Journal of Animal Science Research /vol.34, No.2; 2024/pp 77-85

The effects of EDTA and propylene glycol on sperm quality and levels of extender elements during cryopreservation

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Received: October 19, 2022 Accepted: April 26, 2023

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Running head: The effects of EDTA and PG on sperm quality
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Journal of Animal Science Research /vol.34 No.2/ 2024/pp 77-85
https://animalscience.tabrizu.ac.ir

DOI: 10.22034/as.2023.53840.1681

Abstract
The present study was designed to evaluate the effect of adding ethylene diamine tetra acetate (EDTA) and propylene glycol (PG) on extender calcium and magnesium and quality traits of frozen-thawed Ghezel ram sperm. Semen samples were collected from five rams (3-4 years) during the non-reproductive season once a week with 15 replicates. Samples were diluted with Tris-based extender without additive (control) and supplemented with 1.75 mM EDTA, 2% PG, and 7% PG (instead of glycerol). Sperm quantitative characteristics were studied on 0, 20, 40, and 60 days of the storage after freeze-thawing. Results indicated a negative correlation between the amount of calcium in seminal plasma and sperm parameters ($P<0.01$). Also, there was a negative correlation between magnesium and sperm membrane integrity ($P<0.01$). Furthermore, 1.75 mM EDTA and 2% PG significantly decreased calcium and magnesium levels in freeze-thawed samples compared to the control group ($P<0.05$). Adding of 1.75 mM EDTA and 2% PG also significantly improved sperm quality parameters compared to the control group ($P<0.05$). It may be concluded that the addition of 1.75 mM EDTA and 2% PG to diluent significantly decreased the amount of calcium and magnesium of seminal plasma and improved the sperm parameters after the freeze-thawing process.

Keywords: Calcium, EDTA, Magnesium, Propylene glycol, Sperm quality

Introduction
The fluid medium of semen that spermatozoa are suspended within it is called seminal plasma. There was the very complex and variable biochemical composition of seminal plasma among species that is made up of energy substrates (fructose, sorbitol, glycerylphosphocholine), organic compounds (citric acid, amino acids, peptides, low and high-molecular-weight proteins, lipid, hormones, cytokines), and also ions that the sperm function is highly dependent on the ionic environment (Hamamah and Gatti 1998; Juyena *et al.* 2012). Cations such as calcium and magnesium that belong to the same family in the periodic table, are used in osmotic equilibrium (Cevic *et al*. 2007; Liang *et al*. 2016). They have similar homeostatic regulatory systems and can potentially antagonize each other in many physiological activities and are components of many

important enzymes (Cevic *et al*. 2007; Liang *et al.* 2016).

Magnesium is the second most prevalent intracellular cation and is involved in the metabolic activity of the cell as the main cofactor for kinase enzymes (Eghbali *et al*. 2010; Hashemi *et al.* 2017). Magnesium acts as an intracellular calcium antagonist so that increased magnesium levels in human seminal plasma compared with calcium improve erection and ejaculation processes (Hashemi *et al.* 2017). Within the cell, most of the magnesium is bound to proteins and negatively-charged molecules, 80% of cytosolic magnesium is bound to ATP, which is the substrate for numerous enzymes (Eghbali *et al*. 2010). Depletion of intracellular magnesium affects activities dependent on this ion, such as glycolysis, protein synthesis, respiration and reproduction (Wong *et al*. 2001).There is an evidence that alteration in magnesium and calcium levels of seminal plasma can affect sperm quality with decreasing fertility potential (Bassey *et al.* 2013).

Calcium may have a role in steroidogenesis by influencing delivery, utilization of cholesterol by mitochondria. Calcium also stimulates the conversion of pregnenolone to progesterone (Hurley and Doane 1989). Calcium plays an important role in sperm physiology including motility, capacitation and signaling pathways in the acrosomal reaction (Aisen *et al.* 1999; Liang *et al.* 2016). Since calcium influx into sperm cells is involved in the acrosome reaction, it seems that high calcium concentrations in sperm freezing media could increase the risk of premature reactions (Braud *et al.* 2016).

Sperm storage is a useful way to store genetic resources for some endangered species (Hurley and Doane 1989). Freezing is a branch of cryobiology that includes long-term protection and preservation of cells and tissues under very low conditions (Mortimer 1994).

However, investigations have shown that freezing and thawing processes not only generate oxygen free radicals that impair post-thaw motility, viability, intracellular enzymatic activity, fertility, and sperm function (Peris *et al.* 2007; Ozkavukcu *et al.* 2008 Silva *et al.* 2013) but also changes the membrane permeability to some ions including calcium. Semen cryopreservation has increased levels of intracellular calcium that leads to dysfunction and cell death (Bittencourt *et al.* 2014; Nateq *et al* 2021). Binding to the zona pellucida or progesterone leads to an increase in the intracellular calcium concentration of sperm due to the opening of the calcium channel and also the releasing of calcium from intracellular stores (Keshtgar *et al.* 2016). This process increased the production of free radicals in the cell and leads to de-polymerization and membrane fusion, which the final result is acrosome reaction (Keshtgar *et al.* 2016).

Ethylene diamine tetra acetate (EDTA) is the chelator of divalent metal ions such as calcium, magnesium, copper, zinc, etc. (Bourinbaiar and Lee 1996). The EDTA main function is to chelate the extracellular calcium, reducing its influx to the intracellular environment, which minimizes the deleterious effect of calcium on the sperm (Bittencourt *et al*. 2014).

Cryoprotectants is another factor that influences sperm survival during cryopreservation. Low molecular weight cryoprotectants, such as Ethylene glycol (EG), glycerol, and, 1,2 propylene glycol (PG), may cause less damage to spermatozoa because its low molecular weight allows them to cross the plasma membrane more easily (Li *et al*. 2005; Büyükleblebici *et al.* 2014).

Previous studies indicated that EDTA and PG improved sperm quality. However, there is no report about the interaction of them on calcium and magnesium of semen in rams. The aim of this study was to investigate the effects of calcium and magnesium of semen seminal plasma, as well as the addition of EDTA and PG to the diluent during the cryopreservation on calcium and magnesium of seminal plasma, and the sperm parameters after freezing.

Material and methods

Animals: This research was conducted at the Department of Animal Sciences, Faculty of Agriculture, University of Tabriz, Iran. Semen was collected from five rams (3-4 years) using the artificial vagina during the non-

reproductive season. Rams were kept under natural light conditions and during the research period, rams were kept separately and had free access to water and food and licking salt. The rams were habituated for semen collection for two weeks. Before the start of the project, the wool of the ventral region was shortened to facilitate sperm collection and preventing the entry of excreta and pollution into the artificial vagina.

Semen collection, extender preparation and sperm dilution: Semen collection was performed using an artificial vagina during the non-reproductive season once a week for three weeks. Ejaculates were immediately evaluated primarily for parameters including total motility, progressive motility, non-progressive motility, viability, and acrosome integrity and samples with a concentration of 2.5 billion sperm and a progressive motility of over 70% and a volume greater than 0.5 ml were selected for dilution with treatments groups included control, 1.75 mM EDTA, 2% PG as an additive, and 7% PG as a constitution. Dilution contained Tris (2.71 g), citric acid (1g), fructose (1.4 g), penicillin (100000 IU), and, streptomycin (100 mg) in 100 ml distilled water. Then, 73 ml of this solution was mixed with egg yolk (20 ml) and glycerol (7 ml). Ejaculated samples were diluted (1:10; v/v) with each of the four extenders, and then 72 straws (250 µl) were filled with extenders and they were placed in a refrigerator for 1.5 to 2 hours to reach 5°C. After cooling they are placed in 4-5 cm above nitrogen for 8-10 minutes and ultimately, they are immersed in liquid nitrogen. Sperm parameters were investigated in 0, 20, 40 and 60 days of frozen storage. During the thawing, samples were incubated at 38°C for 30 seconds.

Measurement of calcium and magnesium levels of fresh seminal plasma: After collection, a portion of fresh semen was centrifuged for 15 minutes at 3500 rpm to separate seminal plasma (Rangraz *et al*. 2016). After separation, seminal plasma was stored at -20°C to measure. Calcium and magnesium of seminal plasma were measured using the appropriate kits (Pars Azemon Co, Iran) and spectrophotometer (Geneus 20, USA).

Measurement of calcium and magnesium levels of frozen-thawed seminal plasma: In order to determine the effect of EDTA and PG on the amount of frozen-thawed seminal plasma's calcium and magnesium, contents of straws of each groups were poured into glass tubes at different time of thawing (days 0, 20, 40 and 60) separately, then were centrifuged at 3500 rpm for 15 minutes. Seminal plasma of samples was stored at -20°C to measure. Calcium and magnesium of seminal plasma were measured using the appropriate kits and spectrophotometer (Pars Azemon Co, Iran) and spectrophotometer (Geneus 20, USA).

Sperm parameters after thawing

Analysis of standard semen parameters by CASA: Sperm motility (total and progressive motility) was estimated by computer-assisted sperm motility analysis (CASA, VideoTest-Sperm 3.1, St. Petersburg, Russia). One straw per each replicate of treatments was randomly selected and placed individually in a pre-warmed chamber and the loaded chamber placed on the thermal plate of the microscope (37°C) and the motility of 200 sperm were photography and analyzed with CASA system. The thawed semen samples were analyzed for the Total sperm motility (TM, %) and progressive motility (PM, using a phase-contrast microscope (Labomed LX400; Labomed Inc., Culver City, CA, USA).

The percentage of viability: Evaluation of live and dead sperm was used eosin-nygrosin staining. In this method, nigrosin is used to increase the background contrast and sperm head, which makes it easier to detect (WHO 2010). Eosin also penetrates the dead sperm. Viability was assessed by counting 200 cells at magnification of ×400 under light microscope. Sperm with strict exclusion of stain were counted as viable and sperm displaying partial or complete purple staining were considered nonviable.

Acrosome integrity: To determine the acrosomal health, an eosin-nigrosin-stained sample is used. This test was done using light microscope with magnification of 1250. Sperm with integrity acrosome was considered as live sperm. The ration of healthy acrosome was

calculated in 20 dead sperm. Finally, the amount of integrity acrosome in the dead cells is accumulated with live cells that this count is the percentage of acrosome integrity in a sample.

Statistical Analysis: Data were analysed using the GLM and corr procedure of SAS 9.2. Differences between Lsmeans were determined by Duncan's test. Differences with values of $P < 0.05$ were considered to be statistically significant. The results were presented as the Lsmean ± SEM.

Results

Descriptive statistics of quantitative traits of fresh sperm: The parameters of total motility, progressive motility, viability, acrosome integrity, levels of calcium and magnesium were used for the first study of sperm quality that showed in Table 1.

Table1- Descriptive statistics of sperm traits of fresh semen before dilution

Variation	Count	Mean	Lowest	Highest	Standard deviation	Coeff variation
Total motility (%)	15	85	75	92	4.97	5.84
Progressive motility (%)	15	78.36	71.11	86	4.74	6.05
Viability (%)	15	86.70	76.26	93.1	4.92	5.68
Acrosome integrity (%)	15	89.86	78.43	94.94	4.60	5.12
Calcium (mg/dl)	14	7.27	5.94	8.76	0.85	11.70
Magnesium (mg/dl)	13	3.59	1.32	4.83	0.89	24.79

Descriptive statistics of extender sperm traits after thawing: The parameters of total motility, progressive motility, viability, acrosome integrity, levels of calcium and magnesium of extender showed in Table 2.

Table2- Descriptive statistics of extender sperm traits after thawing

Variation	Count	Mean	Lowest	Highest	Standard deviation	Coeff variation
Total motility (%)	358	55.988	25	78	11.560	20.648
Progressive motility (%)	358	50.502	22.78	74.90	11.379	22.532
Viability (%)	358	58.93	25.83	81.90	11.801	20.024
Acrosome integrity (%)	268	61.074	29.54	87.46	11.873	19.440
Calcium (mg/dl)	150	15.05	9.03	20.09	2.57	17.10
Magnesium (mg/dl)	153	2.04	0.46	3.63	0.66	32.30

Correlation between sperm parameters of fresh semen: According to Table 3, there was a significant correlation between total motility with viability and progressive motility ($P<0.01$) but there was no significant correlation among the other parameters ($P>0.05$).

Table3- Correlation between sperm parameters of fresh semen

Parameters	Total motility	Progressive motility	Viability	Acrosome integrity	Calcium of semen	Magnesium of semen
Total motility						
Progressive motility	0.75**					
Viability	0.97**	0.62				
Acrosome integrity	0.49	0.13	0.53			
Calcium of semen	0.01	0.17	-0.03	-0.21		
Magnesium of semen	-0.34	-0.006	-0.39	-0.38	0.08	

** The relationship between the two variables is significant at a level of 1%.

Correlation between calcium and magnesium of frozen-thawed seminal plasma with sperm parameters after thawing: Correlation between calcium and magnesium of frozen-thawed seminal plasma with sperm parameters after thawing was

showed in Table 4. As shown, there was a negative and significant correlation between the calcium level of seminal plasma and sperm parameters (total motility, progressive motility, viability, and, acrosome integrity) (P<0.01). Also, there was negative and significant correlation between magnesium level of seminal plasma and sperm membrane integrity (P<0.01) but there was any correlation between magnesium level of seminal plasma and other sperm parameters (total motility, progressive motility, viability, and, acrosome integrity).

Table4- Correlation between calcium and magnesium of frozen-thawed seminal plasma with sperm parameters after thawing

Parameters	Total motility	Progressive motility	Viability	Acrosome integrity
Calcium of seminal plasma	-0.34**	-0.36**	-0.32**	-0.38**
Magnesium of seminal plasma	-0.04	-0.05	-0.05	-0.06

** The relationship between the two variables is significant at a level of 1%.

The effects of adding EDTA and PG to diluent on calcium and magnesium of frozen-thawed seminal plasma: The effects of adding 1.75 mM of EDTA, 2% PG, and 7% PG (instead of glycerol) on calcium and magnesium level of seminal plasma are shown in Table 5. Adding of 1.75 mM of EDTA and 2% PG significantly decreased calcium and magnesium level of seminal plasma (P<0.05). Both groups of 1.75 mM of EDTA and 2% PG had a significant difference with the control group (P<0.05) and 2% PG had a significant reduction in calcium levels than other groups (P<0.05). However, adding 7% PG to tris-base extender instead of glycerol did not make a significant difference compared to the control group (P>0.05). Also, the amount of magnesium in the 1.75 mM of EDTA and 2% PG significantly decreased (P<0.05) and 1.75 mM of EDTA had a significant reduction in magnesium levels than other groups (P<0.05). However, there was no significant difference between the 7% PG and the control group (P>0.05).

Table5- The effect of adding EDTA and PG to extender on calcium and magnesium of frozen-thawed seminal plasma (Lsmean±SE)

Seminal plasma(mg/dl)	Diluents			
	EDTA 1.75 mM	2% PG	PG7%	Control
Calcium	15.14±0.19[b]	11.45±0.18[c]	16.76±0.19[a]	17.31±0.20[a]
magnesium	1.32±0.06[c]	1.76±0.07[b]	2.61±0.06[a]	2.51±0.06[a]

⁎Different superscripts within the same rows demonstrate significant differences among groups(P<0.05).

The effects of different levels of diluents on sperm parameters after frozen-thawed: The effect of adding treatments on total motility, progressive motility, viability, and acrosome integrity after the freezing-thawed process is presented in Table 6. As shown, diluent containing 2% propylene glycol has the best function and significant difference in all parameters among other treatments (P<0.05).

Tabe6- The effect of different diluents on sperm parameters after frozen-thawed (Lsmean±SE)

Parameters	Diluents			
	EDTA 1.75 mM	2% PG	PG7%	Control
Total motility	54.73±1.36[b]	63.40±1.36[a]	46.80±1.36[c]	50.67±1.36[bc]
Progressive motility	50.40±1.31[b]	57.76±1.33[a]	41.00±1.27[c]	44.41±1.27[c]
Viability	59.20±1.30[b]	65.85±1.28[a]	49.42±1.25[d]	54.23±1.25[c]
Acrosome integrity	60.81±1.72[b]	68.20±1.72[a]	52.41±1.77[c]	56.85±1.72[bc]

⁎Different superscripts within the same rows demonstrate significant differences among groups(P<0.05).

Discussion

The present study investigated the effect of the amount of calcium and magnesium of semen on sperm parameters (including total motility, progressive motility, viability, and acrosome integrity) after thawing. In addition, the effect of adding EDTA as a chelator and PG as a cryoprotectant on amount of calcium and magnesium of frozen-thawed samples and sperm parameters were evaluated. Different reports have described the range of quantitative characteristics of ram semen as follows: total motility (70-90%), progressive motility (45-90%), viability (60-90%), amount of calcium (6-15 mg/dl) and amount of magnesium (2-13 mg/dl) (Karagiannidis *et al.* 2000; Moghaddam *et al*. 2012; Juyena *et al.* 2012; Tavakoli *et al.* 2018) that these findings complied with our descriptive statistics of quantitative traits in Table 1.

The results showed that there was no significant correlation between the amount of calcium of semen and sperm parameters before freezing (total motility, progressive motility, viability, and, acrosome integrity). Correlation between the calcium level of extender and sperm parameters after thawing (total motility, progressive motility, viability, and, acrosome integrity) was significant. Results show that the calcium level of fresh seminal plasma was lower than seminal plasma of frozen-thawed samples. The amount of intracellular calcium was increased during freezing and thawing process. In fresh sperm, intracellular calcium is low compared to the frozen-thawed sperm. Frozen-thawed sperm has significantly higher intracellular calcium than fresh sperm (Kadirvel *et al.* 2009). Furthermore, the addition of 1.75 mM EDTA and 2% PG to the diluent reduced the calcium content of extender in comparison with the control group. PG facilitates calcium influx through the voltage – dependent calcium channels in cell (Hottori *et al.* 1999). The results showed that the addition of 2% PG reduced extracellular calcium content more than 1.75 mM EDTA. 1.75 mM EDTA was more effective on magnesium ions and significantly reduced the magnesium content of seminal plasma. The best performance was related to the 2% PG group, but the 1.75 mM EDTA group showed better performance than the control group. Accordingly, Kaya *et al.* (2002) have shown that calcium induces acrosome reactions in mammals and also correlates with sperm motility. Wong *et al.* (2001) have reported neither beneficial nor adverse associations between calcium and sperm motility. In another study, Hong *et al*. (1984) showed that calcium had an adverse effect on the motility of mature spermatozoa in ejaculated semen. Magnesium is found in almost all enzymatic systems and as a marker for seminal vesicular secretion and as an intracellular calcium antagonist can play a role in spermatogenesis and sperm motility (Wong *et al*. 2001; Jobim *et al*. 2004). There is a positive correlation between the amount of magnesium and apoptosis free cells in rams (Juyena 2011).

According to our findings, the addition of 1.75 mM EDTA and 2% PG to diluent of ram semen significantly reduced the calcium and Magnesium content of seminal plasma in comparison with the control group, which can improve the parameters after the freeze-thawing generalization of this issue. 2% PG was also able to reduce intracellular calcium in comparison with 1.75 mM EDTA. However, in the case of magnesium, a diluent containing 1.75 mM EDTA compared with 2% PG showed a further decrease in the amount of plasma Magnesium. Moreover, 1.75 mM EDTA and 2% PG improved total motility, progressive motility, viability, and acrosome integrity after freeze-thawing compared with 7% PG and control group. Broad *et al*. (2016), studied the effect of EDTA on dog frozen sperm. They found that the addition of EDTA significantly reduced the calcium content of seminal plasma and improved the motility. Adding EDTA in semen diluents chelate the calcium and reduces its concentration in the plasma membrane. EDTA also chelates other ions and may also contribute to inhibiting lipid peroxidation (Holt 2000). Several studies have reported that the addition of EDTA as a chelator to diluent improved the progressive motility and acrosome integrity (Juang *et al.*

1990; Aisen *et al*. 1999; Keshtghar *et al.* 2016). After exposure to EDTA, the calcium ion concentration in seminal plasma decreased with increasing EDTA concentration (Lee *et al.* 1996). Kaneko and Nakagata (2006) reported that a chelating agent such as EGTA and EDTA are necessary to protect spermatozoa from damage by freeze-drying.

Acknowledgements: The authors would like to thank all employees of Khalatpushan Research Station for their kind cooperation during the experiment.

Conflict of interest: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. I would like to assure you, there is no potential conflict of interest among the authors. Furthermore, all authors have read and approved this manuscript and their names were mentioned in the correct order.

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تأثیر ادیتات سدیم و پروپیلن گلیکول بر کیفیت اسپرم و سطح عنا صر رقیق‌کننده در نگهداری انجمادی

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تاریخ دریافت : ۱۴۰۱/۷/۲۷ تاریخ پذیرش: ۱۴۰۲/۲/۶

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چکیده

زمینه مطالعاتی: برای افزایش بهره‌وری در تلقیح مصنوعی دام‌ها با اسپرم منجمد، ضرورت دارد که صفات کیفی اسپرم و مقادیر عناصر سمینال پلاسما اسپرم در فرایند انجماد و نگهداری انجمادی حفظ شود. این مطالعه جهت ارزیابی تأثیر افزودن اتیلن دی آمین تترا استات (EDTA) و پروپیلن گلیکول (PG) بر کلسیم و منیزیم رقیق‌کننده و صفات کمی اسپرم فریز- یخ‌گشایی شده‌ی قوچ قزل انجام گرفت. **روش کار:** نمونه‌های منی از ۵ رأس قوچ قزل ۳ تا ٤ ساله در طول فصل غیرتولیدمثلی یکبار در هفته با ۱۵ تکرار گرفته شد. نمونه‌ها بعداز ارزیابی اولیه و کسب دامنه‌های صفات مورد نظر با رقیق‌کننده‌ی بر پایه‌ی تریس بدون افزودنی (گروه شاهد)، ۱/۷۵ میلی‌مولار EDTA، ۲ درصد PG و ۷ درصد PG (به عنوان جایگزین گلیسرول) رقیق‌سازی شدند. صفات کیفی وکمی اسپرم در روزهای صفر، ۲۰، ٤۰ و ٦۰ بعد از فرآیند فریز- یخ‌گشایی بررسی شدند. **نتایج:** نتایج نشان داد که ارتباط منفی بین مقادیر کلسیم و پارامترهای اسپرم وجود دارد (P<۰/۰۱). همچنین رابطه‌ی منفی بین منیزیم و یکپارچگی غشا وجود داشت (P<۰/۰۱). علاوه بر این ۱/۷۵ میلی‌مولار EDTA و ۲ درصد PG به طور معنی‌داری سطوح کلسیم و منیزیم را در نمونه‌های فریز- یخ‌گشایی شده نسبت به گروه شاهد کاهش دادند (P<۰/۰۵). افزودن ۱/۷۵ میلی‌مولار EDTA و ۲ درصد PG پارامترهای کیفی اسپرم را در مقایسه با گروه شاهد بهبود بخشید (P<۰/۰۵). **نتیجه‌گیری نهایی:** می‌توان نتیجه گرفت که افزودن ۱/۷۵ میلی‌مولار EDTA و ۲ درصد پروپیلن گلیکول به عنوان جایگزین گلیسرول به طور معنی‌داری مقادیر کلسیم ومنیزیم سمینال پلاسما را کاهش داده و خصوصیات اسپرم را بعد از فرآیند فریز-یخ‌گشایی بهبود می‌بخشد.

واژگان کلیدی: پروپیلن گلیکول؛ کلسیم؛ کیفیت اسپرم؛ منیزیم؛ EDTA