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Influence of sugars and osmoregulators on the motility and viability of cooled and frozen-thawed ram semen

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The aim of the study was to determine the effect of sugars and osmoregulators on the viability of frozen-thawed ram semen in a Tris-based diluent. Two experiments were conducted to evaluate the effect of sugars (fructose, glucose, sucrose and raffinose) and two osmoregulators (hypotaurine and taurine) on the viability of ram sperm. In experiment 1, each of the four sugars (fructose, glucose, sucrose and raffinose) was added (500 mg%) to the diluents. Semen was collected from 15 rams once a week using an artificial vagina, diluted, cooled slowly to 5°C over 2 h, frozen in the form of pellets, and plunged into liquid nitrogen. The frozen semen was thawed, and sperm viability was calculated 3 h after thawing. In experiment 2, two osmoregulators, hypotaurine (0.20 and 0.40 mg/ml) and taurine (2 and 4 mg/ml), were added to Tris-based raffinose diluent. Motility and viability rates were calculated. The results showed that motility was gradually significantly ($P < 0.05$) improved by using 500 mg% raffinose in the Tris-based diluents after dilution at 30°C and before freezing at 5°C. Post-thawing motility and viability rates were highest ($P<0.05$) when raffinose was used in Tris-based diluent for cryopreservation of ram semen. *In vitro* supplementation of the semen diluent with 4 mg/ml taurine had a beneficial effect on the motility and viability of ram spermatozoa after dilution at 30°C and before freezing at 5°C. The same trend was observed after freezing-thawing and 3 h post-thawing. In conclusion, 500 mg% raffinose was a more suitable sugar component for treatment of ram spermatozoa in Tris diluent than fructose or glucose, while 4 mg/ml of taurine in Tris-raffinose medium exerted a beneficial effect on the motility and viability of ram sperm at cooling and post-thawing.

KEY WORDS: ram semen, sugars, osmoregulator, dilution, freezing

Studies on artificial insemination (AI) of sheep were first conducted at the beginning of the twentieth century by Soviet scientists, whose research on diluting media and reproduction

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led to the development and practical application of AI in farm animals. The need to use ram semen over extended periods or at different times of the year prompted research on storage of spermatozoa under artificial conditions. This could be achieved by methods that reduce or arrest the metabolism of sperm and thereby prolong their fertile life. Accordingly, researchers studied storage of semen in both a liquid (unfrozen) state, using reduced temperatures or other means to depress sperm metabolism (Maxwell and Salamon, 1993), and a frozen state, which involved preservation at sub-zero temperatures (Salamon and Maxwell, 1995). AI with frozen ram semen yields lower fertility rates, as the freezing and thawing of semen cause serious damage to ram spermatozoa. Such damage is less pronounced in diluted and chilled semen (Paulenz et al., 2002). Moreover, AI with frozen semen requires proper thawing and intrauterine insemination by laparoscopy, for which skilled technicians and proper equipment are indispensable, whereas chilled ram semen does not pose such difficulties. The success of artificial insemination depends on the management of semen collection and its storage and use (Leboeuf et al., 2000). Frozen-thawed semen has advantages over fresh or refrigerated semen due to its long shelf-life and transportability. However, cryopreservation causes greater damage to spermatozoa, resulting in lower fertilizing capacity compared to fresh and refrigerated semen. Successful preservation of ram sperm depends on a number of factors, such as the type and composition of the extender and pre-freezing dilution and processing of semen (Salamon and Maxwell, 2000; Azevedo, 2006; Maia et al., 2010). The composition of semen extenders is one of the most important factors affecting the degree of sperm cryodamage. Sodium citrate and Tris egg yolk extenders have been widely used for chilling and freezing of ram semen. The cryoprotectant effect of egg yolk is reported to derive from the low-density lipoprotein fraction (Moussa, 1999), which prevents the loss of membrane phospholipids and thereby increases the tolerance of sperm to the chilling and freezing process (Parks and Graham, 1992). The effects of sugars and osmoregulators on the post-thawing motility of mammalian sperm have previously been discussed in the literature (Maia et al., 2010). Both monosaccharides and disaccharides have been extensively used for storage of ram semen. Sugar has several functions, serving as the energy substrate for sperm cells during incubation (Fukuhara and Nishikawa, 1973) and maintaining the osmotic balance of the extenders (Aboagla and Terada, 2003). In view of the above, the present study was designed to study the effect of several sugars and osmoregulators on the viability of frozen–thawed ram semen in a Tris-based extender.

Materials and methods

Ethics approval. The experiment was conducted with the consent of the Institutional Animal Care and Use Committee of the Animal Production Research Institute, Sakha, Kafr Al-sheikh, Ministry of Agriculture, Egypt, without interfering with animal welfare.

Chemical reagents. All sugars and chemical reagents used in the study were purchased from Sigma-Aldrich Co., Germany.

Experimental animals, location and feeding

The study was performed on 15 mature cross rams (3/8 Finnish x 5/8 Rahamani). Their ages ranged from 2 to 3 years and they weighed 33-42 kg. The rams were raised at the Sakha

Experimental Station, Animal Production Research Institute, Egypt. The station is located in the north part of the Nile Delta (31° 5' 47.976'' N; 30° 55'20.8272'' E). The average temperature, relative humidity and wind speed were 26ºC, 56% and 9.8 km/h. The animals were housed in semi-open yards. The experiment was begun in December 2017 and lasted for 6 months. All animals were healthy and clinically free of external and internal parasites.

Semen collection and sperm evaluation

Semen was collected from rams once a week using an artificial vagina. This regime was begun three weeks before the start of the experiment to stabilize semen characteristics. Three consecutive ejaculates were obtained from each ram at 5-10-minute intervals on each day of collection. Semen samples were transferred to the laboratory and placed in a water bath at 30°C for evaluation. Only ejaculates with a volume of 0.5-2.0 ml, a minimum sperm concentration of 3000 $x10^6$/ml, total motility higher than 70%, and <10% abnormal sperm were used, according to Chemineau et al. (1991). The ejaculates were pooled to yield one semen sample with a total volume of 2.0-2.5 ml in order to avoid the effect of individual variability in rams. Fifteen trials were carried out for each experiment.

Experimental design

Experiment 1. This experiment was carried out to study the effect of sugars on the freezability of ram sperm in Tris-based extender. This extender was composed of Tris (3.634 g), citric acid monohydrate (1.990 g), fresh egg yolk (15 ml), penicillin G sodium (100,000 IU), streptomycin sulfate (100 mg), and glass distilled water to 100 ml. Four types of sugars (fructose, glucose, sucrose and raffinose) added (500 mg%) to the Tris-based extender were compared in terms of sperm motility and viability. The pooled sample was diluted with these sugars at the start of the experiment (1 part semen + 4 parts diluent). The semen was cooled slowly to 5°C within 2 hours. Then the cooled semen was frozen in the form of pellets (0.3 ml/pellet) on a Fluorethene plate cooled by immersion in liquid nitrogen inside a foam box (34x22x17 cm, containing 5 l of LN_2) for 15 minutes and then raised and lifted onto a liquid nitrogen surface. Pellets were transferred into the liquid nitrogen container and stored for 3 hours before thawing. Pellet-frozen ram semen was thawed in a Tris-buffer solution (Tris (3.634 g), citric acid (1.999 g), and glass distilled water to 100 ml. The motility and viability percentages of fresh-diluted, cooled and frozen-thawed semen were calculated according to Rawash et al., 2018.

Experiment 2. This experiment was carried out to study the effect of the osmoregulators hypotaurine and taurine on the freezability of ram semen. Pooled semen samples from each ram were divided and diluted 1:4 at 30°C with Tris-based extender supplemented with the best of the sugars tested in Experiment 1 (500 mg% raffinose). Hypotaurine at levels of 0.20 and 0.40 mg/ml and taurine at 2.0 and 4.0 mg/ml were added to the extenders. The diluted semen samples were processed for freezing and thawing as previously described for Experiment 1. The motility and viability percentages of fresh-diluted, cooled and frozen--thawed semen were calculated.

Statistical analysis

Data from 15 replicates were presented as means ± SEM and subjected to analysis of variance (two-way ANOVA) using SPSS software (SPSS, 2016).

Results and discussion

When spermatozoa are frozen and thawed, they are subject to various stressors (physical, physiological, osmotic and chemical) that result in disruption of the transbilayer phospholipid asymmetry of mammalian sperm. This causes damage to the plasma membrane and increases its susceptibility to lipid peroxidation (Rawash et al., 2018), predisposing the sperm to gross morphological damage and decreasing motility and fertilization capacity. The results of the present study indicate that the addition of raffinose and taurine to ram semen extender improved its freezability. The addition of 500 mg% raffinose to semen samples in the Tris-based extenders after dilution at 30°C and before freezing at 5°C clearly resulted in a significant ($P < 0.05$) increase in motility (82.0 ±1.2; 82.0 ±1.5, respectively) as shown in Table 1.

Table 1
Effect of different sugars on motility percentages in ram semen (mean ±SEM)

Motility	Diluents				
	Tris-based extender (T)	T+Fructose	T+Glucose	T+Sucrose	T+Raffinose
After dilution at 30°C	80.0 ±1.4^{a}	81.6 ±1.2^{ab}	81.0 ±1.2^{ab}	81.6 ±1.2^{ab}	82.0 ±1.2^{bc}
Before freezing at 5°C	78.3 ±1.5^{abc}	80.6 ±1.4^{d}	79.0 ±1.4^{ab}	80.6 ±1.4^{d}	80.6 ±1.5^{dc}

Means with different superscripts a, b, c in the same rows are significant at $P < 0.05$

In addition, the highest motility and viability were detected in sperm samples treated with raffinose in the Tris-based extenders for cryopreservation of ram semen (46.0 ±2.3; 176.3 ±7.0, respectively). The second-best motility and viability were found in samples in extenders supplemented with sucrose (Table 2).

Table 2
Effect of different sugars on post-thawing sperm motility and viability of ram semen (mean ±SEM)

Post-thawing sperm motility %	Diluents				
	Tris-based extender (T)	T+Fructose	T+Glucose	T+Sucrose	T+Raffinose
0 h	43.3 ±1.6^{a}	45.6 ±1.8^{a}	47.6 ±1.8^{a}	54.3 ±1.8^{a}	56.0 ±2.0^{a}
1 h	36.3 ±2.0^{abc}	40.3 ±1.9^{a}	45.6 ±1.6^{a}	49.3 ±1. 8^{a}	52.0 ±2.1^{a}
2 h	30.6 ±2.2^{abc}	37.3 ±2.2^{abc}	42.3 ±2.2^{abc}	47.6 ±2.3^{abc}	50.3 ±2.1^{abc}
3 h	26.3 ±2.2^{abc}	31.3 ±2.8^{abc}	37.3 ±2.4^{abc}	41.6 ±2.3^{abc}	46.0 ±2.3^{abc}
Viability indices	114.8 ±6.5^{abc}	131.8 ±7.2^{abc}	148.8 ±6.5^{abc}	165.8 ±6.5^{abc}	176.3 ±7.0^{abc}

Means with different superscripts a, b, c in the same rows are significant at $P < 0.05$

The beneficial effect of raffinose was due to its ability to stabilize the protein-liquid complex of the sperm cell membrane (Nauk, 1991) and to ensure the optimum osmotic pressure of the extender for successful freezing of ram semen by the pellet method (Salamon and Lightfoot, 1969). Raffinose, a trisaccharide, plays a cryoprotective role by decreasing intracellular ice crystal formation through its interaction with membrane lipids and proteins during cryopreservation (Salamon and Maxwell, 2000). Bucak et al. (2013) reported that 10 mM of raffinose in a Tris-based extender maintained motility, viability, mitochondrial activity, and acrosome integrity in frozen-thawed ram sperm. The addition of raffinose to Tris-based extender at a level of 70 or 100 mM has been found to increase sperm viability and motility, while reducing the level of acrosome and total sperm abnormalities in frozen-thawed ram sperm (Jafaroghli et al., 2011). In contrast, glucose was a more suitable sugar component in the Tris medium than fructose or raffinose for pellet freezing of ram sperm (Salamon and Visser, 1972). At the same time, post-thawing recovery of ram sperm after freezing in Tris- fructose- yolk and Tris-glucose-yolk extenders were higher than in glucose-citrate-yolk or raffinose-citrate-yolk media (El-Gaafary, 1990). The in vitro addition of taurine to semen diluents (Tris-based + raffinose) resulted in a significant ($P < 0.05$) increase in the motility % of ram semen after dilution at 30°C and before freezing at 5°C (Table 3).

Table 3

Effect of osmoregulators on motility of ram semen in Tris-raffinose extender (mean ±SEM)

Motility	Diluents				
	Tris-raffinose extender (R)	R+0.20 mg/ml hypotaurine	R+0.40 mg/ml hypotaurine	R+2.0 mg/ml taurine	R+4.0 mg/ml taurine
After dilution at 30°C	79.9 ±1.0^{a}	81.6 ±1.2^{ab}	81.0 ±1.0^{ab}	81.6 ±1.0^{ab}	82.1 ±1.0^{bc}
Before freezing at 5°C	77.5 ±1.4^{abc}	77.5 ±1.3^{d}	75.3 ±1.1^{ab}	77.8 ±1.3^{d}	77.8 ±1.2^{dc}

Means with different superscripts a, b, c in the same rows are significant at $P < 0.05$

The results showed that addition of 4 mg/ml of taurine to the semen extenders significantly ($P < 0.05$) increased the post-thawing motility and viability of ram semen during the freezing process (Table 4).

The results of the present study provide clear evidence that taurine added to Tris-raffinose extender exerted a beneficial influence on the motility and viability of ram sperm at cooling and freezing. This finding supports previous research by Sanchez-Partida et al. (1997), who reported pronounced enhancement of Tris-raffinose extenders through supplementation with taurine. This is may be due to its osmoregulatory effect rather than its antioxidant activity. The inclusion of amino acids (e.g. taurine, hypotaurine, proline, glutamine, glycine, histidine, and cysteine) in extenders reduces DNA degeneration and increases the post-thaw motility, viability, membrane integrity, and fertility of ram sperm (Bucak and Tekin, 2007; Bucak et al., 2009; Rather et al., 2016). Taurine is a major non-

Table 4

Effect of osmoregulators on post-thawing sperm motility and viability in ram semen in Tris-raffinose extender (mean ±SEM)

Post-thawing sperm motility %	Incubation time				
	Tris-raffinose extender (R)	R+0.20 mg/ml hypotaurine	R+0.40 mg/ml hypotaurine	R+2.0 mg/ml taurine	R+4.0 mg/ml taurine
0 h	41.0 ±1.8^{d}	44.6 ±1.8^{bcd}	47.5 ±2.4^{b}	48.9 ±1.5^{b}	53.2 ±1.8^{a}
1 h	34.2 ±2.1^{ab}	40.3 ±1.9^{cd}	45.6 ±1.6^{ac}	44.6 ±2.1^{dc}	49.2 ±1.6^{c}
2 h	26.7 ±2.9^{a}	37.3 ±2.2^{b}	42.3 ±2.2^{ab}	36.7 ±2.6^{bc}	40.3 ±2.2^{cd}
3 h	21.4 ±3.0^{a}	31.3 ±2.8^{b}	37.3 ±2.4^{ab}	30.0 ±3.0^{b}	35.3 ±2.9^{c}
Viability indices	103.0 ±8.1^{a}	123.0 ±8.3^{b}	119.1 ±10.8^{ab}	138.7 ±7.8^{bc}	151.2 ±6.7^{c}

Means with different superscripts a, b, c in the same rows are significant at $P < 0.05$

-enzymatic scavenger that has an important role in protecting sperm against ROS and lipid peroxidation (Saleh and Agarwal, 2002). In addition, it protects sperm against high potassium ion concentrations by reducing Na-K-ATPase activity and consequently the influx of extracellular potassium ions, which have been shown to be detrimental to sperm motility *in vitro* (Dumoulin et al., 1992).

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