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Cryoprotective Effects of Royal Jelly Extenders on Buffalo Bull Spermatozoa

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Abstract

THE purpose of the current investigation was to explore the impact of the three extenders Tris-royal jelly (TRJ), Tris-royal jelly Dimethyl Sulphoxide **(**TRJ DMSO) and Tris-royal jelly Ethylene Glycol (TRJ EG) on freezability of buffalo bull semen. Semen from five mature buffalo bulls was gathered. The main extender was Tris-citric acid- fructose egg yolk (TCFY) . Tris diluent having zero RJ, zero DMSO and zero EG was considered to be the control.

The other extenders were Tris containing Royal Jelly TRJ(0.05%RJ), Tris containing Royal Jelly and Dimethyl Sulfoxyde TRJ DMSO(0.05%RJ+1.5%DMSO) and Tris containing Royal Jelly and Ethylene glycol TRJ EG(0.05%RJ+1.5%EG). The semen specimens were supplementary and neat concentration of spermatooa 60 × 10^6/mL was obtained. Diluted semen was frozen. The frozen post-thawed extended buffalo semen revealed significant amelioration in all the studied parameters of the three extenders (TRJ, TRJ DMSO and TRJ EG) If compared to the control. TRJ DMSO was the best ameliorating of sperm cryosurvivability followed by TRJ and TRJ EG. Significant decrease in post-thawing sperm motility with the advance of time in all the extenders used. Significant elevation of the total antioxidants (TAC) and non- significant decrease of malondialdehyde (MDA) of the three extenders relative to the control. The superior conception rate(CR) was obvious in TRJ DMSO(64.6%) followed by TRJ(60%) and TRJ EG(57%) if compared to the control(40%).

It can be fulfilled that, Royal Jelly DMSO is the best ameliorating for sperm cryosurvivability and fertility followed by Royal Jelly and Royal Jelly Ethylene glycol.

Keywords: Royal Jelly, Dimethyl Sulphoxide, Ethylene Glycol, Semen, Buffalo.

Introduction

Distribution of the super genetic characteristics to ameliorate the genetic structure of farm animals could be achieved via artificial insemination which is considered an essential tool for dissemination of the superior genes [1,2]. The subfertility of bulls used in AI program is the main factor for immense economic losses especially when these bulls have high genetic constitution [3].The normal capacitation and potential to fertilize the oocytes takes place during the journey of the spermatozoa in the female genital tract post various changes involving rearrangement of the spermatozoal membrane and enhancing of sperm motility and metabolic activities [4]. Capacitation is potentiated in the female reproductive tract by the action of bicarbonate and calcium ions[5].The premature capacitation and hazardous acrosome reaction occurring during cryopreservation is attributed to protein and lipid changes of the sperm membrane consequential from excessive permeation of calcium ions with subsequent decrease in vitro fertilizing capacity[6].The laboratory evaluation for sperm capacitation is of an immense value for revealing the normality of spermatozoa post cryopreservation[3].

The objective of preservation of bovine semen is mainly to maximize the outcomes of genetically superior males through semen storage for long periods and increasing the number of doses of semen obtained from each ejaculate with positive effect on animal production, and product quality. Freezing results in deterioration of nearly half of the preserved sperm cells [7] mainly due to the intracellular ice crystalization during cryopreservation [7, 8].So the ingredients of the semen diluent is of an essential value to minimize this damage [9].The diluents of semen storage of animal species must include appropriate pH and buffering ability, proper degree of osmolality and keep the sperm cells from damage during freezing [10, 11].

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Royal Jelly (milk of honey bees) is manufactured by means of the pharyngeal and mandibular glands of the honey bees workers and this is measured as the main nutrients of the bee larvae and queen through the initial seventy two hours of their living. The ingredients of Royal jelly are proteins, sugars, lipids, essential amino acids and vitamins [12, 13]. Royal jelly has valuable metabolic effects in diverse cells and tissues of laboratory animals [14]. Alreshoodi and Sultanbawa[15], recorded antibacterial property of Royal jelly. The cryoprotective outcome of Royal jelly is mainly to its high contents of essential amino acids ingredients having strong antioxidant property via eliminating the excess harmful oxygen free anions [16].

The cryoprotectant Ethylene glycol (EG) with the low-molecular-weight results in little stress on spermatozoa than Glycerol as its low molecular weight renders it liable to traverse the sperm membrane easily and reduces spermatozoal membrane damage through decreasing the ice crystalization [17]. EG has revealed as well superior post-thaw bull sperm motility compared to Glycerol and Dimethyl Sulphoxide, consequential from the decrease in the osmotic stress [18]. The cryoprotectant Ethylene glycol has little deleterious impact on liveability, motility and acrosomal integrity if compared with glycerol [19].

The penetrating cryoprotectant decreases the physicochemical stresses consequential from the semen freezing practice [20]. Dimethyl Sulphoxide (DMSO) is a penetrating substance through the sperm membrane. These penetrating ingredients can pass through the sperm membranes and regulate the degree of sperm dehydration occurring by freezing through transitions of the sperm membrane. Penetrating cryoprotectants supply sperm inside cell safety since these are derived from the outer structure of biomolecules, thus fixing the native normal state [21]. Dimethyl Sulphoxide (DMSO) is a penetrating substance that permeate the sperm membrane simply to substitute the water substance of the spermatozoa and decrease the freezing damage resulting from the ice crystals [22].

The semen freezing results in morphological and vital damages due to excessive release of reactive oxygen species (ROS) [18]. The spermatozoa membrane unsaturated fatty acids are liable to peroxidation causing oxidative deterioration with consequent decreased forward motility, percent of alive sperms and integrity of DNA [23, 24]. So, the objective of the current study was to clarify the impact of the three diluents TRJ, TRJ DMSO and TRJ EG on freezability of buffalo bull spermatozoa.

Material and Methods

Buffalo bulls

Five buffalo bulls (aged 3- 5 years, 600-800 Kg body weight) kept at the Buffalo Semen Freezing Center were chosen as the supply of semen. The buffalo bulls were fed on the ordinary ration and normal management was practised. They were kept under superior general health environment, free from common and reproductive diseases. Feeding: in summer, selected bulls were reserved cool by sprinkling water of minimum humidity, fed during cool hours and have abundance of drinking fresh water. The animals were rationed: in summer,6 kg dried matter+2kg roughage and 3.5 kg dried barseem per animal per day. In winter, 6 kg dried matter+2kg roughage and28 kg barseem/animal/day. Temperature humidity index was :72-78.

Semen gathering for initial Evaluation

Sample of semen from the bulls was harvested by the prepared artificial vagina every week for eighteen weeks. Ejaculates were mainly evaluated for sperm forward motility and sperm concentration. Semen samples with (70%) minimum spermatozoal forward motility and normal morphological spermatozoal percent were collective to get enough semen to eliminate the bull individual variation. Semen samples were kept for ten minutes at 37°C in the water bath prior to extension.

Semen freezing procedures

The crucial diluent was Tris-citric acid- fructose (TCF) which was set as recorded by de Paz *et al.* [25] and Roof *et al.* [26]. The TCFYG diluent was set by adding 3.028 g Tris, 1.678 g citric acid and 2.000 g fructose in 100 mL bi-distilled water, 20% egg yolk and 7% glycerol with a penicillin-streptomycin mixture as antibiotic at 0.01 mL/mL of the extender were added according to Ijaz *et al.* [27]. Tris extender with zero RJ , zero DMSO and zero EG was reserved as a control .The other extenders were Tris containing Royal Jelly TRJ (0.05%RJ), Tris containing Royal Jelly and Dimethyl Sulfoxyde TRJ DMSO (0.05%RJ+1.5%DMSO) and Tris containing Royal Jelly and Ethylene glycol TRJEG (0.05% RJ+1.5% EG) . The seminal specimens were diluted to have neat spermatozoal concentration 60×10^6/mL. Diluted semen specimens having zero Royal Jelly, zero DMSO and zero EG represent the control and the other extenders were considered to be the experimental. Diluted semen was gradually refrigerated (roughly for two hours) up to 5°C and subjected to two hours equilibratation. Semen was put into 0.25 ml polyvinyl French straws and then, the straws were located in a horizontal manner on a special holding rack and exposed to vapor of liquid nitrogen 4 cm far from its top surface about ten minutes and were at once dipped in nitrogen liquid [2].

Assessment of Semen Quality Criteria

The evaluation was carried out after cooling and freezing of buffalo bull semen. Frozen semen straws were exposed to thawing at 37°C for sixty seconds. The characteristics examined were (sperm motility, liveability, morphological abnormalities, sperm membrane and acrosome integrities).

Progressive forward motility:

Sperm motility was evaluated using a drop of diluted semen gently mixed with warmed 2.9% Sodium Citrate dehydrate solution. This drop was put on a glass slide and covered by a clean cover slip, then was investigated by the research microscope (X400). Minimum two hundred spermatozoa from at least four microscopical fields were calculated. Motility was calculated on a standard scale of 0 to 100% [28].

Living sperm percent and morphological abnormality:

Live sperm percent was investigated by eosin-nigrosine stain of standardized smears using bright field microscope (X400). Abnormal spermatozoa were estimated in the same smear, at least two hundred sperms were investigated in five microscopical fields [29].

Sperm membrane integrity [Hypo-osmotic swelling test, (HOST)]:

The hypo-osm: The hypo-osmotic solution (125mOsm/1) was set by adding 6.25 gm of sodium citrate dihydrate and 11.25 gm of fructose in one liter distilled water. A volume of 10µl semen was gently mixed with 1 ml of solution and incubated for sixty minutes at 37°C. Post incubation, a drop of the solution having semen was put on a glass slide, covered with a cover slip and inspected with the research microscope (X400). A total of two hundred spermatozoa were calculated; the percent of spermatozoa positive to the test (HOST) [with curled or swollen tail] was estimated [30].

Acrosome morphology:

Samples of semen were examined by Trypan blue/Giemsa staining with minor modifications [31]. Designed for staining, Trypan blue was applied with a concentration of 0.27%., one drop (five µl) of extended semen and one drop (five µl) of Trypan blue were thorough mixed on a slide and two smears were set from each semen droplet. Stained slides were air-dried in vertical location and then placed in 10% buffered formol saline (9 gm Nacl,6.5gm $Na_2H PO_4$,4 gm NAH_2PO_4 for fixing at 37^0C for 30 minutes. The slides were put into jars containing the Giemsa solution and left for 12 hours. The Giemsa staining solution was newly set by addition of 14.3%(v/v) of Giemsa stock solution (Sigma GS-500) to bidistilled water. Slides were washed once more in bidistilled water, air-dried in vertical manner and coverslipped. Intact acrosomes were purple, the frontal part of the sperm head with no acrosome was light purple.

Viability index:

The post- thawing viability indices were assessed as recorded by Milovanov [32] to be equal to half of the post-thawing sperm forward motility plus the sum of sperm forward motilities post first, second, and third hours of thawing.

Assessment of oxidant/antioxidant parameters

Semen was gathered then centrifuged at 2773 ×g for 5 min at 40^0C using a cooling centrifuge (Sigma 3-18KS, Germany). The seminal plasma was separated and stored at -80°C. The level of total antioxidant capacity (TAC) in the seminal plasma was estimated in accordance with the method of Koracevic *et al.* [33], and lipid peroxidation levels as malondialdehyde (MDA) according to the method of Satoh [34] using test kits of Biodiagnostic Co., Egypt. All measurements were assessed using Double Beam UV/Visible Spectrophotometer, Model T80, UK.

In vivo conception rate (CR)

Artificial insemination was carried out for number of buffalo females (n=245) with the TRE frozen post-thawed semen control diluted in TCFY. Conception rate was done through rectal palpation post two months from the artificial insemination. The artificially inseminated females were chosen through the cooperation with Beni-Suef Governorate. The insemination of females was perfomed using the insemination gun and semen was deposited inside the uterus. The inseminated females were examined via rectal palpation 2 months post-insemination. CR was calculated following the equation:

$$CR = \frac{\text{no.of pregnant buffaloes}}{\text{total no.of inseminated buffaloes}} \times 100$$

Statistical analysis

Statistical analysis data were carried out using the SPSS [35] computerized program v. 14.0 to calculate the analysis of variance (ANOVA) for the different parameters between control and supplement replications. Significant difference between means was computed using Duncan test at $P<0.05$.

Results

The frozen post-thawed extended buffalo semen (Table1) revealed significant amelioration in all the studied parameters of the three extenders (TRJ, TRJ DMSO and TRJ EG) if compared to the control. TRJ DMSO was the best ameliorating of sperm cryosurvivability followed by TRJ and TRJ EG if compared to the control.

Table (2) exhibited significant decrease in post-thawing sperm motility with the advance of time in all the extenders used.

Table (3) showed significant elevation of the total antioxidants (TAC) and non- significant decrease of malondialdehyde (MDA) the three extenders relation to the control.

Table (4) showed the superior conception rate(CR) in TRJ DMSO(64.6%) followed by TRJ(60%) and TRJ EG(57%) if compared to the control(40%).

Discussion

Many causes were recorded to control the freezability of the sperm cells relating to ice crystallization, osmotic stress, deleterious property of the supplemented cryoprotectants and the individual difference [36, 37]. Oxidative damage is the major cause affecting the fertilizing potential and vitality of post-thawed frozen spermatozoa [38,39,40]. Oxidative damage takes place a result of improper ratios of reactive oxygen species (ROS) release and the antioxidant enzyme levels [41]. Over accumulation of oxygen free radicals are hazardous to the spermatozoa [42] despite low levels of these ROS are necessary capacitation of spermatozoa in human, a vital process which is indispensable for the spermatozoa to gain the power of ova fertilization [43]. Upon oxidative effect, spermatozoa are suffering from severe deterioration including peroxidation of membrane fatty acids, DNA damage, [44], decreased mitochondrial function [45,46] and lowered levels of the enzymes linked to motility [47].

Variable antioxidant enzymes are involved in the spermatozoa and seminal plasma mainly the antioxidant enzymes (CAT, SOD, and GSH). Their antioxidant ability is inadequate and commonly decreases along the freezing protocol, so enrichment of the antioxidant ingredients is indispensable to be a fraction of the semen diluent [48].

An immense international interest with the valuable property of hebal products and their different ingredients is remarkable [49]. Freezing-thawing of semen results in deterioration to spermatozoa and the oxygen free radicals are harmful to the sperm plasma membrane mostly the polyunsaturated fatty acids, acrosome and sperm DNA and membrane proteins producing lipid peroxidation of membrane lipids and sperm DNA damage with subsequent decline in the sperm value [7, 50]. However, it is essential for conserving the superior genetic structure of our native buffalos. Semen freezing procedures are accompanied with cryoinjury due to the over production of ROS [51], so, the natural herbal supplement to the diluent improves the antioxidant capacity and as a result ameliorating the fertilizing capacity of frozen-thawed sperm cells [52]. Premature harmful capacitation of sperm applied for artificial insemination is a principal agent of male lowered fertility and is considered a strong indicator for determination of low fertile bulls [3]. The results of the current study clarified improved conception rate in Royal Jelly DMSO followed by Royal Jelly and Royal Jelly Ethylene glycol if compared to the control. The higher CR in these extenders is due to the superior sperm motility, the lowest percent of premature capacitation with the higher post freezing overall antioxidant levels. These findings are in accordance with Mahmoud *et al.* [53] who documented that sperm motility may be an important parameter for semen quality, showing that considerable correlations were originated between sperm motility and both sperm morphological abnormalities and membrane fluidity. Sperm motility may be a good marker for DNA integrity of the sperm cells [54]. Vale [55] reported a conception rate exceeding fifty percent as a satisfactory outcome post artificial insemination with post frozen - thawed semen in buffalo bulls. Al Naib *et al.* [56] recorded bulls with conception rate 50% as super fertile, and the sperm of these bulls is more motile and capable to penetrate mucus and have high potential of oocyte fertilization in vitro.

The improved semen cryopreservation upon using royal jelly enriched extender in the current study is referred to its essential amino acids content that induces an antioxidant outcome by removal of the excess accumulated oxygen free radicals [57, 58]. Bansal and Bilaspuri [59] recorded that low concentrations of these ROS play a vital task in sperm functional activities including capacitation, acrosome reaction, and signaling processes to ensure fertilization. The amino acids fraction in royal jelly improves the sperm forward motility, acrosome capacitation and as a result the fertility potential [60]. The unsaturated fatty acids in royal jelly ameliorate the sperm forward motility [61, 62]. Royal jelly reduces the fatty acid peroxidation as observed from the decreased concentration of MDA [60, 16]. Enrichment of RJ (0.1%) in freezing diluent has saved superior sperm cryoprotection to the sperm membrane. The cryoprotection to spermatozoal membrane by Royal jelly during freezing procedures is linked with the occurrence of essential amino acids such as aspartic acid, cystine, cysteine, tyrosine, lysine, glycine, leucine, isoleucine, and valine. It has been reported that cystine, proline and cysteine act as antioxidants that reduce the oxygen free radicals and generate the enzyme glutathione throughout the freeze-thawing procedure protecting the spermatozoal membranes [63]. Moreover, the Royal

jelly addition of 0.4% RJ to the semen extender in the incubation media maintained superior quality and longevity of spermatozoa, improved sperm quality and fertility and have a fundamental role in the protection of sperm acrosome [64]. The improved results in this study are due to the combined effects of RJ and EG. The inclusion of the cryyoprotectant ethylene glycol in the present study ameliorated semen freezing. The cryyoprotectant ethylene glycol enhanced post-thaw freezing sperm parameters in bull semen [65], in ram [66], in stallion [67,68, 69]and in buffalo [70].

Ethylene glycol has a minor molecular weight and an inferior toxic effect and elevated spermatozoal permeability relation to glycerol [71, 72] with decline of the sperm osmotic damage during preservation [73]. These findings are compatible with the enhanced sperm forward motility at these levels. These findings are compatible with Mahmoud *et al.* [53] who recorded that motility is a real indicator for semen value, exhibiting that considerable relations were significant between sperm motility and both sperm abnormalities and membrane status. However, Buyukleblebici *et al.* [74] recorded no enhancement of post-thaw sperm forward motility in bulls by using ethylene glycol in cryoprotection.

DMSO is a permeating agent into the sperm cells that can penetrate across cellular membranes and change the rate and degree of sperm dehydration during the freezing membrane transition phase. DMSO is a permeable cryoprotectant and get easily through the spermatozoal membrane to replace the water fraction of the sperm cell and decrease the freezing injury caused by ice crystallization [22, 75]. The inclusion of DMSO in the present study improved semen freezing.

El-Harairy *et al.* [76]explored that, the frozen-thawed semen enriched with 3.5% GL plus 3.5% DMSO when added with GSH at levels of 0.2, 0.4 and 0.8mM obviously improved the percentage of frozen-thawed sperm forward motility and sperm cryosurvival and reduced the percent of spermatozoa acrosomal damage and level of extracellular AST, ALT,ACP, ALP and LDH enzymes levels outside the sperm cells and added that, the maximum conception rate was obvious in the cows artificially inseminated with the frozen-thawed bull semen enriched with a mixture of 3.5% DMSO and 3.5% glycerol . Post-thawing sperm forward motility, viability and normal acrosome improved upon using 1.75% DMSO in goat semen diluent [77].

Conclusion

In the present investigation, it could be accomplished that, Royal Jelly DMSO is measured as the best ameliorating for sperm cryosurvivability and fertility followed by Royal Jelly and Royal Jelly Ethylene glycol.

Authorship:

The author had performed all the items of the experimental design, the collection of semen, the diluting concentrations, the freezing process, semen evaluation and the preparing of the manuscript.

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Conflict of interest

The author announce that, there isn't any conflict of interest.

Ethical Approval

The experimental plan was approved by the Medical Research Ethics Committee of the National Research Centre, Dokki, Egypt and its registration number is 19/104 and its date is 10/10/2019.

TABLE 1. Effects of Tris Royal Jelly, Tris Royal Jelly DMSO and Tris Royal Jelly EG extenders on frozen post- thawed extended buffalo bull Semen (Mean±SE).

Diluent	Motility	Alive	Abnormalities	HOST	Acrosome	Viability index	Capacitation
Control (TCFYG)	43.20±.916^{a}	85.40±1.11^{b}	10.00±.63^{b}	57.30±.200^{a}	84.50±1.5^{c}	81.63±.32^{a}	22.67±1.20^{c}
TRJ(0.05%RJ)	62.00±1.22^{b}	86.00±.77^{b}	10.26±.19^{b}	83.45±2.61^{bc}	91.67±.91^{d}	117.33±0.88^{b}	10.67±0.67^{a}
TRJ DMSO (0.05% RJ+1.5% DMSO)	66.00±1.00^{c}	86.92±.77^{b}	10.26±.19^{b}	84.41±1.05^{c}	80.26±.48^{b}	109.00±0.58^{c}	8.33±0.33^{a}
TRJ EG (0.05% RJ+1.5%EG)	57.60±1.12^{d}	71.46±1.02^{a}	17.50±.80^{a}	79.02±.847^{b}	75.67±1.24^{a}	133.50±0.50^{d}	14.00±1.00^{b}
P-value	.000	.000	.000	.000	.000	.000	.000

Means bearing different superscripts between different extenders and differ at 5% of probability. Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG); TRJ (Tris RJ); TRJ DMSO; TRJ EG.

TABLE 2. Effects of Tris Royal Jelly, Tris Royal Jelly DMSO and Tris Royal Jelly EG extenders on post-thaw total motility % of frozen-thawed bull spermatozoa. (Mean±SE).

Hours	Control Tris extender	Tris Royal jelly	Tris Royal Jelly DMSO	Tris Royal Jelly EG	p-value
0	43.20±0.92^{a}	62.00±1.22^{b}	66.00±1.00^{c}	57.60±1.12^{d}	0.00
1	27.00±1.22^{a}	33.00±3.00^{ab}	30.00±4.47^{ab}	39.00±1.90^{b}	0.057
2	19.40±4.45^{a}	29.00±1.80^{ab}	27.00±3.70^{ab}	35.00±2.04^{b}	0.041
3	14.00±2.40^{a}	23.00±1.20^{b}	18.00±3.70^{b}	32.50±1.40^{b}	0.001

Means bearing dissimilar alphabetical superscripts (a, b, c, d) within row are significantly variable at least at P <0.05.

TABLE 3. Effects of Tris Royal Jelly, Tris Royal Jelly DMSO and Tris Royal Jelly EG extenders on Antioxidant concentration-TAC (mM) and MDA concentration (µM).

Diluent	TAC	MDA
Control (Tris extender)	0.22±0.02^{a}	8.60±0.06^{a}
Tris Royal jelly	0.29±.003^{a}	7.8233±0.43^{a}
Tris Royal jelly Dimethyl Sulfoxyde	0.31±.038 ^{ab}	8.09±0.85^{a}
Tris Royal jelly Ethylene glycol	0.32±.03^{b}	8.08±.22^{a}
p-value	.090	.731

Means bearing different superscripts between different extenders and differ at 5% of probability. Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG); TRJ (Tris RJ); TRJ DMSO, TRJ EG.

TABLE 4 . Effects of Tris Royal Jelly, Tris Royal Jelly DMSO and Tris Royal Jelly EG extenders on a field conception rate test in buffalo

Treatment	No of inseminated buffaloes	No of conceived buffaloes	In vivo fertility rate (CR, %)
Control (TCFYG	55	22	40 %
TRJ	60	36	60%
TRJ DMSO	65	42	64.6%
TRJ EG	65	37	57%

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تأثير الحفظ بالتجميد لمخففات الرويال جلى للحيوانات المنوية لطلائق الجاموس

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قسم التكاثر في الحيوان والتلقيح الصناعى - معهد البحوث البيطرية - المركز القومى للبحوث بالدقى – الجيزة – مصر.

استهدفت الدراسة الحالية تقييم كفاءة حفظ الحيوانات المنوية لطلائق الجاموس باستخدام مخففات الرويال جلى اثناء التجميد. تم تخفيف السائل المنوى باستخدام مخفف التريس المحتوى على مخففات الرويال جلى وقد تم تبريد السائل المنوى فى قصيبات وترك 4 ساعات عند درجة5 مئوية ثم تعريضه 10 دقائق وغمره فى النيتروجين السائل للتجميد .تم التقييم أظهرت النتائج تحسن فى صفات السائل المنوى بعد التجميد وكذلك نسبة الحمل وكانت أحسن النتائج فى مخفف الرويال جلى دمسو.

الكلمات الدالة: الجاموس –السائل المنوى-رويال جلى - دمسو - اثيلين جليكول- تجميد