https://doi.org/10.52973/rcfcv-e35595 Revista Científica, FCV-LUZ / Vol. XXXV

Comparison of the Preservative Effects of Trehalose, Iodixanol and EDTA in the Cryopreservation of Ram Sperm Collected by Electroejaculation

Comparación De los Efectos Protectores de la Trehalosa, El iodixanol y EDTA en la Criopreservación De Semen De Carnero Recolectado Mediante Electroeyaculación

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ABSTRACT

Improving spermatological quality in frozen ram semen is very important for the development and dissemination of artificial insemination in sheep. For this purpose, researchers have added many substances in various proportions to sperm extenders and investigated the effects of these substances. The presented study compares the protective effects of adding Trehalose, Iodixanol, and EDTA, both individually and in different combinations, to the diluent for freezing ram sperm. The study concluded that; (1) trehalose, iodixanol or EDTA significantly contribute to the preservation of the functional and morphological integrity of spermatozoa, (2) while the addition of iodixanol or EDTA alone to the basic extender failed to increase total and progressive motility after thawing, when used together they produced a synergistic effect and significantly increased total motility, (3) the use of trehalose, iodixanol and EDTA together at appropriate concentrations or the addition of 5% iodixanol or 1.5 g/L EDTA alone to the extender increased spermatozoon persistence after thawing.

Key words: Ram semen; EDTA; Iodixanol; trehalose

RESUMEN

Mejorar la calidad espermatológica del semen congelado de carnero es crucial para el desarrollo y la difusión de la inseminación artificial en ovinos. Para ello, los investigadores han añadido muchas sustancias en diferentes proporciones a los extensores de esperma y han investigado los efectos de estas sustancias. El estudio presentado compara los efectos protectores de añadir Trehalosa, Iodixanol y/o EDTA, tanto individualmente como en diferentes combinaciones al diluyente, para congelar el semen de carnero. El estudio concluyó que: (1) la trehalosa, el iodixanol o el EDTA contribuyen significativamente a la preservación de la integridad funcional morfológica de los espermatozoides, (2) mientras que, la adición de iodixanol o EDTA por sí sola al diluyente básico no aumentó la motilidad total progresiva después de la descongelación, cuando se usaron juntos produjeron un efecto sinérgico y aumentaron significativamente la motilidad total, (3) el uso de trehalosa, iodixanol y EDTA juntos en concentraciones apropiadas o la adición de 5% de iodixanol o 1.5 g/L de EDTA por sí sola al diluyente aumentó la persistencia de los espermatozoides después de la descongelación.

Palabras clave: Semen de carnero; EDTA; iodixanol; trehalosa

Recibido: 19/12/2024 Aceptado: 29/03/2025 Publicado: 02/05/2025

INTRODUCTİON

It is well-documented that sperm freezing induces biochemical, ultrastructural and functional alterations in spermatozoa [1]. Because the plasma and acrosome membranes of spermatozoa are sensitive to freezing, the permeability of the cell membrane increases, which may negatively impact sperm motility, morphology, and chromatin integrity [2 , 3]. Furthermore, hyper-oxidation and the generation of reactive oxygen species take place, potentially leading to damage to the mitochondrial sheath and tail axoneme. Numerous scientific studies have been conducted to reduce these adverse effects and enhance fertilization capacity in frozen-thawed ram semen. To achieve this, permeable cryoprotectants (glycerol, dimethyl sulfoxide), non-permeable cryoprotectants (egg yolk, skimmed milk), sugars (lactose, raffinose, trehalose, glucose), salts (sodium citrate, citric acid), and antioxidants (amino acids, enzymes, vitamins), herbal extracts (ginkgo biloba, capsaicin) were incorporated into the sperm diluent and evaluated [4 , 5 , 6 , 7 , 8].

Trehalose, a disaccharide, protects spermatozoa by creating a hypertonic environment during the glycerolization and equilibration phases and by influencing intracellular crystallization after dehydration. This cryoprotective effect is explained by trehalose's ability to regulate membrane fluidity [9 , 10]. It was also reported that trehalose could change the protein structure of ram sperm during cryopreservation and have antioxidant effect on frozen spermatozoa, participation in glycolysis and increase the tolerance of spermatozoa to various stress factors [11]. Ethylenediaminetetraacetic acid (EDTA) reduces the freezing temperature of disaccharide solutions [1]. Aisen *et al.* [12] indicated that combining trehalose and EDTA in a tris extender provided better protection against the effects of freezing on sperm compared to using trehalose alone. Originally developed as an x-ray contrast agent, iodixanol is now commonly used as a centrifugation medium for isolating live cells due to its non-toxic nature to cells [13 , 14]. The successful results in studies using iodixanol for centrifugation in sperm freezing suggest that the minimal residual iodixanol, which remains with the pellet at the bottom after centrifugation and cannot be aspirated, may have provided a cryoprotective effect. In order to investigate this hypothesis, Saragusty *et al*. [15] tried iodixanol for the first time in the freezing of bull semen and obtained highly positive results.

Although the exact mechanism of action is not yet completely understood, the study revealed that the addition of iodixanol to the bull sperm extender raised the glass transition temperature and altered the structure of ice crystals formed during freezing. This modification transformed the crystals into a form that would either not damage or cause less damage to spermatozoa [15].

No other similar study was found examining the effects of using iodixanol, EDTA and trehalose together or separately on frozen ram semen. However, there are studies investigating the effects of using two of these three substances together or separately [16 , 17 , 18 , 19].

The primary aim of the current study was to create new semen diluent formulations for freezing ram semen collected through electroejaculation. For this purpose, the protective effects of adding Trehalose, Iodixanol, and EDTA separately and in various combinations to the extender were compared in the freezing of ram semen.

MATERIALS AND METHODS

The study received approval from the Ethics Committee of Dicle University Health Sciences Education and Research Centre (2018-51333).

Preparation of extenders

Semen was obtained from five Awassi rams (*Ovis aries*) using an electroejaculator (P-T Electronics, Model 302, Boring, OR, USA). The samples were transported to the laboratory within 30 min of collection. Sperm concentration was measured using an automatic concentration device (IMV, Accucell). High-quality ejaculates (concentration: ≥ 2 × 10^9 /mL; mass motility: ≥4; motility: ≥70%) were combined [16] and then split into 9 equal portions. Each part was prepared with its own diluent. All chemicals, except iodixanol, were obtained from Sigma Chemical Co. Visipaque 320 TM (652 mg/mL of iodixanol in water, Opakim) was used as the origin of iodixanol.Tris diluent was utilized as the basic diluent in the study. The study utilized a two-step dilution method, with glycerol introduced at +5°C [20]. The basic glycerol-free diluent was separated into 9 equal portions and the following diluent groups were formed by adding trehalose (Tr), iodixanol (Io) and EDTA alone or different combinations of these substances:

- Tris (control),
- Io5 (%5 Io, v/v)
- Io10 (%10 Io, v/v),
- Tr50 (50 mM Tr),
- EDTA (1.5 g/L),
- Io5/EDTA (%5 Io + 1.5 g/L EDTA),
- TR50/EDTA (50 mM Tr + 1.5 g/L EDTA),
- TR25/Io5/EDTA (25 mM Tr + %5 Io + 1.5 g/L EDTA)
- TR50/Io5/EDTA (50 mM Tr + %5 Io + 1.5 g/L EDTA)

Spermatological examinations

Sperm motility was evaluated after first dilution, second dilution at +5°C and equilibration using a phase-contrast microscope (X 400; OlympusNBX51) with a warm stage at 37°C. Motility estimates were made in three different microscopic fields for each sample, and the estimates were averaged for the final motility rate. After equilibration, straws were frozen in nitrogen vapour (4.0 cm above the liquid nitrogen) for 10 min and placed in liquid nitrogen until evaluation [20].

Sperm motion parameters were evaluated using a computer-assisted sperm analyser (CASA$^{(TM)}$) system (IVOS 12.3, Hamilton Thorne Biosciences) with the set-up values of Demir *et al* [21]. Each group had three frozen straws thawed at 37°C for 30 s in a water bath and then combined. A 10 µL sample was placed in a pre-heated 10-µm-deep Makler counting chamber (Sefi Medical Instruments), where sperm motility and motion parameters were analyzed at 37°C. About 600–800 sperm cells were examined across ten fields. Each CASA assessment was performed three times per sample. Following the initial CASA analysis conducted immediately after thawing (0 h), the same semen samples were incubated at 37°C for 4 h to assess longevity. Sperm motion parameters were re-evaluated after 1, 2, and 4 h of post-thaw incubation.

Thermal stress test (TST)

After thawing, semen samples were incubated at 46°C for 15 min [16] and immediately analyzed using CASA to assess motility and motion parameters.

Hypo-osmotic swelling test (HOST)

The functional integrity of the sperm plasma membrane was assessed using the modified hypo-osmotic swelling test. In summary, 20 µL of thawed semen was mixed with 350 µL of a sodium sulfate–fructose solution (75 mOsm), incubated in a water bath at 41°C for 45 min, and fixed with 50 µL of formalin-buffered saline. A total of 200 cells were examined under a phase-contrast microscope (X 400; Olympus, BX51) [16].

Morphological examinations

Abnormalities in the acrosome, head, mid-piece, and tail were evaluated using an eosin–nigrosin stain mixture. A total of 200 spermatozoa were examined under a light microscope (X 1000).

Statistical analysis

The study was conducted ten times. Results were presented as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's test to identify differences between groups, utilizing SPSS version 11.0 for Windows (SPSS Inc.). Differences with P-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The results of the study was presented in 6 tables. Motility values after firs dilution, after cooling to 5 degrees and after equilibration are presented in TABLE I.

TABLE I. Comparison of motility rates in fresh semen after initial dilution, cooling to +5 °C and equilibration between groups

No	Group Name	Motility after first diluation(%)	Motility at +5°C (%)	Motility after equilibration (%)
1	Control	78.8±1.11^{ab}	75.2±1.09^{cd}	72.3±2.11^{cde}
2	Io5	82.3±1.35^{b}	78.3±1.42^{d}	75.1±1.68^{e}
3	Io10	82.1±1.16^{b}	79.3±1.30^{d}	74.5±1.67^{e}
4	TR50	78.9±1.38^{ab}	70.8±2.61^{bc}	67.5±1.31^{bcd}
5	EDTA	81.5±1.05^{b}	78.6±1.27^{d}	73.3±1.83^{de}
6	Io5/EDTA	83.5±1.20^{b}	80.4±1.14^{d}	74.7±2.04^{e}
7	TR50/EDTA	79.6±1.54^{ab}	67.9±2.66^{ab}	63.5±2.48^{ab}
8	TR25/Io5/EDTA	79.8±1.46^{ab}	70.7±2.47^{bc}	67.2±1.12^{bc}
9	TR50/Io5/EDTA	75.8±2.27^{a}	64.2±2.97^{a}	58.3±2.85^{a}
P	Value	P<0.05	P<0.0001	P<0.0001

Values are expressed as Mean±Standard Error (Mean±SE). abcd: Differences between values with different letters in the same column are statistically significant

Motility of the groups containing 50 mM Trehalose after + 5°C cooling and equilibration was lower compared the control (tris) and other study groups (P<0.0001). This may be due to the increase in osmotic pressure of trehalose, which may adversely affect spermatozoa. The decrease in our results obtained from the Tr 50 group after equlibration is similar to the results of Cirit *et al* [16] and Özmen *et al* [17]. However, while the total motility rate in the Tr 50 group after equlibration was 80% in Cirit *et al* [16], this rate was found to be 67%. This may be due to the fact that the rams used in these studies were of different breeds (Dorset, Awassi).

The results of the Hypoosmotic Swelling Test (HOST) performed after thawing are presented in TABLE II.

After thawing, the Hypo-osmotic Swelling Test (HOST) results were found to be significantly higher in all study groups (P<0.0001) compared to the control group. This shows that the substances used in the study have a positive effect on membrane functions. In studies conducted with sperm extenders containing different doses of iodixanol and trehalose, it was determined that the control group value in the HOST tests performed after thawing was lower than the other study groups, similar to our study [15 , 16].

The results of the morphological disorder tests performed post thawing are presented in TABLE III.

TABLE II. Comparison of Hypoosmotic Swelling Test (HOST) results between groups after thawing

No	Group İsmi	HOST Positive (%)
1	Control	42.0±2.22^{a}
2	Io5	63.3±3.62^{b}
3	Io10	60.1±1.96^{b}
4	TR50	63.6±1.60^{b}
5	EDTA	63.6±2.78^{b}
6	Io5/EDTA	67.2±1.64^{b}
7	TR50/EDTA	66.5±2.99^{b}
8	TR25/Io5/EDTA	64.6±3.88^{b}
9	TR50/Io5/EDTA	61.6±1.98^{b}
P	Value	P<0.0001

Values are expressed as Mean±Standard Error (Mean±SE). ab: Differences between values with different letters in the same column are statistically significant

TABLE III. Comparison of post-thawing morphological abnormality rates between groups

No	Group Name	Acrosome (%)	Head (%)	Midpiece (%)	Tail (%)	Total (%)
1	Control	22.5±2.37^{b}	1.9±0.40^{b}	1.6±0.30^{a}	2.3±0.45^{a}	28.3±2.52^{b}
2	Io5	14.8±1.03^{a}	0.5±0.23^{a}	1.3±0.43^{a}	1.4±0.45^{a}	17.9±1.21^{a}
3	Io10	14.8±1.51^{a}	0.8±0.28^{a}	1.3±0.30^{a}	1.4±0.31^{a}	18.2±1.48^{a}
4	TR50	11.3±2.20^{a}	0.8±1.22^{a}	1.5±0.44^{a}	2.1±0.29^{a}	15.6±2.23^{a}
5	EDTA	13.9±0.85^{a}	0.3±0.14^{a}	0.7±0.22^{a}	1.7±0.31^{a}	16.6±0.80^{a}
6	Io5/EDTA	13.8±1.33^{a}	0.3±0.19^{a}	0.8±0.34^{a}	1.3±0.33^{a}	16.3±1.07^{a}
7	TR50/EDTA	11.1±1.14^{a}	0.6±0.29^{a}	1.1±0.34^{a}	1.6±0.29^{a}	14.3±1.23^{a}
8	TR25/Io5/EDTA	12.4±1.22^{a}	0.9±0.40^{a}	0.8±0.18^{a}	1.7±0.40^{a}	15.8±1.24^{a}
9	TR50/Io5/EDTA	12.3±0.54^{a}	1.0±0.43^{a}	1.0±0.30^{a}	1.5±0.62^{a}	15.8±0.86^{a}
P	Value	P<0.0001	P<0.05	P>0.05	P>0.05	P<0.0001

Values are expressed as Mean±Standard Error (Mean±SE). ab: Differences between values with different letters in the same column are statistically significant

When the post-thaw acrosomal, head and total abnormality rates were compared, it was found that they were significantly lower in all study groups compared to the control group (P<0.0001). There was no statistical difference between the groups in terms of midpiece and tail abnormalities (P>0.05). Considering these data, it can be said that the substances used provided protection in general. Cirit *et al* [16] obtained the lowest total abnormality rates in ram sperm after thawing from 2.5% iodixanol, 5% iodixanol and 50 mm trehalose groups. The difference between these groups and the control group was found to be significant. Swami *et al* [22] reported in their studies on murrah buffalo that 1.25%; 2.5%; 5% iodixanol did not create any difference with the control group after thawing. In our study, differences were found between all groups and the control group. These results indicate that the effect of iodixanol on the morphology of spermatozoa may be species-specific.

The results of the Thermal Stress Test (TST) performed after thawing were presented in TABLE IV.

TABLE IV. Comparison of Thermal Stress Test (TST) results between groups after thawing

No	Group Name	Total Motility (%)	Progressive Motility (%)
1	Control	43.2±3.98^{bc}	16.1±2.15^{ab}
2	Io5	47.0±5.05^{c}	21.7±2.24^{b}
3	Io10	35.8±3.32^{ab}	15.8±2.14^{ab}
4	TR50	36.8±2.02^{ab}	16.5±1.21^{ab}
5	EDTA	36.1±1.79^{ab}	17.3±1.41^{ab}
6	Io5/EDTA	39.8±2.69^{abc}	22.1±1.83^{b}
7	TR50/EDTA	36.3±3.48^{ab}	14.9±2.11^{a}
8	TR25/Io5/EDTA	32.4±2.14^{a}	14.1±1.54^{a}
9	TR50/Io5/EDTA	30.8±4.25^{a}	12.8±3.02^{a}
P	Value	P<0.05	P<0.05

Values are expressed as Mean±Standard Error (Mean±SE). abc: Differences between values with different letters in the same column were statistically significant.

The highest total motility values in the post-thawing Thermal Stress Test (TST) were obtained in the Io5 group and the difference between this group and all other groups except the control and Io5/EDTA groups was significant (P<0.05). Similarly, the highest progressive motility values were obtained from the Io5/EDTA and Io5 groups. There was a statistical difference between these groups and the TR50/Io5/EDTA, TR25/Io5/EDTA and TR50/EDTA groups, where the lowest values were obtained (P<0.05). Özmen *et al*.[17] found that the group containing 5% iodixanol had the best motility value after TST. When TABLE IV is examined, the best motility values after TST were obtained from the Io5 and Io5/Edta groups. These results also show that there is no negative interaction between iodixanol and edta.

Total and progressive motility values after thawing (0 h) are presented in TABLE V.

TABLE V. Comparison of total and progressive motility of spermatozoa at 0 h after thawing between groups

No	Group Name	Total Motility (%)	Progressive Motilite (%)
1	Control	42.0±1,90^{ab}	22.0±1.13^{cd}
2	Io5	41.8±2.65^{ab}	20.3±1.67^{bc}
3	Io10	44.2±3.21^{b}	18.5±1.82^{bc}
4	TR50	37.1±2.08^{ab}	17.0±1.69^{abc}
5	EDTA	35.9±2.74^{a}	17.2±2.13^{abc}
6	Io5/EDTA	51.9±1.82^{c}	25.7±1.78^{d}
7	TR50/EDTA	39.5±2.48^{ab}	16.2±1.72^{ab}
8	TR25/Io5/EDTA	41.4±2.06^{ab}	16.0±1.17^{ab}
9	TR50/Io5/EDTA	36.3±3.41^{ab}	12.1±2.15^{a}
P	Value	P<0.01	P<0.001

Values are expressed as Mean±Standard Error (Mean±SE). abcd: Differences between values with different letters in the same column are statistically significant

The highest total motility was obtained from the Io5/EDTA group after thawing (at the 0 h) and the difference between this group and all other groups was significant (P<0.01). The Io5/EDTA group had the highest progressive motility value after thawing and the difference between this group and all other groups except the control group was significant (P<0.001). These results are the most significant findings of presented study. When added to the extender individually, iodixanol and EDTA did not have a positive effect on motility after thawing. However, when used together, they significantly increased total motility after thawing. In the study, no positive effect of 5 or 10% iodixanol addition was found on total and progressive motility after thawing compared to tris diluent. This was an unexpected result because Cirit *et al*. [16] reported that the addition of 5%

iodixanol significantly increased total and progressive motility after thawing in rams. This discrepancy between the results may be due to the different iodixanol sources used. While OptiPrepTM (Axis-Shield PoC AS, Oslo, Norway) was used in the study by Cirit *et al*. [16], Visipaque 320TM (Opakim Tıbbi Ürünler Tic. Ltd. Şti., Turkey) was used in the presented study. When these 2 iodixanol sources are compared, it is seen that some of their chemical properties different. For example, OptiPrepTM and Visipaque 320TM densities are 1.320 ± 0.001 g/ml, 320 mg I/ml; osmolality 170 ± 15 mOsm, 290 mOsm respectively. This differences may have affected the results.

Within the scope of the study, total and progressive motility rates of spermatozoa were determined in the study groups at 1, 2 and 4 hours after thawing and the results were presented in TABLE VI.

TABLE VI. Comparison of total and progressive motility of spermatozoa between groups at 1, 2 and 4 hours after thawing.

		1st hour 1h		2st hour 2h		4st hour 4h	
No	Group Name	Total Motility (%)	Progressive Motilite (%)	Total Motility (%)	Progressive Motility (%)	Total Motility (%)	Progressive Motility (%)
1	Control	21.5 ±3.25^{a}	12.2±1.99	19.6±2.49	5.3±0.75	7.2±0.84^{a}	1.4±0.28^{ab}
2	Io5	26.0±2.66^{ab}	12.1±1.58	25.4±4.90	8.1±1.46	17.5±4.42^{bc}	2.7±0.63^{abcd}
3	Io10	32.3±3.72^{abc}	13.8±1.64	23.8±3.62	6.9±1.31	12.1±2.91^{abc}	1.7±0.37^{abc}
4	TR50	28.8±3.68^{abc}	10.8±2.46	27.4±4.41	5.0±0.82	8.5±1.60^{ab}	1.2±0.25^{a}
5	EDTA	33.5±4.58^{bc}	16.0±2.59	22.1±3.48	10.1±2.30	16.4±3.16^{abc}	5.6±1.74^{d}
6	Io5/EDTA	34.1±4.08^{bc}	15.3±2.42	20.0±4.00	8.8±2.05	9.5±1.73^{ab}	3.4±0.98^{abcd}
7	TR50/EDTA	38.8±4.05^{c}	13.9±2.71	22.7±4.76	9.0±2.08	15.1±2.96^{abc}	4.3±1.09^{bcd}
8	TR25/Io5/EDTA	35.8±2.88^{bc}	14.7±2.24	27.3±5.22	11.2±2.89	17.6±3.63^{bc}	4.4±1.18^{bcd}
9	TR50/Io5/EDTA	30.2±4.15^{abc}	13.4±2.87	24.8±4.83	8.9±2.07	20.1±3.65^{c}	4.7±1.15^{cd}
P	Value	P<0.05	P>0.05	P>0.05	P>0.05	P<0.05	P<0.01

Values are expressed as Mean±Standard Error (Mean±SE). abcd: Differences between values with different letters in the same column are statistically significant

The highest total motility values at the end of the 4 h were obtained from TR50/Io5/EDTA, TR25/Io5/EDTA and Io5 groups (20.1, 17.6 and 17.5%, respectively) and the differences between these groups and the control group (7.2%) were statistically significant (P<0.05). In the resilience test, EDTA and TR50/Io5/EDTA groups had the highest progressive motility values at the end of the 4 h (5.6 and 4.7%, respectively) and the differences between these groups and control (1.4%) and TR50 groups (1.2%) were found to be significant (P<0.05).

According to the data of Aisen *et al.* [12], the TR50/EDTA group, which was expected to be one of the best, did not produce the claimed synergistic effect. However, the Io5/EDTA group had higher values for motility after equilibration, after TST and after thawing than the TR50/EDTA group (P<0.01). These data indicate that there is a synergistic interaction between EDTA and iodixanol and that this positive effect is more pronounced than the Trehalose/EDTA combination. However, there are also differences between the studies, such as the method of sperm collected (artificial vagina, electroejaculation) and the different breeds of rams (Pampinta, Awessi). In addition, they show a path for further research in this area.

CONCLUSIONS

The addition of trehalose, iodixanol, or EDTA to the extender for freezing ram sperm collected by electroejaculation significantly contributed to the preservation of spermatozoa's functional and morphological integrity

The addition of iodixanol or EDTA alone to the base extender failed to improve total and progressive motility after thawing; however, when used together, they created a synergistic effect, significantly increasing total motility

The use of trehalose, iodixanol and EDTA in appropriate concentrations together or the addition of 5% iodixanol or 1.5 g/L EDTA alone to the extender increased the durability of spermatozoa after thawing.

Conflict of interest

The authors declare that they have no conflict of interest.

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