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Dietary flax seed oil and/or Vitamin E improve sperm parameters of cloned goats following freezing-thawing

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ABSTRACT

Semen cryopreservation is affected by individual differences and use of clones animal from the same source is the main tool to eliminate genetic variation. Among many nutrients that are necessary for fertility, essential fatty acids and antioxidants are vital for production of healthy sperm by improving sperm membrane integrity and protecting sperm from oxidative stress. The goal of the current study was to investigate whether a flax seed oil or/and Vitamin E dietary supplementation could improve semen quality of cloned bucks following semen cryopreservation. Accordingly, eight adult cloned Bakhtiari bucks were divided randomly into four groups. Bucks were offered a base diet of hay and concentrate. The concentrate was enriched with flax seed oil, 30 gr/kg body weight/day (OIL), Vitamin E (VIT), 3 gr/kg body weight/day, or combined flax seed oil and the vitamin E (OIL-VIT). The concentrate with no supplements was considered as control group (CONT). Both flax seed oil and Vitamin E supplements were added to the total diet. The bucks were fed with their corresponding diets for a total of 9 weeks while sperm collection was carried out within 10–14 weeks. Ejaculates were diluted with Andromed® and were frozen in liquid nitrogen. Sperm parameters and reactive oxygen species (ROS) contents were evaluated following freezing/thawing. According to the results of our study, dietary supplementation with flax seed oil, or/and Vitamin E can improve sperm motility, vitality and number of sperm with intact plasma membrane following freezing-thawing. But the degree of improvement in these parameters was significantly higher when Flax seed oil and vitamin E were co-supplemented.

1. Introduction

Intra-class variances due to differences in genetic and environmental factors are unavoidable nuisance factors that can substantially affect results of any experiment on animals. Even though this problem has been theoretically solved by providing a large homogenous sample size, this intra-class discrepancy has remained the main problem for evaluation of independent factors in animal science and medical experiments. Use of identical twins or high number of clones from the same sources was suggested as the main tool to eliminate the genetic source of individual differences [4].

Artificial insemination (AI) in goat is a powerful tool that can allow breeders to use superior bucks and subsequently to improve rate of genetic gain, decreasing risk of sexually transmitted diseases and accuracy of time of kidding [5,6]. Although a doe can be inseminated with fresh semen, success of AI mainly depends on sperm cryopreservation technique [7]. A variety of injuries occur at cellular and molecular levels during semen cryopreservation which may impair sperm function and fertilization potential when compared to fresh semen. Reduction in sperm fertilization potential during semen cryopreservation can be partly accounted by excessive production of reactive oxygen species (ROS) which plays a central role in induction of cryo-injury through oxidative stress [8–10]. Like other cells, sperm plasma membrane is made of a phospholipid bilayer which contains large amounts of polyunsaturated fatty acids (PUFA). This lipid composition naturally

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undergoes a series of modifications in sperm head during capacitation and acrosome reaction [11,12]. This provides sperm membrane fluidity which is needed for committing membrane fusion event during fertilization process [13]. However, the sperm PUFAs are extremely vulnerable to oxidative damages which are mainly generated by ROS [14]. To overcome this problem, antioxidants are added in semen extenders during semen cryopreservation of most of farm animal species [15–17]. On the other hand, semen quality depends on season, breed, genetic value of buck, buck health, age, and most importantly the diet. It seems that feeding specific supplements can improve semen quality by increasing sperm quantity, motility, viability as well as antioxidants capacity in cellular and seminal plasma. Among many nutrients that are necessary for fertility, essential fatty acids are vital for production of healthy sperm by improving sperm membrane structure and protecting sperm from oxidative stress [18–20].

With regard to effect of individual variations on sperm freezing-thawing outcome, we have a great opportunity of having nine cloned bucks, which were produced by somatic cell nuclear transfer (SCNT) from the same cell line, to test different hypothesis while eliminating genetic source of variation [20]. The aim of the present study was to evaluate the effect of dietary supplementation of flax seed oil as a PUFA, or/and Vitamin E as an antioxidant, on sperm parameters of cloned bucks following freezing-thawing.

2. Material and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma Chemical CO. (St. Louis, MO, USA) unless stated otherwise. Andromed® as semen extender was obtained from Minitube, Germany.

2.2. Animals

This experiment was conducted at the animal farm of Reproductive Biotechnology research center at Royan Institute (Isfahan, Iran latitude 32°39′N). Eight adult cloned Bakhtiari bucks were randomly selected from nine cloned bucks aged 2–3 years, and then were divided randomly into four groups (two per group).

Bucks were offered a base diet according to small ruminant nutrition system(SRNS) Cornell University (Version:1,9,4468). Treatments groups were contained flax seed oil, 30 gr/kg body weight/day (OIL), Vitamin E (VIT), 3 gr/kg body weight/day, or combined flax seed oil and vitamin E (OIL-VIT). In addition, the concentrate with no supplements was considered as the control group (CONT). Both flax seed oil and Vitamin E supplements were added to basic diet. Diets were formulated as percent of Dry Matter (100 ± 5). Control group received a basic diet (Alfalfa; 22.4, Straw; 44.9, Barley; 7.67, Corn; 22.7, Salt; 1.1, Mineral supplements; 0.55, Vitamin supplements; 0.55, Mono Calcium Phosphate; 0.14,, Calcium; 0.42 and potassium 0.21% of dry matter containing 7.07% Crud protein and 1.74 mcal metabolism energy), whereas, VIT group received basic diet supplemented with vitamin E, 3 gr/kg body weight/day contain 1.74 mcal metabolism energy, OIL group received basic diet supplemented with flax seed oil, 30 gr/kg body weight/day, containing 1.91 mcal metabolism energy and OIL-VIT group received basic diet supplemented with both vitamin E and flax seed oil, 3 and 30 gr/kg body weight/day, respectively containing 1.91 mcal metabolism energy.

The bucks were fed with their corresponding diets for a total of 9 weeks while the sperm collection was carried out within 10–14 weeks.

2.3. Semen collection, processing, and freezing-thawing

Semen collection and processing were carried out according to Forouzanfar et al. [17]. In brief, totally 10 ejaculates were obtained by artificial vagina from the bucks twice a week, started from week-10 to week-14, which was corresponded to the breeding season (April/May, 2015). The collected samples of raw semen from each cloned bucks were kept separate and transported at 35 °C for approximately 30 min to the laboratory for microscopic evaluations. A total of 6 from 10 ejaculates which showed at least 70% motile, 80% morphologically normal sperm and 1–2 ml volume in each treatments and control groups were mixed and used for further steps. For freezing, all of the treatments were repeated for at least 6 times with the mixed semen samples from the two bucks in each group. Each semen sample was diluted to final concentration of 400 × 106 spermatozoa/ml (200 × 106 spermatozoa per straw) with Andromed® extender, cooled to 4 °C over a period of 2 h, drawn into 0.5 ml French straws (Biovet, L'Agile France), and heat-sealed. Then the straws were kept at 4 °C for one hour followed by exposure to liquid nitrogen (LN2) vapor for 12 min, plunged into LN2, and stored in LN2 until being thawed, and used for evaluation of sperm parameters. Thawing step was carried out by plunging the straws in a 37 °C water bath for 30 s. The thawed samples were kept at room temperature and individually evaluated by a single trained individual.

2.4. Measurement of sperm motility after freezing-thawing process

Measurement of sperm motility was carried out according to Shafiei [14]. After thawing, 4–5 straws from each replicate were diluted with fertilization medium (Tyrode's albumin lactate pyruvate medium- Fert-TALP) to final concentration of 1×10^6spermatozoa/ml. The percentages of rapid progressive that swim 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 movement were assessed using a computer-assisted sperm analysis (CASA) system (Video Test, ltd: version Sperm 2.1© 1990-2004, Russia) [21]. For each sample, 5 μl of sperm suspension was loaded on a pre-warmed slide and then covered by an 18 × 18 mm coverslip, a minimum of 500 sperm per sample were analyzed in at least three different microscopic fields.

2.5. Assessment of sperm plasma membrane integrity

Sperm plasma membrane integrity was estimated by the hypo-osmotic swelling test (HOST) based on curled and swollen tails [22]. Following 30 min incubation of 25 μl sperm suspension and 200 μl of HOST solution (100 mOsm/l, 57.6 mM fructose and 19.2 mM sodium citrate) at room temperature, 5 μl of homogenized mixture was mounted and directly investigated using an inverted microscope (Olympus, CKX41 - Japan). The percentage of intact-membrane spermatozoa was estimated by recording of at least 200 spermatozoa in more than five different microscopic fields.

2.6. Assessment of sperm viability

Sperm viability was evaluated using the eosin-nigrosin staining following the thawing process as described previously [15]. In brief, 50 μl of diluted semen was mixed with 100 μl of eosin (1%) in a test tube for 30 s and stained with nigrosin (10%) for 30 s. One drop of stained semen was loaded on a slide and a semen smear prepared. The smears were air dried and examined directly. At least 200

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sperm were assessed at a magnification of 1000× under oil immersion using light microscope. Sperm head that were unstained were considered as “live sperm” while sperm with pink or red coloration in the head region were considered as “dead sperm”.

2.7. Assessment of oxidative stress in sperm using flow cytometer

We used flow cytometer (FAC Scan; Becton Dickinson, San Jose, CA) to determine the ROS content in thawed and diluted semen as described previously [23]. In brief, samples from each group were centrifuged at 700 g for 10 min, and pellet was resuspended in 1 ml of phosphate buffer saline (PBS). The percentage of ROS-positive spermatozoa was measured following incubating 1×10^6 sperm/ml with 5 μM of 2′, 7′-Dichlorofluorescin diacetate (DCF-DA) for 30 min at room temperature. Regarding the mechanism of ROS reaction with DCF, it is note-worthy that once DCF-DA enters a 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, sperm become DCF positive. In order to differentiate between low or high ROS production, we reduced the concentration of DCF-DA to 5 μM, so that only sperm cells which produced high concentration of DCF-DA were detectable.

2.8. Statistical analysis

The results are reported as the mean ± standard error (SE) for each experiment. The analysis of variance (ANOVA) was used for treatments comparisons. One-way ANOVA with the Tukey multiple comparison post-hoc test was used to compare the different stimuli. $P \leq 0.05$ was considered statistically *significant*.

3. Results

Table 1 shows dietary supplementation with flax seed oil and/or Vitamin E on sperm motility of Bakhtiari cloned bucks following freezing-thawing process. Supplementation with flax seed oil and/or Vitamin E significantly improved total motility. Furthermore, class A + B motility was significantly improved with co-supplementation with flax seed oil and Vitamin E (OIL-VIT) over control and other treatment groups.

Table 2 presents the percentage of sperm viability, sperm membrane integrity and DCF-positive sperm in semen samples of Bakhtiari cloned bucks following freezing-thawing procedure. Percentages of sperm viability were significantly higher in the treated compared to control bucks. However, combined application of OIL and VIT, only significantly improved the viability compared to VIT group. Similarly, supplementation of the flax seed oil and/or Vitamin E led to a significantly higher percentage of sperm with intact membrane ($HOST^+$) compared to the control group ($P \leq 0.05$). Additionally, sperm in the OIL-VIT treatment presented a higher percentage of sperm with intact membrane compared to the VIT group ($P \leq 0.05$), while the difference was not significant for the OIL group.

For ROS production, the highest percentage of DCF positive sperm was observed in the control cloned goats compared to all of the treatment groups ($P \leq 0.05$) although no significant difference was observed among the treatment groups.

4. Discussion

Investigating the general roles of dietary supplements on reproduction performance is of particular interests for assisted reproductive technologies (ART). However, genetic variation as an inevitable annoyance factors, can substantially affect the results of any experiment. The great advantage of using cloned animals for evaluation of dietary treatments may attenuate this unavoidable factor. So far, several in vitro experiments, by addition of antioxidants, vitamins, fish oil, and cholesterol, into the semen diluents/extenders, have been carried out to increase sperm cryopreservation competency. It has already been accepted that diet can directly or indirectly influence male fertility. However, in our experiment we designed an in vivo study to change sperm structural constitute using dietary supplementation aiming to increasing the sperm cryopreservation potential/resistance. For this reason, we have coined the “in vivo sperm engineering” which referred to any in vivo manipulation of sperm/semen using diet supplementation to modify structure and content of spermatozoa and ejaculates, respectively.

The results of present study indicated that overall sperm

Table 1
Effect of dietary supplementation with flax seed oil, or/and Vitamin E, on the sperm motility of Bakhtiari cloned goats following freezing-thawing.

Post thawing motility (%) ± SE					
Group	Class A	Class B	Class C	Class A + B	Total
CONT	5.34 ± 0.62^{ab}	13.73 ± 0.83^{a}	15.82 ± 1.41^{a}	18.8 ± 1.07^{a}	34.57 ± 1.21^{a}
VIT	5.54 ± 0.41^{ab}	18.35 ± 0.91^{a}	22.28 ± 2.41^{b}	23.89 ± 1.15^{b}	46.18 ± 2.82^{b}
OIL	4.2 ± 0.45^{a}	18.6 ± 0.64^{ab}	24.86 ± 1.32^{b}	22.9 ± 0.86^{ab}	47.78 ± 1.52^{b}
OIL-VIT	7.36 ± 0.74^{b}	22.1 ± 1.25^{b}	20.43 ± 1.31^{ab}	29.45 ± 1.45^{c}	49.89 ± 2.12^{b}

Different superscripts within the same column demonstrate significant differences at $P < 0.05$. Sperm motility characteristics in computer - assisted semen analysis (CASA): class A, represents bucks 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. Groups contains: Control; No feed supplemtation, VIT; feed supplemented with vitamin E 3 gr/kg body weight/day and OIL; feed supplemented with flax seed oil 30 gr/kg body weight/day, and OIL-VIT; feed supplemented with vitamin E and flax seed oil,3 and 30 gr/kg body weight/day respectively.

Table 2
Percentage of sperm viability, intact membrane integrity (HOST +) and DCF-positive (ROS contents) in semen samples of Bakhtiari cloned bucks in post thawing conditions.

Group	Viability (%) ± SE	Membrane integrity−$HOST^+$ (%) ± SE	ROS content (%) ± SE
CONT	36.04 ± 1.07^{a}	38.39 ± 1.22^{a}	60.6 ± 2.96^{b}
VIT	42.14 ± 1.3^{b}	45.68 ± 1.17^{b}	42.2 ± 2.49^{a}
OIL	45.21 ± 1.15^{bc}	47.97 ± 1.28^{bc}	34.9 ± 3.03^{a}
OIL-VIT	48.59 ± 1.09^{c}	50.91 ± 1.83^{c}	36.5 ± 5.55^{a}

Different superscripts within the same column demonstrate significant differences at $P < 0.05$.Groups contains: Control; No feed supplemtation, VIT; feed supplemented with vitamin E 30 gr/kg body weight/day and OIL; feed supplemented with flax seed oil 30 gr/kg body weight/day, and OIL-VIT; feed supplemented with vitamin E and flax seed oil, 30 and 3 gr/kg body weight/day respectively.

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motility, viability, and membrane integrity were improved in the cloned goats which received vitamin E and/or flax seed oil compared to those identical peers which did not receive any supplements. In addition, co-supplementation of vitamin E and flax seed oil showed strong synergistic effects on sperm motility, viability and membrane integrity. It has been evident that membranes of sperm cell consist of a high concentration of polyunsaturated of fatty acids (PUFA) [24] and for this reason susceptible to oxidative stress [25], especially during the process of freezing and thawing [26]. This lipid composition of sperm membranes, which is considered as important structural and functional component, may undergo a series of modifications during capacitation and acrosome reaction events [27]. Essential fatty acids are vital for production of healthy sperm and their inadequate intake associated with the production of abnormal sperm [28,29]. Supporting our data, it has been fully documented that PUFAs contributes to sperm membrane fluidity and flexibility by altering the lipid composition of spermatozoa and seminal plasma [30,31]. Moreover, it has been shown that dietary fatty acid sources affected PUFAs composition of the ram's spermatozoa [32,33]. On the other hand, it has been shown that spermatozoa from different species exhibit different resistance to the cryo-injury process [34]. Considering this issue, the presence of higher fatty acid content in sperm membrane resulted in higher sperm membrane flexibility and cryosurvival [35]. Therefore, the observed improvement in sperm quality after freeze-thawing in the current study can be attributed to improvement in sperm membrane quality.

It has been well-documented that low concentration of ROS is essential for sperm cells to acquire fertilizing capability [1] while high concentrations of ROS in spermatozoa can be detrimental for sperm motility, viability, and DNA integrity [2]. On the other side, it has been reported that low antioxidant content of seminal plasma also decreased sperm motility, viability, and normal morphology [28,29]. Vitamin E, which presents in mammalian cell membranes and seminal plasma, act as primary ROS scavenger and subsequently attenuator of oxidative stress [3]. Implementation of synthetic analogue of vitamin E also substantially preserved membrane ultrastructure of spermatozoa and increased intact plasma and mitochondria membranes [36]. Post-thawing sperm parameters were improved when methionine, cysteamine and buthylated hydroxytoluene (BHT) were supplemented in the sperm cryopreservation medium [37]. However, it has yet been determined the exact interrelations of oxidants and antioxidants in seminal plasma of domestic animal [36].

The results of present study suggested that ROS content of sperm diluents was significantly higher in the control group compared to treatments groups after the freeze-thawing process. Although the exact mechanism by which feed supplementation of flaxseed oil and vitamin E upgraded goat sperm parameters after freeze-thawing is yet to be elucidated, decreasing semen ROS content while improving PUFA content of sperm membranes can subsequent to increasing sperm membrane resistance to cryoinjuries and finally amending sperm motility, viability, and integrity parameters (21). However, further experiments could be designed to modify the lipid composition or/and antioxidant capacity of sperm or semen to make it more resistant to the cryopreservation process. Indeed, sperm fatty acid composition has this potential to be used as an index to predict sperm capacity for cryopreservation.

This study benefited from eight cloned bucks which were produced by somatic cell nuclear transfer (SCNT) technology. The cloned animals are generally believed to have no genetic variation, although it has been reported that cloned animals through SCNT inherit their mitochondria exclusively from the oocyte recipient [38]. Nonetheless, we cannot rule out the importance of mitochondrial variations, which are maternally inherited, among the cloned bucks on sperm parameters and seminal plasma.

5. Conclusion

According to the results of our study, dietary supplementation with flax seed oil, or/and Vitamin E can improve sperm motility, vitality and number of sperm intact plasma membrane following freezing -thawing. This means that flax seed oil, or/and Vitamin E in diet are important factors for successful semen cryopreservation. The results of this study provided an alternate pathway for improving the sperm attributes by using the in vivo sperm engineering technology. Moreover, this approach can be used to improving the sperm quality of sires from other mammalians which are experimentally or economically important.

Statement of funding

There is no funding for the present study.

Conflict of interest

There is no conflict of interest to declare.

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