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Cryoprotective Effects of Royal Jelly Ethylene Glycol Diluent on Buffalo Bull Spermatozoa

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Abstract

THE target of this research was to measure the influence of Tris-extender augmented with a combination of Royal Jelly (RJ) and Ethylene glycol (EG) on buffalo sperm preservability. Collective (February –may 2023) buffalo bull semen was extended in Tris extender (Tris with Zero RJ and Zero EG (TCFYG) was reserved as a control. EG was supplementary in the Tris diluent at a concentration1.5% and RJ was added at concentrations (0.05, 0.1, 0.2, 0.3 or 0.4%) . Tris containing EG and the diverse RJ concentrations were reserved as investigational. Diluted semen samples were exposed to semen freezing program. Semen estimation including motility, alive%, abnormality%, intact sperm membrane and acrosome status have been carried out for both refrigerated and frozen-thawed spermatozoa. Regarding Tris Royal jelly Ethylene glycol (TRE) Post- cooling, semen quality was significantly higher in TRE_1 (Tris containing 0.05% RJ) andTRE2 (Tris containing 0.1% RJ). Post freeze-thawing, sperm forward motility was noticeably increased in TRE_4 (Tris containing 0.3% RJ) relative to the control and the other applied RE concentrations. The entire used concentrations of RE considerably elevated the spermatozoal membrane flexibility (HOST) in comparison with the control.

In conclusion, in cooled semen the second concentration pocesses the best semen characteristics and in the after freeze-thawing results the most enhanced sperm criteria and the conception field rate (CR) were in TRE_4.

Keywords: Buffalo; Semen; Preservation; Royal Jelly; Ethylene glycol.

Introduction

Artificial insemination (AI) is the fundamental method for distribution of the best genetic characteristics to ameliorate the genetic structure of the livestock [1 , 2] The objective of preservation of bovine semen is mainly to extend the outcomes of genetically superior males through semen storage for long periods and maximizing the number of doses of semen harvested from a given ejaculate with encouraging influence on cattle productivity, and the product value [3] Freezing results in deterioration of nearly fifty percent of the preserved sperm cells[4] mostly due to the intracellular ice crystals formation through cryopreservation [4, 5],So the ingredients of the semen diluent is of an important value to decrease this stress [6]The diluents of semen storage of animal species are essential to have satisfactory power of Hydrogen and buffer capability, proper Osmol degree and guard sperm cells from cryoinjury[7,8]. Ethylene glycol improved post freezing boar sperm [9]. The semen freezing results in morphological and vital damages due to excessive release of oxygen free anions[10]. The sperm cell outer membrane unsaturated fatty acids are liable to peroxidation causing oxidative deterioration with consequent decreased forward motility, liveability and integrity of DNA [11, 6].

The mandibular and pharyngeal glands of bee workers create royal jelly, also known as bee's milk, which serves as the queen and larvae's primary source of nutrition for the first seventy-two hours of their lives. Lipids, carbohydrates, proteins, vitamins, and essential amino acids are all abundant in royal jelly [12, 13]. In a variety of lab-experimental animal cells and tissues, royal jelly exhibits extremely valuable natural properties [14].Royal jelly has antiseptic impact [14]. The cryopreservative effect of Royal jelly is attributed to its vital amino acids ingredients which applies an antioxidation consequence via removal of extra oxygen free anions [15].

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Ethylene glycol as a cryoprotectant (EG) with the tiny molecular mass results in lower stress on spermatozoa than Glycerol as its low molecular weight renders it vulnerable to pass through the sperm membrane easily and minimize spermatozoal membrane damage via lowering ice crystal formation [16].EG has explored in addition superior post-thaw bull sperm motility relative to Dimethyl Sulphoxide and Glycerol, resulting from the decrease in the osmotic damage [17]. Therefore, the goal of the current study was to determine how buffalo bull sperm preservability was affected by Tris-extender enhanced with a combination of Royal Jelly and ethylene glycol (EG).

Material and Methods

Buffalo males

Five buffalo bulls (matured 3- 5 a long time) kept up at the Abassia Buffalo Semen Solidifying Center, Central Organization for Veterinary Administrations, Service of Agribusiness, Egypt, were chosen as the source of semen. The creatures were kept up beneath uniform standard nourishment and administrative hones. They were in great common wellbeing conditions(600-800 Kg body weight), free from common and genital infections.

Bolstering: among summer, breeding bulls were kept cool and comfortable by sprinkling water at slightest 3-4 times a day, ensured from coordinate wind impacts, housed in a put with comfortable microenvironment with slightest mugginess, encouraged amid cool hours with get to bounty cool drinking water. They (February –may 2023) were nourished:

In summer, 6kg dry matter+ 2kg roughage and 3.5kg dried barseem/animal/day. In winter, 6kg dry matter+ 2kg roughage and28kg barseem/animal/day. Temperature stickiness list:72-78.

Semen Collection and essential Assessment

Semen tests were collected utilizing arranged counterfeit vagina each week for four and half months. The semen ejaculates were mainly assessed for spermatozoal forward motility and sperm concentration. Semen tests with (70%) at slightest sperm motility and ordinary morphological sperm rate were pooled to have sufficient semen to kill the person bull variety. Ten minutes were spent holding the collected semen at 37°C in the water shower. sometime recently weakening.

Semen handling

The essential extender was Tris citric corrosive fructose (TCF) and was arranged concurring to Foote [18].Twenty per cent egg yolk (TCFY) was included. Tris extender with zero RJ and zero EG was saved as a control .EG was included to the Tris extender at a concentration1.5% and RJ was included at concentrations (0.05, 0.1, 0.2, 0.3 or 0.4%). Semen tests were included and flawless sperm concentration 60 × 106/mL was accomplished. Other semen samples containing EG and the various amounts of RJ were retained as tests, and weakened semen tests with zero RJ and zero EG speak to the control. Weakened semen was allowed to equilibrate for two hours after being progressively cooled to 5°C. Pressing semen into 0.25 ml polyvinyl French straws, the full straws were uniformly placed on an unusual rack, exposed to vaporize 4 cm above the surface of fluid nitrogen for 10 minutes, and then immediately submerged in fluid nitrogen.

Evaluation of Semen Quality Factors

Bull spermatozoa that had been thawed and chilled were the subject of the evaluation. One miniature was made by defrosting solidified straws at 37°C. The following features were examined: motility, living percentage, morphological abnormalities, sperm layer, and acrosome status. *Spermatozoal motility:*

A drop of weaker semen was used to calculate dynamic motility using a pre-warmed 2.9% sodium citrate dry out setup. The drop was examined under the magnifying lens (X400) after being placed on a glass slide and fastened with a sanitized cover slip. A minimum of 200 spermatozoa were examined, drawn from at least four minuscule regions. A continuous scale from 0% to 100% was used to measure motility [19]. Live sperm extent and morphological anomalies: Eosin-nigrosine recolor in uniform smears was used to measure the amount of live spermatozoa using shining field optics (X400). At most 200 sperm were counted in 5 small sections, with abnormal spermatozoa being counted within the same spread [20].

Test for hypoosmotic swelling:

By dissolving 6.25 grams of sodium citrate dihydrate and 11.25 grams of fructose in 1000 milliliters of purified water, the hypo-osmotic arrangement (125mOsm/1) was created. After carefully blending 10µl of semen with 1 ml of arrangement, the mixture was hatched for 60 minutes at 37°C. A drop of the semen-containing arrangement was placed on a glass slide, covered with a cover slip, and examined under a microscope (X400) after it had been left to stew. A total of 200 spermatozoa were examined, and the proportion of spermatozoa that tested positive for having a twisted or enlarged tail was calculated [21].Acrosome morphology: It was assessed using Giemsa recolor in accordance with Watson's methodology [21]. On a heated slide, a drop of weak semen was applied and allowed to dry in the sun. After immersing the smears in 10% buffered formal saline for fifteen minutes, they were cleaned under running water. The smears were allowed to dry completely before being immersed in a buffered Giemsa setup in a coplin jar for ninety minutes. Following this, they were quickly

rinsed in refined water and allowed to dry again. Using an oil submersion focal point and a light magnifying lens set to 1000x magnification, the dried smears were examined. In 200 spermatozoa, the rate of regular acrosomal cap was computed. When the recolor was evenly and visibly distributed, the acrosome was regarded as common.

Assurance of oxidant/antioxidant parameters

Semen was collected and centrifuged at 2773 ×g for 5 min at 40C employing a cooling centrifuge (Sigma 3-18KS, Germany). The collected seminal plasma was put away at -80°C. The level of antioxidant capacity (TAC) and lipid peroxidation divisions as malondialdehyde (MDA) in the seminal plasma was decided agreeing to the strategy agreeing to the method of Koracevic et al.[22], using test packs that were provided by Biodiagnostic Co., Egypt. All estimations were performed utilizing Twofold Pillar UV/Visible Spectrophotometer, Demonstrate T80, UK.

Conception rate in vivo

A total of 320 buffalo females were artificially inseminated using TRE solidified post-thawed semen control that was diluted in TCFY. Two months after artificial insemination, rectal palpation was used to determine the pregnancy rate. The Beni-Suef Governorate's affiliation allowed for the use of the inseminated females. Semen was stored inside the uterus during the insemination of females using the insemination weapon. The inseminated females were inspected by means of rectal palpation 2 months post-insemination. CR was calculated taking after the condition:…… CR = no. of pregnant buffaloes / total number of inseminated buffaloes ×100……?

CR=???????

Statistical analysis

The SPSS [24] computer software version 14.0 was used to evaluate factual examination data and conduct the analysis of variance (ANOVA) for the variable criteria comparing the control and enhancement replications. The Duncan test was used to calculate the noteworthy variation between suggests at P <0.05.

Results

Cooled semen

In Tris Royal jelly Ethylene glycol (TRE) Post cooling (Table1), sperm motility was significantly (P<0.004) higher inTRE_2, alive percent was significantly (P<0.000) higher inTRE_1, sperm morphological anomalies were markedly (P<0.000) inferior in all applied concentrations and acrosome percentage was noticeably (P<.011) greater as the control.

Post-thawed semen

Post – thawing (Table 2), sperm forward motion was markedly (P<0.000) elevated in TRE_4 as relative to the additional experimental concentrations of RE and the control. Every one of the applied concentrations of RE were obviously (P<0.000) superior in spermatozoal membrane flexibility comparative to the control.

Table (3) showed significant elevation of the total antioxidants(TAC) and significant decrease of malondialdehyde(MDA) of the applied extenders relative to the control. The best was TRE_4extender.

Conception rate.

The conception rate (Table 4), was the superior in TRE4.

Discussion

Many factors have been postulated to control the spermatozoal cryosurvival comprising ice crystal formation, osmotic stress, harmful effects of the added cryoprotectants and the individual variation [23, 24].Among a variety of factors, oxidative damage affects the fertilizing capacity and vitality of frozen post-thawed spermatozoa [25, 26,27].Oxidative injury occurs a consequence of inappropriate ratios of oxygen anions release and the antioxidation enzymatic levels [28].Extreme production of free anions of oxygen are hazardous towards spermatozoa [29].low ratios of these ROS are essential for spermatozoal human capacitation,a vital activity which is indispensable aimed at the sperm cells to gain their capacity of oocyte fertilization [30]. Upon oxidative effect, spermatozoa are exposed to severe deterioration including peroxidation of membrane fatty acids, DNA damage [31], decreased activity of mitochondria [32] and decreased activation of the enzymes related to motility [33].

Variable antioxidant fractions are incorporated in semen mainly the antioxidant enzymes (CAT, GSH and SOD). Its antioxidative potential is inadequate and regularly decreases along the freezing protocol, So antioxidants enrichment is indispensable to be involved in the semen diluent [34].

There is an immense international attention for the valuable synergistic possessions of natural products and their various components relative to the particular vital ingredient [35].Freezing-thawing of semen results in deterioration to spermatozoa and the oxygen free radicals deteriorate the sperm plasma membrane particularly the polyunsaturated fatty acids, acrosome and sperm DNA and membrane proteins causing lipid peroxidation of membrane lipids and sperm DNA damage with subsequent decline in the sperm characteristics [4,36]. However, it is important for preserving the good genetic properties of the native buffalo breeds. Freezing plan of semen is accompanied with cryoinjury due to the excess production of ROS [37]. Therefore, the natural

supplement to the diluent improves the antioxidant capacity and for that reason enhancing the fertility potential of freeze-thawed sperm cells [38].

In the current research, Tris extender enriched with Ethylene glycol and concentrations of Royal jelly (TRE) after cooling, sperm motility was considerably greater inTRE_2, alive and acrosome percent were significantly higher inTRE_1, sperm abnormalities were significantly lower in all the applied concentrations. Freeze–thawed sperm forward motility was imminently elevated in TRE_4 as correlated to the control and other used concentrations of RE as this concentration gave the maximum cryoprotection of spermatozoa ,while the lower concentrations yielded moderately high protection and the elevated concentration than 0.3% RJ was deleterious to spermatozoa. All applied concentrations of RE were substantially superior in sperm membrane flexibility (HOST) as compared to the control. The pregnancy percent was superior in TRE_4 and this consequence was in accordance with the superior forward sperm motility at this applied concentration with the higher post freezie-thawed total antioxidant levels. These findings are compatible with Elnagar [39], Zahmatkesh et al. [14]and Ghanbari et al. [15] who reported the improving outcome of Royal jelly on male fertility in laboratory animals and also with Moradi et al. [40]who postulated improved sperm quality in rams during cooling .In this regard, Shahzad et al. [41]documented upgraded buffalo spermatozoal motion after application of Tris–extender augmented by 0.05, 0.1, 0.2 and 0.3% than 0.4% royal jelly and the control ones. These authors added that, the sperm liveability, acrosome and plasma membrane fluidity were markedly better in 0.1 royal jelly enriched assembly as compared to other groups. They established their consequences by applying In vitro fertilization and field insemination where the multiplication and conception rates were enhanced with 0.1royal jelly concentration. The improved semen cryopreservation upon using royal jelly enriched extender in the current study is due to its indispensable amino acids fractions that applies an antioxidative outcome via removal of the extra oxygen free anions [42]. Bansal and Bilaspuri[43] documented that reduced levels of these ROS have a vital action in sperm functional activities including capacitation, acrosome reaction, and signaling processes to ensure fertilization. The royal jelly amino acids ingredients advance the sperm forward motility, acrosomal reactivity and as a result the fertilization capacity [44].The essential lipid fraction in royal jelly advance the spermatozoal forward dynamics [45]. Royal jelly upgraded the lipid oxidation as observed by the dropped percent of MDA [14,15].The enclosure of RJ (0.1%) in freezing diluent has preserved superior sperm cryoprotection to the sperm membrane. The protection to spermatozoal membrane by Royal jelly during freezing steps is accompanied with the occurrence of essential amino acids such as aspartic acid, cystine, cysteine, glycine, tyrosine, lysine, leucine, isoleucine and valine. It has been documented that proline protects the spermatozoal membranes and cellular proteins counteracting the damaging effect and proline, cystine and cysteine act as antioxidants that eliminate ROS and produce the glutathione enzyme during the freeze-thawing protocol [46].Furthermore, the Royal jelly biochemical features, Presence of vital amino acids (10-hydroxy-2-decenoic acid) and vitamins (EandC) also have a vital role in the protection of sperm membrane [11].The improved results in this study are attributed to the combined effects of RJ and EG. The addition of ethylene glycol in the present study ameliorated semen freezing. Ethylene glycol upgraded post freeze-thaw sperm parameters in semen of bulls [17] , in ram [47], in stallion [48]and in buffalo [49].

Ethylene glycol has a minor molecular weight and an inferior toxic effect and elevated spermatozoal permeability relative to glycerol with decline of the sperm osmotic damage during preservation [50]. However, Buyukleblebici et al. [51] observed no enhancement of post freeze-thaw sperm cells forward motion in bulls upon using ethylene glycol in cryoprotection. We can come to the conclusion that, in cooled semen the second concentration has the superior sperm characteristics. Sperm parameters of frozen-thawed semen displayed that, the best was particular to the fourth concentration. The post freeze-thawing findings exhibited that, the most ameliorated sperm criteria and pregnancy rate were found in TRE_4.The importance of my study is to improve the buffalo semen preservability and consequently ameliorating the semen fertilizing capacity and the field pregnancy rate after using this frozen-thawed semen in artificial field insemination .

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.

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

TABLE 1. Effect of Tris extender enriched with combination of Royal Jelly and Ethylene glycol on the cooled extended buffalo bull semen (Mean ±SE) .

Diluent	Motility	Alive	Abnormality	HOST	Acrosome
TRE1	81.66±1.66^{a}	91.00±1.00^{c}	7.00±1.15^{a}	78.16±.87^{b}	84.33±.66c
TRE2	95.00±2.88^{c}	80.66±.66^{a}	13.66±.88^{b}	58.21±1.22^{a}	78.33±.66a
TRE3	91.66±1.66^{bc}	86.00±1.00^{b}	7.33±.33^{a}	79.13±1.48^{b}	81.33±1.33b
TRE4	93.33 ±1.66^{bc}	86.33±1.33^{b}	7.33±.33^{a}	72.18±5.87^{b}	80.66±.66ab
TRE5	93.33±1.66^{bc}	84.66±.88^{b}	12.66±.66^{b}	76.07±.90b	81.00±1.00ab
Control	88.33±1.66^{b}	85.66±1.20^{b}	18.33±1.66^{c}	80.69±.73b	80.00±.00ab
P-value	.004	.000	.000	.001	.011

Means bearing different superscripts between different extenders and differ at 5% levels of probability. Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG); TRE1 (Tris RE1); TRE2 (Tris RE2); T RE3 (Tris RE3) , TRE4(Tris RE4) and TRE5(Tris RE5) .

TABLE 2. Effect of Tris extender enriched with combination of Royal Jelly and Ethylene glycol on the post- thawed extended buffalo bull Semen (Mean±SE).

Diluent	Motility	Livability	Abnormality	Membrane integrity	Acrosome
TRE$_1$	45.00±2.88^{b}	69.66±2.60^{b}	18.33±.88^{b}	81.65±.12^{c}	71.00±1.00^{b}
TRE2	48.33±1.66^{b}	66.00±1.00^{b}	18.33±.88^{b}	78.60±.24^{c}	73.33±1.66^{b}
TRE3	48.33±1.66^{b}	58.33±1.66^{a}	21.33±1.33^{b}	79.75±.48^{c}	63.66±1.33^{a}
TRE4	58.33±1.66^{c}	71.33±1.85^{b}	17.66±1.45^{b}	79.04±1.54^{c}	75.66±1.76^{b}
TRE5	6.66±1.66^{a}	70.00±2.88^{b}	17.33±1.76^{b}	60.37±.06^{b}	72.33±2.33^{b}
Control	43.33±3.33^{b}	86.66±3.33^{c}	6.66±.33^{a}	48.66±7.51^{a}	86.66±1.66^{c}
P-value	.000	.000	.000	.000	.000

Means bearing different superscripts between different extenders and differ at 5% levels of probability. Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG); TRE1 (Tris RE1); TRE2 (Tris RE2); T RE3 (Tris RE3) , TRE4(Tris RE4) ,and TRE5 (Tris RE5).

TABLE 3. Effect of Tris extender enriched with combination of Royal Jelly and Ethylene glycol on Antioxidant TAC (mM) and MDA concentrations (µM).

Diluent	TAC	MDA
TRE1	0.32±0.06^{b}	7.93±0.52^{b}
TRE2	0.34±0.04^{b}	7.86±0.46^{b}
TRE3	0.34±0.04^{b}	7.96±0.40^{b}
TRE4	0.36±0.02^{b}	7.33±0.33^{b}
Control (Tris extender)	0.22±0.01^{a}	8.93±0.17^{a}
p-value	0.02	0.01

Means bearing different superscripts between different extenders and differ at 5% levels of probability. Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG); TRE1 (Tris RE1); TRE2 (Tris RE2); T RE3 (Tris RE3) , TRE4(Tris RE4) and TRE5(Tris RE5) .

TABLE 4. Effect of Tris extender augmented with mixture of Royal Jelly and Ethylene glycol on buffalo conception ratio.

Treatment	Number of inseminated animals	Number of conceived animals	In vivo fertility ratio(CR, %)
TRE$_1$	55	30	55 %
TRE2	40	23	58%
TRE3	67	44	66%
TRE4	58	39	68%
TRE5	50	4	7%
Control (TCFYG)	50	26	55.2%

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كفاءة حفظ الحيوانات المنوية بالتجميد لطلائق الجاموس باستخدام مخفف الرويال جيلى ايثيلين جليكول

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الملخص

استهدفت الدراسة الحالية تقييم كفاءة حفظ الحيوانات المنوية لطلائق الجاموس بالتجميد باستخدام مخفف الرويال جيلى ايثيلين جليكول .تم تخفيف السائل المنوى باستخدام مخفف التريس المحتوى على تركيزات مختلفة من الرويال جيلى (0.05, 0.1, 0.2, 0.3, 0.4%) مع اضافة ايثيلين جليكول بتركيز ثابت 5% .1واحتوى التريس على صفر الرويال جيلى و صفر ايثيلين جليكول(الكنترول).تم تعريض السائل المنوى المخفف لبرنامج التجميد . تم التقييم. أظهرت النتائج تحسن فى صفات السائل المنوى بعد التبريد والتجميد وكانت أحسن النتائج مع تركيز 1و% في التبريد ومع التركيز 3و% بعد التجميد وأيضا كانت نسبة العشار هى الأعلى مع نفس التركيز.

الكلمات الدالة: الجاموس، السائل المنوي، الحفظ، غذاء الملكات، الايثيلين جليكول.