

[8] Lemma A. Effect of cryopreservation on sperm quality and fertility. INTECH Open Access Publisher; 2011.

[9] Barbas J, Mascarenhas R. Cryopreservation of domestic animal sperm cells. Cell Tissue Bank 2009;10:49–62.

[10] Dorado J, Munoz-Serrano A, Hidalgo M. The effect of cryopreservation on goat semen characteristics related to sperm freezability. Anim Reprod Sci 2010;121:115–23.

[11] Gürler H, Malama E, Heppelmann M, Calisici O, Leiding C, Kastelic J, et al. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. Theriogenology 2016;86:562–71.

[12] Baumber J, Ball BA, Linfor JJ, Meyers SA. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J Androl 2003;24:621–8.

[13] Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang J-A, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod 2009;24:2061–70.

[14] Tremellen K. Oxidative stress and male infertility: a clinical perspective. Studies on Men's Health and Fertility. Springer; 2012. p. 325–53.

[15] Sanocka D, Kurpisz M. Reactive oxygen species and sperm cells. Reprod Biol Endocrinol 2004;2:12.

[16] Neild DM, Gadella BM, Chaves MaG, Miragaya MH, Colenbrander B, Agüero A. Membrane changes during different stages of a freeze–thaw protocol for equine semen cryopreservation. Theriogenology 2003;59:1693–705.

[17] Aitken R, Irvine D, Wu F. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. Am J Obstet Gynecol 1991;164:542–51.

[18] Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil 1987;81:459–69.

[19] Mandal R, Badyakar D, Chakrabarty J. Role of membrane lipid fatty acids in sperm cryopreservation. Advances in Andrology 2014;2014.

[20] Meizel S, Turner KO. Stimulation of an exocytotic event, the hamster sperm acrosome reaction, by cis-unsaturated fatty acids. FEBS Lett 1983;161:315–8.

[21] Wassall SR, Stillwell W. Polyunsaturated fatty acid–cholesterol interactions: domain formation in membranes. Biochim Biophys Acta Biomembr 2009;1788:24–32.

[22] Towhidi A, Zeinoaldini S, Ardebili R, Dadashpour Davachi N, Nasiri AH. Combined n-3 fatty acids and î±-tocopherol supplementation improved the ovine sperm cryosurvival. Iran J Biotechnol 2013;11:238–43.

[23] Kargar R, Forouzanfar M, Ghalamkari G, Esfahani MHN. Dietary flax seed oil and/or Vitamin E improve sperm parameters of cloned goats following freezing-thawing. Cryobiology 2017;74:110–4.

[24] Amri Z, Zaouay F, Lazreg-Aref H, Soltana H, Mneri A, Mars M, et al. Phytochemical content, Fatty acids composition and antioxidant potential of different pomegranate parts: comparison between edible and non edible varieties grown in Tunisia. Int J Biol Macromol 2017;104:274–80.

[25] Jing P, Ye T, Shi H, Sheng Y, Slavin M, Gao B, et al. Antioxidant properties and phytochemical composition of China-grown pomegranate seeds. Food Chem 2012;132:1457–64.

[26] Kornsteiner M, Wagner K-H, Elmadfa I. Tocopherols and total phenolics in 10 different nut types. Food Chem 2006;98:381–7.

[27] Capannesi C, Palchetti I, Mascini M, Parenti A. Electrochemical sensor and biosensor for polyphenols detection in olive oils. Food Chem 2000;71:553–62.

[28] Nasr-Esfahani M, Hosseini S, Hajian M, Forouzanfar M, Ostadhosseini S, Abedi P, et al. Development of an optimized zona-free method of somatic cell nuclear transfer in the goat. Cellular Reprogramming (Formerly" Cloning and Stem Cells"), vol. 13; 2011. p. 157–70.

[29] França L, Becker-Silva S, Chiarini-Garcia H. The length of the cycle of seminiferous epithelium in goats (Capra hircus). Tissue Cell 1999;31:274–80.

[30] Júnior AM, Oliveira L, Neto AA, Alves F, Miglino MA, Carvalho M. Spermatogenesis in goats with and without scrotum bipartition. Anim Reprod Sci 2012;130:42–50.

[31] Leal M, Becker-Silva S, Chiarini-Garcia H, França L. Sertoli cell efficiency and daily sperm production in goats (Capra hircus). Anim Reprod 2004;1:122–8.

[32] Forouzanfar M, Sharafi M, Hosseini S, Ostadhosseini S, Hajian M, Hosseini L, et al. In vitro comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen. Theriogenology 2010;73:480–7.

[33] Forouzanfar M, Ershad SF, Hosseini SM, Hajian M, Ostad-Hosseini S, Abid A, et al. Can permeable super oxide dismutase mimetic agents improve the quality of frozen–thawed ram semen? Cryobiology 2013;66:126–30.

[34] Shafiei M, Forouzanfar M, Hosseini SM, Esfahani MHN. The effect of superoxide dismutase mimetic and catalase on the quality of postthawed goat semen. Theriogenology 2015;83:1321–7.

[35] Shi L, Ren Y, Zhou H, Hou G, Xun W, Yue W, et al. Effect of rapid freezing–thawing techniques on the sperm parameters and ultrastructure of Chinese Taihang black goat spermatozoa. Micron 2014;57:6–12.

[36] Fonseca J, Torres C, Maffili V, Borges A, Santos A, Rodrigues M, et al. The hypoosmotic swelling test in fresh goat spermatozoa. Anim Reprod 2005;2:139–44.

[37] Sharafi M, Zhandi M, Shahverdi A, Shakeri M. Beneficial effects of nitric oxide induced mild oxidative stress on post-thawed bull semen quality. International journal of fertility & sterility 2015;9:230.

[38] Kiani-Esfahani A, Tavalaee M, Deemeh MR, Hamiditabar M, Nasr-Esfahani MH. DHR123: an alternative probe for assessment of ROS in human spermatozoa. Syst Biol Reprod Med 2012;58:168–74.

[39] Forouzanfar MAA, Hosseini SM, Hajian M, Nasr Esfahani MH. Supplementation of sperm cryopreservation media with cell permeable superoxide dismutase mimetic agent (MnTE) improves goat blastocyst formation. Cryobiology 2013;67:394–7.

[40] Brito L, Silva A, Rodrigues L, Vieira F, Deragon L, Kastelic J. Effect of age and genetic group on characteristics of the scrotum, testes and testicular vascular cones, and on sperm production and semen quality in AI bulls in Brazil. Theriogenology 2002;58:1175–86.

[41] Esmaeili V, Shahverdi A, Moghadasian M, Alizadeh A. Dietary fatty acids affect semen quality: a review. Andrology 2015;3:450–61.

[42] Snoj T, Kobal S, Majdic G. Effects of season, age, and breed on semen characteristics in different Bos taurus breeds in a 31-year retrospective study. Theriogenology 2013;79:847–52.

[43] Yunsang C, Wanxi Y. Functions of essential nutrition for high quality spermatogenesis. Adv Biosci Biotechnol 2011;2011.

[44] Kermack AJ, Macklon N. Nutritional supplementation and artificial reproductive technique (ART) outcomes. Reprod Fertil Dev 2015;27:677–83.

[45] Rassu SPG, Enne G, Ligios S, Molle G. Nutrition and reproduction. Dairy sheep nutrition 2004:109.

[46] Bucak MN, Sarıozkan S, Tuncer PB, Sakin F, Ateşşahin A, Kulaksız R, et al. The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. Small Rumin Res 2010;89:24–30.

[47] Hu J-H, Tian W-Q, Zhao X-L, Zan L-S, Wang H, Li Q-W, et al. The cryoprotective effects of ascorbic acid supplementation on bovine semen quality. Anim Reprod Sci 2010;121:72–7.

[48] Nasiri A, Towhidi A, Zeinoaldini S. Combined effect of DHA and α-tocopherol supplementation during bull semen cryopreservation on sperm characteristics and fatty acid composition. Andrologia 2012;44:550–5.

[49] Moore AI, Squires EL, Graham JK. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology 2005;51:241–9.

[50] Oliveira CHd, Vasconcelos ABd, Souza FA, Martins-Filho OA, Silva MX, Varago FC, et al. Cholesterol addition protects membrane intactness during cryopreservation of stallion sperm. Anim Reprod Sci 2010;118:194–200.

[51] Moce E, Blanch E, Tomás C, Graham J. Use of cholesterol in sperm cryopreservation: present moment and perspectives to future. Reprod Domest Anim 2010;45:57–66.

[52] Jacyno E, Kołodziej A, Kawęcka M, Pietruszka A, Matysiak B, Kamyczek M. The relationship between blood serum and seminal plasma cholesterol content in young boars and their semen qualitative traits and testes size. Arch Tierz 2009;52:161–8.

[53] Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agric Food Chem 2000;48:4581–9.

[54] Seeram NP, Aviram M, Zhang Y, Henning SM, Feng L, Dreher M, et al. Comparison of antioxidant potency of commonly consumed polyphenol-rich beverages in the United States. J Agric Food Chem 2008;56:1415–22.

[55] Gholami H, Chamani M, Towhidi A, Fazeli MH. Improvement of semen quality in holstein bulls during heat stress by dietary supplementation of omega-3 fatty acids. Int J Fertil Steril 2011;4.

[56] Schmid-Lausigk Y, Aurich C. Influences of a diet supplemented with linseed oil and antioxidants on quality of equine semen after cooling and cryopreservation during winter. Theriogenology 2014;81:966–73.

[57] Fair S, Doyle D, Diskin M, Hennessy A, Kenny D. The effect of dietary n-3 polyunsaturated fatty acids supplementation of rams on semen quality and subsequent quality of liquid stored semen. Theriogenology 2014;81:210–9.

[58] Dolatpanah M, Towhidi A, Farshad A, Rashidi A, Rezayazdi A. Effects of dietary fish oil on semen quality of goats. AJAS (Asian-Australas J Anim Sci) 2008;21:29.
[59] Esmaeili V, Shahverdi A, Alizadeh A, Alipour H, Chehrazi M. Saturated, omega-6 and omega-3 dietary fatty acid effects on the characteristics of fresh, frozen–thawed semen and blood parameters in rams. Andrologia 2014;46:42–9.
[60] Coşkun N, Karaca F. Effects of cryopreservation on sperm quality and fertility. Lalahan Hayvancilik Arastirma Enstitüsü Dergisi 2016;56:32–9.
[61] Romão R, Marques C, Baptista M, Vasques MI, Barbas JP, Horta A, et al. Evaluation of two methods of in vitro production of ovine embryos using fresh or cryopreserved semen. Small Rumin Res 2013;110:36–41.
[62] Watson P. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60:481–92.