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Evaluation of the effects of cysteine and trehalose on long-term cryopreservation of ram semen

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ABSTRACT

The sheep artificial insemination with frozen-thawed semen results in a low fertility rate because cryopreservation of ram semen is difficult. The objective of this study was to cryopreserve ram semen with cysteine and trehalose for a long time. This study carried out at the Agricultural Research Station of Tabriz University in the breeding season for 3 months. Semen collected from 4 Ghezel (redish-brown) rams using artificial vagina. Samples immediately transported to the laboratory and after a primary evaluation in 37°C all samples were pooled. Semen samples diluted (1:4) with a Tris-based extender containing additives including 50 mM trehalose (T), 4 mM cysteine (Cys), 50 mM trehalose plus 4 mM cysteine (Cys+T) and an extender containing no additives (control), then cooled to 5°C and frozen in 0.25 ml French straws, and finally stored in liquid nitrogen. The frozen straws were individually thawed in 0, 15, 30, 45, 60, 75 and 90 days for evaluation. The results showed that adding cysteine and trehalose (cysteine+trehalose) significantly (P<0/05) improved frozen-thawed semen characteristics. The highest sperm motility, progressive motility and viability belonged to Cys+T and the lowest to control group. Abnormal sperms in cysteine were lower than the control group (P <0/05). This study showed that the combination of trehalose and cysteine improves long-term cryopreservation of ram semen.

Keywords: artificial insemination, cryopreservation, cysteine, trehalose.

INTRODUCTION

The aim of storage of semen is to prolong the fertilizing capacity of spermatozoa by reducing or detaining their motility and metabolic reaction (Evans and Maxwell, 1987)**.** The main changes that occurred during storage included reduction in motility and morphological integrity of spermatozoa. These changes may be contributed to the accumulation of the toxic products of metabolism, mainly of reactive oxygen species (ROS) formed through lipid peroxidation of the membranes of spermatozoa (Salamon and Maxwell, 2000). Improvement of the extender is necessary because the main injury to sperm occurs during dilution and cooling (Tasseron et al., 1977). Factors affecting the proportion of survivors are: cold shock susceptibility, cooling rate, diluent composition and osmotic stress. Factors influencing functional status of survivors include: membrane stability, oxidative damage, membrane receptor integrity and nuclear structure (Watson, 2000). It has been suggested that intracellular ice formation in the sperm cell is one of the detrimental factors that reduces the viability and membrane integrity of frozen-thawed sperms (Jafaroghli et al., 2011). The cryosurvival of spermatozoa increased significantly in hypertonic diluents compared with that in isotonic diluents, regardless of their composition (Fiser et al., 1982). Sugars have several functions in sperm extenders, including providing energy substrate for the sperm cell during incubation, maintaining the osmotic pressure of the diluents, acting as a cryoprotectant, and decreasing the extent of cell injury by reducing the intracellular ice formation (Ahmad and Aksoy, 2012). Trehalose (a non-reducing and non-permanent disaccharide in which tow glucose molecules are linked together in a 1,1-glucosidic linkage (α-d-glucopyranosyl 1-1, 1-α-d-glucopyranoside), commonly found in high concentration in many organisms such as yeast and fungal spores) has a protective action related both to osmotic effect and specific interactions with membrane phospholipids, rendering hypertonic media, causing cellular osmotic dehydration before freezing, and decreasing the amount of cell injury by ice crystallization (Badr et al., 2010; Bucak et al., 2007). The section of trehalose appears to be associated with its ability to replace water at the membrane-solution interface (Aisen et al., 2002). Antioxidative and cryoprotective effects of

additives such as cysteine, glutamine, taurine and trehalose, which improve sperm function, such as motility, membrane integrity, endogenous antioxidant activities and fertilizing ability, have been demonstrated in various species (Bucak et al., 2013). Cysteine and ergotinine play an important role in maintaining metabolic functions and cell motility of sperm (Aisen et al., 2000). Cysteine is a low molecular weight amino acid containing thiol; which has antioxidant capacity, penetrates the cell membrane easily, stimulates glutathione synthesis and thus enhancing the maintenance of intracellular glutathione levels and scavenging ROS. Additionally, cysteine has been shown to prevent loss of sperm functions during sperm liquid storage or in the frozen state (Sarıözkan et al., 2009; Tuncer et al., 2010; Uysal and Bucak, 2007). Membrane integrity requires an adequate level of reduced glutathione (GSH) to remove H_2O_2 and organic hydroperoxides produced by ROS. The addition of antioxidants to freezing media has been proposed to prevent some of these events (Aisen et al., 2005). Cysteine has been shown to improve the post-thawed motility and morphology of bull, ram and goat sperm, and also to maintain the viability, chromatin structure, and membrane integrity of boar sperm during liquid storage at 5 ºC (Sarıözkan et al., 2014).There was little knowledge on reproductive characteristics of Ghezel rams particularly semen cryoperesevtion. Thus, the objective of this study was cryopreservation of ram semen with cysteine and trehalose for 3 months., early cultivars with high economic seed yield. They added that it is difficult to select an ideal cultivar in most crop species when acceptance is conditioned by several traits.

In general, selection indices provide a useful method for quantifying selection potential as well as providing a good chance for more efficient selection. For more accurate calculations and explanation of the results, correlated response between a primary and a secondary trait can be exploited to increase the expression of the primary trait when the selection of the secondary trait produces greater genetic gain than the direct selection of the primary trait (Hallauer *et al*., 2010). Consequently, direct selection for a primary trait, such as seed yield or oil in sesame, is not sufficient to generate sesame genotypes useful as commercial cultivars.

The application of the selection index to improve the lines of sesame was rare. Although most researcher in the developed world made benefit from the restricted selection index, research on sesame in developing countries like Korea and Egypt used only the conventional selection index.

Lee and Chang (1986) in Korea, practiced conventional selection index to 14 traits including yield plant^{-1} using 82 cultivars. He stated that the highest genetic advance was for index that included all traits. However, for reasons of expensive and time consumed. He suggested only 3 characters to be included in the index (days to maturity, length of stem with capsules and capsules plant^{-1}) for future selection indices.

In Egypt, El-Shimy (1995) used 9 conventional selection indices and reported that after two cycles of selection improved yield plant^{-1} by 46.34% of the overall mean of the selected families from the better parent. Samar *et al.* (2002) applied only one conventional index and compared it to direct selection for yield plant^{-1}. They found that the selection index improved seed yield by 16.1% of F_5-derived families.

Most recently, Hidalgo-Countreras (2014) in Nebraska, USA used a multi–trait genomic selection index. He used the principle component analysis via 250 principle components which, in his opinion, explained approximately 99% of the total variability. His method could represent a new era in the use of selection indices to increase selection efficiency in self – pollinated crops.

In view of the above, the objectives of the present study were: i) To generate new forms of sesame cultivars having high seed yield potential and one or more desired by using either conventinoal or restricted selection indices in advanced breeding generations ; ii) Estimation of variances and covariances analysis for each variable and /or each pair of variables to caculate phenotypic (r_p) and genotypic (r_g) correlation coefficients and efficiency of each selection index compared to direct selection for yield; iii) Estimation of heritability percentages, expected and actual genetic advances when selecting for a single trait and expected advance from conventional and restricted selection indices to detect the optimum selection procedures.

MATERIALS AND METHODS

2.1. Animals and location

This study carried out at the Agricultural Research Station of Tabriz University, East Azerbaijan province, Iran (38° 07´ N and 46º 29´ E) with altitude of 1567 m. In a present study, four fertile Ghezel rams , 3–4 years age and 60–80 kgs weight were used at this experiment. The animals were daily fed at a diet consisting of concentrate 200 g and alfalfa hay 1250 g.

2.2. Semen collection, Evaluation and dilution

Semen collected twice weekly using an artificial vagina (37-40 °C) during the breeding season. the ejaculates were holed in a warm water bath at 37 °C until their assessment.After semen collection and a primarily evaluation, only samples more than 70% motile sperm and a sperm concentration of higher than 3×10^9 sperm/ml were used for cryopreservation. The semen was subjected to the following tests.Volume evaluation (ml) measured by calibrated semen collection tube, mass sperm motility by light microscopy (100× magnification) and subjectively scored (1 to 5) pH by pH meter papers, and progressive motility assessed

using diluted semen (1:100) by light microscopy (400× magnification) (Bucak et al., 2008; Khalili et al., 2009). The percentage of live sperm and morphology was evaluated using diluted semen (1:100), stained with eosin-nigrosin and calculated at least different 5 regions on slide (400× magnification). The sperm concentration determined after dilution (1:300) by tuma hemocytometer (Evans and maxwell, 1987).

2.3. Extending and processing of frozen sperm

In this study a Tris-based extender used which consisted of 2.71 g tris (hydroxymethylaminoethane, Merch 64271, Germany), 1.4 g citric acid (Appli chem Gmbh 64291,Germany), 1 g fructose (Daejung Chemicals and Metals, 0043, korea), in 100 ml distilled water, and diluent was supplemented with 7% (v/v) glycerol (Merch 6100, Germany), 20% (v/v) egg yolk, penicillin (100,000 IU) and streptomycin (100 mg). The extender divided into four equal portions and supplemented with (50 mM) trehalose (Merch 64271, Germany), (4 mM) cysteine (Merch 64271, Germany) and 50 mM trehalose + 4 mM cysteine, and the control group went without trehalose and cysteine. Samples, after dilution (1:4) at 37 °C, aspirated into 0.25 ml straws ,then cooled to 5° C for 90 min , thereafter frozen above (4 cm) nitrogen vapor, and finally placed in liquid nitrogen for storage. The frozen straws were thawed at 37°C for 30s for evaluation of motility, progressive motility, percentage of live sperm, and morphology every 15 days.

2.4. Statistical analysis

The present experiment was studied in a completely randomized design. The statistical analyses of the data performed by the General Linear Model (GLM) procedure of SAS (2003). Differences were taken as significant for $P<0.05$.

RESULTS AND DISCUSSION

Results

After semen collection and a primarily evaluation, only samples with more than 70% motile sperm and a sperm concentration of higher than 3×10^9 sperm/ml were used for cryopreservation. The measuring parameters were set out in Table 1.

Table 1. Characteristics of ejaculates before pooling

	Motility (%)	Live sperm (%)	Volume (ml)	Color	Sperm concentration ($\times10^9$ cells /ml)
Mean	90	92	1.2±0.064	Creamy	4.1

Comparison of average percentage of live sperm after thawing showed that the extender containing of Cys+T had a positive significant effect on sperm viability during preservation (0, 15, 30, 45, 60, 75 of 90 days after frozen). So, the extender containing Cys + T kept sperms alive longer than other groups ($P < 0.05$).

Table 2. Viability of sperm cells after thawing (%) (LSM±SE)

During of storage (day)	Cys+T	T	Cys	Control
0	75.5±2.64^a	71.57±1.64^b	69.67±0.95^b	69.35±1.65^b
15	71.77±1.6^a	69.98±1.79^{ab}	68.5±1.02^b	64.97±1.45^c
30	70.30±0.62^a	69.3±0.81^a	66.6±1.41^b	60.41±0.53^c
45	69.32±0.86^a	68.96±1.72^a	63.73±0.97^b	57.4±2.89^b
60	68.31±0.95^a	67.88±0.30^a	64.85±1.98^b	55.27±2.23^c
75	67.35±1.10^a	64.58±1.5^b	62.65±1.06^b	54.4±1.38^c
90	66.1±0.83^a	63.47±0.37^b	63.11±1.61^b	52.10±0.98^c

within a row dissimilar letters show significantly different ($P < 0.05$)

During 90 days of storage, percentage of alive sperms in extender containing Cys+T was higher than the control group ($P < 0.05$) so, it is important to note that this percentage was sharply decreasing in control group. It may be due to the cryopreservation effect of cysteine and trehalose (Table 2). The best results for sperm motility related to extender containing Cys+T (Table 3).

Table 3. Sperm motility after thawing (%) (LSM±SE)

During of storage (day)	Cys+T	T	Cys	Control
0	61.5±0.81[a]	57.52±0.85[b]	56.75±0.92b[c]	55.4±0.93[c]
15	55.5±1.15[a]	54.6±1.24[a]	54.75±0.87[a]	54.4±0.48[a]
30	55.00±2.16[a]	49.25±0.95[b]	46.50±1.29[c]	45.3±1.70[c]
45	56.2±4.34[a]	50.00±3.55[b]	46.25±1.5[b]	38.75±1.5[c]
60	53.75±2.5[a]	48.75±2.5[b]	48.75±2.5[b]	46.25±2.5[b]
75	58.75±2.5[a]	46.25±2.5[b]	45.00±0.90[b]	43.75±2.5[b]
90	52.95±2.5[a]	47.4±3.3[b]	44.8±3.76[bc]	41.25±2.5[c]

Within a row dissimilar letters show significantly different ($P < 0.05$)

The post-thawed sperm motility after 90 days of storage was highest in the Cys+T extender. spermatozoa motility decreased gradually in diluent containing Cys+T compared to control group. In addition, the progressive motility in the Cys+T was significantly more than control group ($P < 0.05$). In diluent containing Cys+T the average of post-thawed progressive motility were respectively 48.5, 42.5, 43.00, 47.5, 38.75, 43.75, 38.75 for 0, 15, 30, 45, 60, 75 and 90 days that shows significant differences with control group ($P < 0.05$). The progressive motility in extender containing Cys+T which decreased slowly *was* significantly different from control group.

Table 4. Progressive motility after thawing (%) (LSM±SE)

During of storage (day)	Cys+T	T	Cys	Control
0	48.5±0.73[a]	47.57±0.82[ab]	46.97±0.4[b]	47.5±0.91[ab]
15	45.2±0.58[a]	44.55±1.31[a]	42.50±0.71[b]	42.27±0.66[b]
30	43.00±1.82[a]	43.75±1.50[a]	35.50±1.00[b]	36.25±0.95[b]
45	47.5±2.88[a]	42.5±2.88[a]	35.00±5.7[b]	31.25±2.5[b]
60	38.75±2.5[a]	33.75±2.5[b]	36.25±2.5a[b]	28.75±2.5[c]
75	43.75±2.5[a]	36.25±2.5[b]	32.5±2.88b[c]	31.25±2.5[c]
90	38.75±4.78[a]	36.25±2.5[a]	33.75±2.5[a]	27.00±2.88[b]

Within a row dissimilar letters were significantly different ($P < 0.05$)

During 90 days of storage, an increase in percentage of sperm abnormalities in all groups was observed. But in the extenders containing T, Cys and Cys+T sperm abnormalities were significantly lower than control samples ($P < 0.05$). The achieved results showed that through increasing the duration of storage of sperm, extender containing Cys+T, T and Cys became more effective on percentage of sperm abnormalities than the control group (Table 5).

Table 5. Abnormal sperm after thawing (%) (LSM±SE)

During of storage (day)	Cys+T	T	Cys	Control
0	10.13±0.55[b]	10.10±0.29[b]	9.66±1.00[b]	11.49±0.90[a]
15	9.90±0.48[d]	12.02±0.45[c]	13.27±1.02[b]	16.82±0.53[a]
30	11.12±0.48[b]	12.77±0.93[ab]	13.09±1.76[b]	13.82±0.83[a]
45	12.68±0.80[c]	16.07±1.56[b]	14.73±0.61[bc]	19.30±1.91[a]
60	14.64±0.71[c]	14.47±0.79[c]	18.50±1.63[b]	21.23±1.5[a]
75	15.22±0.53[b]	15.43±1.25[b]	17.87±1.75[b]	24.91±2.47[a]
90	17.65±1.08[b]	18.5±1.25[b]	17.8±1.52[b]	23.14±2.47[a]

Within a row dissimilar letters are significantly different ($P < 0.05$)

4. Discussion

Cryopreservation, adversely the cryosurvival of spermatozoa, leads to reduction of sperms fertile life (Badr et al., 2010). More specifically, ram semen has proven to be more difficult to cryopreserve than semen of other farm animals (Nur et al., 2010). Cryopreservation of spermatozoa including the decrease in temperature, enhances oxidative stress due to ice crystallization and lipid peroxidation, and cold shock which irreversibly leads to damage of the sperm organelles, associated with a reduction in motility, viability and fertilizing ability (Çoyan et al., 2011). This study investigated the effects of trehalose and cysteine on the alive sperm motility, progressive motility , and morphological abnormalities of ram semen during 90 days of storage. The present study indicated the cryoprotective capacity of Cys, T and Cys+T. Adding Cys + T to diluent the ram semen had a high effect on sperm parameters. In this study, cystein and trehalose took a positive effect on frozen-thawed ram spermatozoa. The sperm motility, progressive motility and viability in group with Cys+T were higher than in other groups. Also, the results showed that abnormal sperms in extender containing Cys,T and Cys+T were lower than the control group. The exact mechanism of cysteine preserving the sperm during freezing and thawing put forth by Bansal and Bilaspuri (2010) and Bucak et al (2008) explained that cysteine is a low-molecular weight amino acid containing thiol, a precursor of intracellular glutathione, glutathione (l-g-glutamyl-l-cysteinylglycine), a tripeptide thiol compound which has many important functions in the cellular physiology and metabolism including the protection of the cell from oxidative stress, synthesis of protein, DNA, and gamete cell

fertilization. Resent reports have shown that addition of trehalose to extender before freezing leads to improve frozen-thawed semen characteristics in ram (Jafaroghli et al., 2011), bull (Hu et al., 2010), Buffalo (Badr et al., 2010; Reddy et al., 2010), goat (Tuncer et al., 2010), rat (Sarıözkan et al., 2012), dog (Yamashiro et al., 2007), mouse (Storey et al., 1998), and boar (Pérez et al., 2009; Hu et al., 2009). The mechanism by which trehalose preserved the sperm membrane during freezing and thawing was described by Tonieto et al (2010), Aisen et al (2005; 2002; 2000), Ahmad and Aksoy (2012) in which Trehalose promotes cell dehydration,influences the crystallization pattern of the solute channels existing in portions of unfrozen water of the extender, and contributes to the reduction in the formation of ice crystals, in addation to its anti-oxidant activity. As trehalose also reacts a direct interaction with phospholipid polar head groups, confers better cryoprotection. Some researchers believe that hypertonic diluent causes cell dehydration prior to freezing and reducing the intracellular ice formation, causes decrease in cell damages. High concentration of trehalose leads to decrease of motility and acrosome integrity (200 and 400 mOsm) while using trehalose 100, 50 mM improves frozen-thawed characteristics of ram semen (Aisen et al., 2002; Uysal and Bucak, 2009). Trehalose provides better protection for membrane, reduction in the number of damaged membranes, increase in the level of reduced glutathione, and also reduction in the level of lipid peroxide (Aisen et al., 2005). Trehalose (100 mM) leads to reduction in sperm DNA damage and increase in the level of glutathione reductase, superoxide dismutase and total antioxidant (Badr et al., 2010). According to Uysal and Bucak report (2009) about oxidized glutathione (5 mM), bovine serum albumin (10 mM) and cysteine (10 mM) caused significant improvement in ram sperm motility, acrosome integrity and viability after thawing, in comparison with control group.

CONCLUSION

In conclusion, the results of the present study were in accordance with other findings, reporting that trehalose and cysteine improves ram semen characteristic. Furthermore, it is concluded that Cys+T provides best cryopreservation for 90 days of freezing.

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