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# Comparative Study of Royal Jelly, Turmeric and Wheat Germ Extenders on The Cryosurvivability, Sperm Resistance, Antioxidants and Fertility in Cattle Bulls

**Reda I. El-Sheshtawy[1*], Tamer H. Abd El-Aziz[2] and Soad M. Nasr[2]**

[1] *Animal Reproduction and Al Department, Veterinary Division, National Research Center, Dokki, Giza. Box: 12622*

[2] *Department of Parasitology and Animal Diseases, National Research Centre, Dokki, Giza.*

**Experimental Place:** This work was carried out in Semen Freezing Center, General Organization for Vet. Services, Ministry of Agriculture, Abbasia, and National Research Centre, Cairo, Egypt

**Short title**: Comparative study of different extenders on preserved cattle semen.

The present investigation was conducted to improve the post- frozen thawed sperm characteristics, sperm resistance and antioxidant status by adding royal jelly (TRJ), turmeric (TT) and wheat germ (TW) extracts to extender of cattle bulls. Semen from five mature cattle bulls were used (3- 5 years old, 600-800 Kg Bw.). Ejaculates were collected weekly for 12 weeks by artificial vagina**.** Semen was diluted with Tris Citric Fructose Egg Yolk (TCFY) diluent kept as a control and other semen specimens were extended in TCFY diluents with definite concentrations of turmeric extract, wheat germ extract and royal jelly (200 µl /5ml Tris, 250µl stock /3.75ml Tris and 0.05% /Tris, respectively) to achieve concentration of sixty million spermatozoa/ml. Diluted semen samples were then frozen and stored at -196°C for 2 hours. Semen evaluation was implemented. The results showed amelioration in post- frozen thawed sperm characteristics in the three extenders compared to the control (TRIS). The superior sperm resistance and viability index was recorded in the wheat germ extender (TW) .Higher total antioxidant capacity (TAC) and lower malondialdehyde (MDA) were observed in TW and TRJ extenders compared to the control and TT extenders .The highest rate of conception was recorded in TW and TT extenders followed by TRJ and the lowest conception rate was observed in the control group (TRIS). It could be concluded that, adding TW,TT and TRJ to extenders improves the post-frozen thawed sperm characteristics, the sperm resistance and conception rate relative to the control and the superior was the TW extender.

**Keywords:** Cattle, Semen, Preservation, Royal jelly, Turmeric , Wheat germ.

## Introduction

Semen freezing and artificial insemination have a vital, beneficial effect on cattle productivity and the principal mean for spreading of the superior genetic characteristics to ameliorate the genetic structure of the livestock. Semen from the super genetic bulls can be utilized in insemination of numerous number of cows all over the world via the use of frozen semen and artificial insemination and despite cryopreservation of bull sperm has been advanced, knowledge and technological bases are still needed [1].

Sperm cryopreservation has become an essential tool in animal reproduction. Bull semen

Corresponding author: Reda I. El-Sheshtawy, E-mail: Email: rielsheshtawy@gmail.com, Tel.: +202-01099952962
Orcid number : 0000-0001-5016-5696
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cryopreservation causes major reduction in sperm output. The progress of valuable cryopreservation programs in different kinds of animals is important for successful preservation of semen for extended time periods, preserving its fertilizing capability [2].

The considerable decrease of progressively motile spermatozoa post-thawing may be due to incapability of spermatozoa to produce ATP via mitochondrial activity as a result of mitochondrial impairment, so the achievement of proper artificial insemination procedures in bulls is related to the successful persistence of fertilizing capacity of spermatozoa collected and stored from bulls of superior genetic constitution [2].

During Semen freezing, spermatozoa are influenced by drastic osmotic pressure. This takes place through supplementation and deletion of cryoprotective additives, and also during the freezing-thawing processes [3, 4].

During sperm freezing, extracellular ice formation is formed and the sperm cells are exposed to hypertonic environment because the solute concentration is increased extracellularly. This results in permeation of water out of the sperm cells with dehydration, to keep the osmotic balance intra- and extracellularly. During thawing process, the opposite procedure occurs where the spermatozoa are affected by hypotonic medium [5]. Sperm swelling due to water uptake by hypotonic osmotic factor is more hazardous than shrinkage that takes place by hypertonic osmotic stress, particularly post-freezing due to the increased levels of reactive oxygen species. In this regard, Lone et al. [6] stated that oxidants were significantly increased at pre-freeze and post-thaw stages. So thawing is more deleterious than freezing [7, 8]. Therefore, Enhancement of sperm freezing in bulls is a top target that could be attained via enrichment of the diluted semen with natural antioxidants [9,10] . Royal jelly is composed of proteins, lipids, sugars, vitamins and essential amino acids [11, 12]. Royal jelly has antibacterial effect [13].Turmeric extract is rich in the antioxidant curcumin which is added to semen extenders [14] Wheat germ is a superior source of essential unsaturated fatty acids and vitamin E .These ingredients have been commonly used as antioxidant for ameliorating fertility [15].

## Material and Methods

*Cattle bulls*

Five Cattle bulls (aged 3- 5 years, 600-800 Kg body weight) housed at the Semen Freezing Center, Organization for Veterinary Services, Ministry of Agriculture, Egypt, were chosen to be the supply of semen. The bulls were fed on the standard ration and normal management was applied. They were healthy, free from general and genital diseases. Feeding: during summer, breeding bulls were maintained cool by sprinkling water with least humidity, fed during cool hours and have a free access to cool drinking water. They were fed: in summer, 6 kg dry matter2+ kg roughage and 3.5 kg dried barseem/animal/day. In winter, 6 kg dry matter +2kg roughage and 28 kg barseem/animal/day, temperature humidity index:72-78.

*Semen diluents*

The basic extender was Tris-citric acid- fructose (TCF) that was prepared according to Banday et al. [16] . 20% whole egg yolk (TCFY) was added. The Tris extender was kept as a control.

Turmeric extract: Four grams ground turmeric +60ml ethyl alcohol in a test tube. Another 4gm ground turmeric +60ml distilled $H_2O$ in an extra test tube. Good mixing in every tube using stirrer and then filtration. The filtrate is kept at $4^0$C for 24hrs for evaporation. The remaining in the two tubes was well mixed simultaneously before dissolving in 2ml Tris and set aside (reserve solution).

Turmeric enriched diluent [TEE]: Four test tubes (every one includes 5ml TCFY).The first tube has no turmeric extract (control). The turmeric extract concentration as follows (200 µl /5ml, v/v).

Wheat germ extract: 3.75gm wheat germ + 300 ml (70%) methanol to get alcoholic soluble and water soluble fractions together. Daily stirring for one week in the magnetic stirrer was carried out. The mixture was filtrated and evaporated at 40 ˚C. The remaining reserve solution (68ml distilled $H_2O$ having all the extract fractions) [17] with tiny adaptation.

Wheat germ supplement diluent: Five test tubes were used. The first tube was equipped without wheat germ extract in 4ml Tris extender and kept as a control. The extra tube have wheat germ extract (250µlstock / 3.75ml) .

Royal Jelly enriched diluent: RJ was added to the Tris extender at concentration (0.05%).

*Semen Collection and primary assessment*

Semen collection was carried out by means of prepared artificial vagina every week for 12

weeks. The collected semen specimens were primarily estimated for forward spermatozoal motility and sperm cell concentration. Semen samples with minimum (70%) spermatozoal motility and normal morphological structure were pooled to obtain enough semen in order to avoid the bull effect. Pooled semen samples were kept for ten minute in the water bath at 37°C before dilution.

*Semen freezing*

Semen specimens were extended with Tris Citric Fructose Egg Yolk (TCFY) diluent kept as a control and extra semen specimens were extended in TCFY diluents with variable inclusions of turmeric extract , Wheat germ and Royal Jelly to get concentration of 60 million sperm cells/ml. Extended semen samples were slowly refrigerated to attain 5°C ,then kept at equilibration stage up to two hours. Semen samples was put into 0.25 ml polyvinyl French straws. These filled straws were frozen in liquid nitrogen vapor and immediately plunged into -196°C liquid nitrogen for storage [18] for two months.

*Assessment of Semen Quality Criteria*

Semen evaluation was performed after cooling and freezing. Frozen straws were thawed at 37°C for one minute in water bath and immediately examined. After thawing semen characteristics examined were (motility, liveability, morphological abnormalities, and sperm membrane and acrosome integrity.

*Sperm motility*

Forward motility was assayed subjectively using a drop of extended semen in 2.9% warmed Sodium Citrate dehydrate medium. The drop was positioned on a glass slide and a cover slip, and set under the microscope (X400). Minimum two hundred sperm cells of 4 microscopic fields were investigated. Sperm motility was calculated according to the scale of Zero to 100 percent [19].

*Alive sperm percentage and abnormality*

Alive sperm percent and abnormality were estimated with eosin-nigrosine stain in smears using field magnification (X400). Morphologically abnormal spermatozoa were calculated, 200 sperms were examined in five fields [20].

The hypo-osmotic solution (125mOsm/1) was set via dissolving 6.25 grams of sodium citrate dehydrate + 11.25 grams of fructose /L distilled water. A s ample of 10µl semen was mixed with 1 ml of the hypo-osmotic solution and kept for 1 hour at37°C. Post incubation, a drop of this solution was put on a clean glass slide, with a cover slip and 200 spermatozoa were microscopically investigated (X400) ; the percent of spermatozoa (having swelled or curled tail) were considered positive to [Hypo-osmotic swelling test (HOST) [21].

*Acrosome morphology*

Semen samples were examined by Trypan blue/Giemsa staining with little changes [22]. Trypan blue was used at 0.27% concentration, one drop (5 µl) of extended semen and one drop (5 µl) of Trypan blue were mixed on a slide and two smears were prepared. Slides were air-dried in vertical position then put into 10% buffered normal saline(9 gm Nacl,6.5gm $Na_2H\ PO_4$ ,4 gm $NAH_2PO_4$ for fixing at $37^0$C for 30 min .Slides were placed into jars filled with the Giemsa solution and left overnight. The Giemsa staining solution was freshly prepared by adding 14.3% (v/v) of Giemsa stock solution (Sigma GS-500) to distilled $H_2O$. Slides were washed once more in distilled water, air-dried in vertical situation and cover slipped.

Intact acrosomes were purple, the frontal part of the sperm head having no acrosome was pale purple.

*Viability index*

Spermatozoal motility of frozen-thawed semen was investigated and recorded by means of a phase-contrast warm stage microscope (X 200) post thawing for 1, 2, and 3 hours . The post- thawing viability indices were computed as recorded by Milovanov [23] to be equal to half of the post-thaw sperm motility plus the sum of sperm motilities post first, second, and third hours of thawing.

*Determination of oxidant/antioxidant parameters*

Semen samples were collected then centrifuged at 2773 ×g for 5 min at $4^0_C$ using a cooling centrifuge (Sigma 3-18KS, Germany). The seminal plasma was collected and kept at -80℃. The level of total antioxidant capacity (TAC) in the seminal plasma was determined according to the method of Koracevic et al. [24] and lipid peroxidation contents as malondialdehyde (MDA) according to the method of Satoh [25] using test kits that were supplied by Biodiagnostic Co., Egypt. All assays were measured by Double Beam UV/Visible Spectrophotometer, Model T80, UK.

*In vivo fertility rate (CR)*

A total number of cattle females (number=208) were artificially inseminated with the TR, TW and TT frozen post-thawed semen as well as with the post-thawed semen extended in TCFY (control

group). Fertility rate in vivo was measured by calculating the conception rate(CR) via animal rectal palpation post sixty days from insemination. The used artificially inseminated cows were via the collaboration with Beni-Suef Governorate. CR was calculated following the equation:

$$CR = \frac{\text{no.of conceived cattle}}{\text{total no.of inseminated cattle}} \times 100$$

*Statistical analysis*

The obtained data were analyzed using the SPSS computerized program v. 14.0[26] to calculate the analysis of variance (ANOVA) for the different parameters between control and additives replications. Imminent difference between means was calculated using Duncan test at P<0.05.

## Results

As shown in (Table1); the results exhibited significantly (P<0.0001) higher post-thawing sperm motility, alive sperm and sperm membrane(HOST) integrity percent in TRJ,TT and TW extenders compared to the control (TRIS) .The acrosome integrity (HOST) percent was significantly (P<0.0001) higher in TRJ and TT extenders compared to the control and the TW extender. The viability index was considerably (P<0.0001) superior in TW extender, then come the TRJ and the TT diluents where their viability index were imminently (*P<0.0001*) higher than the control (TRIS) extender.

Results of Table (2) showed significantly (P<0.0001) higher sperm motility in TW and TT extenders at 0 hour post-thawing at $37^0$c.After 1 hour post thawing, sperm motility was significantly (P<0.0001) higher in TW and TRJ extenders and deteriorated in the TT and the control .After 2 hours of post thawing, the TW extender preserved the sperm motility while the TT ,the TRJ and the control deteriorated .After 3 hours post thawing TW extender was the superior in preserving the spermatozoal motility relative to the control ,TRJ and TT extenders . In all extenders as well as the control, the spermatozoal motility decreased with the progress of the time of at $37^0$c.

The results of Table (3) showed that, after1 hour of post thawing, the TW and TRJ extenders revealed significantly (*P<0.0001*) higher total antioxidants(TAC) compared to TT extender and TW and TRJ extenders showed significantly (*P<0.0001*) lower malondialdehyde (oxidative biomarker) relative to the control and the TT extenders .After 2 hours of keeping at $37^0$c,TW and TRJ extenders were significantly (P<0.0001) higher in TAC and lower in MDA if compared to the control and the TT extenders. After 3 hours of TW and TRJ extenders exhibited significantly (P<0.0001) higher TAC and lower MDA if compared to the control and the TT extenders.

Table (4) showed that, the highest rate of conception in TW and TT extenders followed by TRJ and the lowest conception rate was observed in the control group (TRIS).

**TABLE 1. Effect of Tris extender enriched with Royal Jelly, Wheat germ extract and Turmeric extract on the post-thawed extended cattle bull Semen (Mean ±SE).**

| Diluent | Motility | Alive | Abnormalities | Host | Acrosome | Viability index |
|---|---|---|---|---|---|---|
| Royal Jelly (0.05% /tris) | 50.00±2.89$^a$ | 82.33±1.45$^a$ | 6.66±0.33$^a$ | 39.33±0.67$^a$ | 86.66±1.00$^a$ | 83.55±٠.73$^c$ |
| Wheat germ extract (250µlstock /3.75ml tris) | 56.66±1.67$^b$ | 72.00±1.20$^b$ | 7.00±1.2$^a$ | 51.00±1.0$^b$ | 50.66±0.67$^b$ | 154.16±4.16$^d$ |
| Turmeric extract (200 µl /5mltris) | 61.66±1.67$^b$ | 80.00±2.89$^a$ | 7.00±0.58$^a$ | 62.33±1.45$^c$ | 80.66±0.67$^a$ | 77.07 09.١±$^b$ |
| Control (tris extender) | 38.33±1.67$^c$ | 56.66±3.33$^c$ | 8.00±0.58$^a$ | 27.66±1.45$^d$ | 68.50±3.67$^c$ | 49.06± 0.58$^a$ |
| p-value | .0001 | .0001 | .615 (NS) | .0001 | .0001 | .0001 |

Means bearing different superscripts (a, b, c, d ) within column significantly differ, non-significant (NS). Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG).

**TABLE 2. Effect of different extenders on post-thaw total motility % of frozen-thawed bull spermatozoa.**

| Hours | Control (tris extender) | Royal jelly | Turmeric | Wheat germ | p-value |
|---|---|---|---|---|---|
| 0 | 38.33±1.67$^{c}$ | 50.00±2.89$^{a}$ | 61.66±1.67$^{b}$ | 56.66±1.67$^{b}$ | 0.00 |
| 1 | 20.00±0.67$^{d}$ | 31.66±1.66$^{a}$ | 23.33±1.66$^{d}$ | 48.33±1.66$^{ef}$ | 0.00 |
| 2 | 8.00±0.01$^{b}$ | 19.33±0.66$^{cd}$ | 16.00±1.00$^{c}$ | 48.33±1.66$^{e}$ | 0.00 |
| 3 | 2.00±0.00$^{c}$ | 6.66±1.66$^{b}$ | 5.00±0.00$^{b}$ | 33.33±2.92$^{a}$ | 0.00 |

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

**TABLE 3. Effect of Royal Jelly, Wheat germ extract and Turmeric extract on Antioxidant concentration-TAC (mM) and MDA concentration (µM).**

| Hours | Control | | Royal Jelly | | Turmeric | | Wheat Germ | | p-Value | p-Value |
|---|---|---|---|---|---|---|---|---|---|---|
| | TAC | MDA | TAC | MDA | TAC | MDA | TAC | MDA | TAC | MDA |
| 1 Hours | 1.70±0.21$^{de}$ | 9.0±0.5$^{c}$ | 1.88±0.13$^{ef}$ | 5.20±0.26$^{a}$ | 0.92±0.8$^{ab}$ | 7.60±1.10$^{b}$ | 1.7 ±0.15$^{de}$ | 5.90±0.24$^{a}$ | 0.001 | 0.001 |
| 2 Hours | 1.17±0.15$^{bc}$ | 15.30±0.35$^{ef}$ | 2.20±0.36$^{fg}$ | 7.40±0.47$^{b}$ | 0.73±0.15$^{a}$ | 12.90±0.66$^{d}$ | 1.3±0.15$^{cd}$ | 7.6±0.46$^{b}$ | 0.001 | 0.001 |
| 3 Hours | 9.13±0.04$^{ab}$ | 15.90±0.90$^{f}$ | 2.26±0.40$^{g}$ | 9.70±0.30$^{c}$ | 0.63±0.23$^{a}$ | 14.70±0.76$^{e}$ | 1.97±0.15$^{efg}$ | 9.30±0.35$^{c}$ | 0.001 | 0.001 |

Means with different alphabetical superscripts (a, b, c, d ,e ,f ,g ) within row are significantly different.

**TABLE 4. Comparison of Royal Jelly, Wheat germ extract and Turmeric extract enriched in Tris extender on a field conception rate test in cattle bulls.**

| Treatment | Inseminated cows | Conceived cows | In vivo fertility rate (CR %) |
|---|---|---|---|
| TR | 92 | 64 | 69.6% |
| Tw | 60 | 45 | 75.6% |
| TT | 56 | 42 | 75.6% |
| Control(TCFYG) | 52 | 26 | 50.2% |

## Discussion

A variety of factors through the freezing procedures, including abrupt temperature alterations, ice crystals creation and osmotic stress, have been responsible as causes for low sperm quality post-thaw. Sperm cryopreservation is an important technique of fertility upon application of artificial insemination, but cryodamage to cellular components may have harmful effects on sperm function. Moreover, Protein degradation, carboxylation, and premature protein phosphorylation could be the major causes of sperm injury post freezing-thawing measures [27, 28].

Sperm DNA deterioration post-thawing could be occurred because of cryooxidative damage and, to a minor degree, to the commencement of an apoptosis [29].

Many causes have been postulated to control the spermatozoal cryosurvival involving ice crystal formation, osmotic stress, harmful effects of the added cryoprotectants and the individual variation [30, 31].Among a variety of factors, oxidative damage affects the fertilizing capacity and functions of frozen post-thawed spermatozoa [32,33,34]. Oxidative damage takes place as a result of improper between the ratios of reactive oxygen species (ROS) release and the antioxidant

enzyme levels [35]. Extreme levels of oxygen free radicals are hazardous to the spermatozoa [36], low ratios of these ROS are essential for spermatozoal capacitation in human, a vital process that is indispensable for the spermatozoa to gain their capacity of ova fertilization [37]. Upon oxidative effect, spermatozoa are exposed to severe deterioration including peroxidation of membrane fatty acids, DNA damage [38] decreased activity of mitochondria [39,40] and decreased activation of the enzymes related to motility [41].

Variable antioxidant fractions are included in the sperm cells and seminal plasma mainly the antioxidant enzymes (CAT, SOD, and GSH). The antioxidant potential is inadequate and regularly decreases along the freezing protocol, So antioxidants enrichment is indispensable to be involved in the semen diluent [42].

There is an immense international attention with the valuable complementally property of natural products and their various components relative to the particular vital ingredient [43]. Freezing-thawing of semen results in deterioration to spermatozoa and the oxygen free radicals resulted in damage of 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 [44,45] . However, it is important for conserving the good genetic properties of our native buffalo breeds. Freezing program of semen is accompanied with cryoinjury due to the over release of ROS [46].So, the herbal supplement in the diluent improves the antioxidant capacity and accordingly ameliorating the fertilizing capacity of frozen-thawed sperm cells [47].

Our results revealed improved post- freeze thaw sperm characteristics and viability index upon addition of royal jelly, turmeric and wheat germ to the Tris extender prior to the freezing process. This improvement could be explained by the significantly higher total antioxidants and the decrease in the level of malondialdehyde in TRJ, TW and TT extenders compared to the control (TRIS only).In this aspect, Lone et al.[6] recorded that ,at post-thaw stage, total antioxidants were positively correlated with sperm motility, sperm membrane integrity and viability and that these correlations are considered as predicting for bull fertility. These findings are also compatible with that of Alyethodi et al. [48] who recorded an elevated unsaturated fatty acids peroxidation and superoxide anions and inferior total antioxidant capacity (TAC) in the inferior freeze-semen samples compared to the superior freeze-samples with the better semen characteristics. RJ exerts an antioxidant effect through elimination of the excess oxygen free radicals [10]. Moreover, RJ ameliorated the fatty acid peroxidation as indicated by the lowered levels of MDA (malondialdehyde) in spermatozoa [12, 49]. Curcumin which is the chief component in turmeric significantly increased the sperm GSH, therefore enhancing the antioxidant potential of the semen diluent [50]. Wheat germ extract has high antioxidant potential relative to its elevated percent of unsaturated lipids , minerals and vitamins .Unsaturated lipids and vitamins[51 , 52] ; minerals [53] are efficient antioxidants that ameliorated the value of cryopreserved semen by elimination of the over oxygen free radicals[54,55] as indicated by decreased malondialdehyde [12] . Wheat germ oil has been included as a strong antioxidant supplement in natural products for improving fertility [14].

The present results exhibited the highest rate of conception in TW and TT extenders followed by TRJ and the lowest conception rate was observed in the control group (TRIS). These findings coincided with the superior sperm motility in these extenders. Vale [56] postulated a pregnancy rate in buffalo exceeding 50%as an excellent result post AI with frozen post-thawed semen Al Naib et al. [57] recorded bulls with conception rate of about 50% to be super fertile, and the semen of high fertile bulls has more motile sperm and capable in penetrating mucus and have high capability of oocyte fertilization *in vitro.*

Sperm morphology, motility, mitochondrial activities and viability are equally susceptible to cryopreservation-induced damage. Protamine-1–DNA alterations started to be significant throughout the cooling stage of the freeze-thawing program. Freeze-thawing exerts changes in the protamine-1/DNA overall construction in boar sperm [58].

Disturbances in a ram sperm proteome post cryopreservation may change the quality of sperm and its specific machinery to sustain capacitation under *in vitro* conditions [59].In this aspect, Zhang et al.[60] found that sperm samples from the infertile men have a significantly higher proportion of Histone to Protamine than did samples from the fertile men.

Antioxidants used as cryoprotectants may be responsible for improving the proteomics which may ameliorate the efficacy of cryopreserved spermatozoa [61]. In this regard, Pardede et al. [62] and Kmal et al. [63] recorded that protamine and many proteins in sperm and seminal plasma are markers of fertility potential as each of the proteins has a vital role in normal sperm function starting from the formation of sperm structure ,motility, capacitation, cell protection, acrosome reaction, good fertilization, egg activation and embryonic growth.

## Conclusions

It could be concluded that, the TW, TT and TRJ extenders were higher in the post- frozen thawed sperm characteristics, the sperm resistance and conception rate relative to the control and the superior was the TW extender.

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*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 authors 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.

*Conflict of interest*

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

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# دراسة مقارنة لمخففات غذاء ملكات النحل والقرقم وجنين القمح على كفاءة الحفظ بالتجميد و الخصوبة ومقاومة الحيوانات المنوية ، مستوى مضادات الأكسدة فى طلائق الأبقار

**رضا ابراهيم الششتاوى[١] ، تامر حلمى عبد العزيز[٢] وسعاد محمد نصر[٢]**

[١] قسم التكاثر فى الحيوان والتلقيح الصناعى - معهد البحوث البيطرية - المركز القومى للبحوث - القاهرة - مصر.

[٢] قسم الطفيليات وأمراض الحيوان - معهد البحوث البيطرية - المركز القومى للبحوث - القاهرة - مصر.

تهدف الدراسة الحالية الى تحسين خصائص السائل المنوى ومقاومة الحيوانات المنوية ،الخصوبة و مستوى مضادات الأكسدة فى طلائق الأبقار٣-٥ سنوات ،٦٠٠-٨٠٠كجم باستخدام مخففات غذاء ملكات النحل والقرقم وجنين القمح وذلك بعد التجميد.تم تخفيف السائل المنوى باستخدام مخفف التريس ويحفظ ككنترول ويضاف الى التريس غذاء ملكات النحل والقرقم وجنين القمح 200 tris /3.75ml 250µlstock ,5ml/ µl ، 0.05% /tris كمعاملات لنحصل على ٦٠ مليون حيوان منوى/مل .تمت علية التجميد ثم التقييم بعد التبريد والتجميد . أوضحت الدراسة أن جنين القمح أعطى أحسن النتائج فيما يتعلق بمقاومة الحيوانات المنوية والحيوية ونسبة الحمل وكذلك نتائج جيدة مع استخدام القرقم وغذاء ملكات النحل مع زيادة مستوى مضادات الأكسدة وانخفاض مستوى المالون داى الدهايد(عامل الاكسدة). نستخلص من تلك الدراسة ان جميع المخففات المستخدمة قد حسنت من خصائص السائل المنوى ومقاومة الحيوانات المنوية والحيوية بعد التجميد.