

## **Cryopreservation and Quality Assessment of Buffalo Bull (*Bubalus bubalis*) Semen Using New Moringa Extender and Antioxidant Co-q10**

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### **ABSTRACT**

Low fertility in buffaloes with frozen-thawed semen is attributed to sperm damage that is related to formation of ice crystals during freeze/thaw process. This study was conducted to evaluate the freezability of buffalo bull semen extended with four types of extenders, including citrate egg-yolk (CEY, control), CEY with ethanolic Moringa Olifera Leaf Extract (eMOLE) as replacement of antibiotics (CEYm), eMOLE and eMOLE with conventional antibiotic (eMOLEa) with or without Coenzyme Q10 as antioxidant. Semen was subjected to calculate progressive motility, livability, abnormality, acrosome status and integrity of sperm membrane in semen after extending, equilibrating and thawing processes. Four sexually mature buffalo bulls (4-5 y and 400-450 kg LBW) were used for semen collection, and semen was collected twice weekly for 4 successive weeks. Semen ejaculates of  $\geq 70\%$  as mass motility percentage were pooled and extended at 37°C by the four types of extenders without or with 30  $\mu\text{M}$  Co-Q10. Results cleared that progressive motility, livability, abnormality, acrosomal damage and plasma membrane integrity percentages improved ( $P < 0.05$ ) in buffalo semen after dilution, equilibration and thawing, while activity of AST, ALT and LDH in seminal plasma of post-thawed semen reduced ( $P < 0.05$ ) by extension of semen with eMOLE as a new extender. All sperm parameters and enzyme activity were maintained by replacing antibiotics in CEY extender with 4 ml eMOLE. All sperm parameters were improved ( $P < 0.05$ ) and enzyme activity was maintained by adding 30  $\mu\text{M}$  of coQ10 as compared to free extenders. Fertility rate of buffalo cows inseminated with semen cryopreserved in extender (eMOLE+coQ10) was the highest (91.6%), followed by eMOLE without coQ10 (83.3%) and the lowest for control CEY (66.6%). In conclusion, eMOLE as a new extender, is promising for the extension and preservation of buffalo semen. Also, adding 4 ml of eMOLE could be a good substitute for the antibiotic component of CEY as a conventional semen extender. Moreover, supplementing semen extenders with antioxidant addition (30  $\mu\text{M}$  CoQ10) is important to improve function of spermatozoa and sperm fertility of cryopreserved semen of buffaloes.

**Keywords:** buffalo semen, extender, Moringa, antimicrobial, coenzyme Q10, cryopreservation.

### **INTRODUCTION**

Semen cryopreservation methods for buffalo have obtained more importance as application of artificial insemination (AI) with frozen thawed spermatozoa and expanded universally. However, the data about standardized protocol for buffalo cryopreservation production is limited (Akhter *et al.*, 2013). During cryopreservation procedures, sperm cells exposed to oxygen and this could generate reactive oxygen species (ROS), leading to oxidative damage to spermatozoa (Chatterjee *et al.*, 2001). Oxidative stress happened because of the imbalance between the productions of ROS and the defensive activity of antioxidant system responsible for their removal (Walczak *et al.*, 2012). These hazards can be minimized by optimizing appropriate diluting media (extenders) in which spermatozoa are frozen and fortification by supplementation with antioxidant (Sansone *et al.*, 2000; Kadirve *et al.*, 2014).

Bacteria is highly influencing the results of viability and fertility of cryopreserved spermatozoa. Bacteria may affect spermatozoa directly, by adhering to spermatozoa, impairing their motility and inducing acrosome reaction, or indirectly by producing toxins. Subsequently, utilizing different antibiotics in the extender is essential to control these bacteria for obtaining the best results of AI (Thibier and Guerin, 2000; Andrabi, 2009; Mughal *et al.*, 2017).

*Moringa oleifera* (MO) Lam, belongs to the monogeneric family *Moringaceae*. Extract of MO (leaves, seeds and roots) have been broadly examined for numerous potential uses as antioxidant, antibacterial, anti-fertility, anti-inflammatory, antifungal etc. (Sadek *et al.*, 2013; Sokunbi *et al.*, 2015). Moringa leaves considered a rich source of antioxidants, in addition to preventing oxidative stress. Moringa as aqueous and ethanolic leaf extracts were shown to contain compounds with wide-spectrum antibacterial activity, competence for repressing the growth

of gram-positive and negative bacteria (Peixoto *et al.*, 2011). Contents of phenolic and flavonoids in Moringa leaves were almost two or three times that of vegetables (Pakade *et al.*, 2013). According to several authors, phenolic and flavonoids are straight forwardly connected to antioxidant potential by scavenging free radicals (Siddhuraju and Becker, 2003; Lukacinova *et al.*, 2008). Moringa treatment enhanced the reproductive potentials, because of low concentration of free radical scavenging enzymes in sperm cytoplasm (D'cruz and Mathur, 2005). Recently, the positive effect of using MOLE as an extender of Friesian bull semen or as an antioxidant additives was reported by El-Nagar (2017).

Coenzyme Q10 (co-Q10) is non-enzymatic antioxidant, lipid-soluble molecule, found in mitochondrial membrane of every mammal cells. This molecule possesses an important role in the synthesis of adenosine triphosphate (ATP) (Pindaru *et al.*, 2013). Such molecule is also involved in gene expression (Tomono *et al.*, 1986). It is also found endogenously in the mitochondria in the mid-piece region of spermatozoa and involved in energy production (Kapoor and Kapoor, 2013). Antioxidants such as coQ10 can neutralize free radicals and may reduce or even help prevent some of the damage they cause like cell membrane damage, tamper with DNA, and cell death. CoQ10 inhibits lipid peroxidation by preventing the production of lipid peroxy radicals. By preventing propagation of lipid peroxidation, coQ10 protects not only lipids, but also proteins from oxidation (Saeed *et al.*, 2016). The successful use of coQ10 as an antioxidant in *in vitro* maturation medium was proved in rabbit oocytes by Abdel-Khalek *et al.* (2016).

Therefore, the current study was undertaken to evaluate the freezability and fertility of buffalo bull semen extended with four types of extenders, including citrate egg-yolk (CEY, control), CEY with ethanolic Moringa Olifera Leaf Extract (eMOLE) as replacement of

antibiotics (CEYm), EMOLE and eMOLE with conventional antibiotic (eMOLEa) with or without Coenzyme Q10 as antioxidant.

## MATERIALS AND METHODS

The experimental work of this study was carried out at Research Station of Animal Production, El-Gemmezah, Gharbiya Governorate, belonging to Animal Production Research Institute, Egypt, and Biotechnology Department, Faculty of Agriculture, Al-Azhar University.

### Animals and management:

Four healthy mature buffalo bulls aging 4-5 years and 400-450 kg live body weight were used as semen donors and individually housed under semi-open sheds. The external genitalia were typically intact and normal. Feeding system of all experimental bulls included daily diet involved 8 kg concentrate mixture, 4 kg clover hay and 4 kg rice straw. At all day times, fresh water and mineral blocks were available during semen collection period.

### Semen collection:

Semen was collected (twice/week) from the bulls for 4 weeks using an artificial vagina (IVM, France) method, before feeding at 8.00 a.m. Two successive

ejaculates were obtained from each bull at each day of semen collection. The collected ejaculates (32 ejaculates with mass motility of  $\geq 70\%$ ) were taken immediately in water bath (37°C) to the laboratory. On each collection day, semen was pooled and divided into 8 replicates for different semen treatments (4 types of extenders x with or without coQ10).

### Types of extenders and treatment:

Semen was extended using four different extenders as shown in table (1), including: Citrate Egg Yolk (CEY) and served as control, CEY with ethanolic Moringa Olifera Leaf Extract (eMOLE) instead of antibiotics (CEYm), eMOLE supplemented with anti-biotics (eMOLEa) or eMOLE without antibiotics as a new extenders. The eMOLE (Egyptian Scientific Society of Moringa, National Research Center, Egypt) was prepared according to the method of Ugwu Okechukwu *et al.* (2013).

The extenders were kept at 37°C until the dilution. Semen extended with different types of extenders were used without or with 30  $\mu\text{M}$  Co-q10 (Sigma-Aldrich, 1g). Each extender was prepared and warmed in water bath (37°C), and semen was extended for each extender type at a rate of 1:20.

**Table 1. The composition of the four extenders (CEYa, CEYm, MOLEa and eMOLEE).**

| Extender              | Sodium Citrate (g) | eMOLE (ml) | Fructose (g) | streptomycin sulphate (mg) | Penciline (IU) | Saline Solution (ml) |
|-----------------------|--------------------|------------|--------------|----------------------------|----------------|----------------------|
| CEY <sup>(1)</sup>    | 2.9                | -          | 0.5          | 1.0                        | 100.000        | -                    |
| CEYm <sup>(1)</sup>   | 2.9                | 4          | 0.5          | -                          | -              | -                    |
| eMOLEa <sup>(2)</sup> | -                  | 10         | 0.7          | 1.0                        | 100.000        | 100                  |
| eMOLE <sup>(2)</sup>  | -                  | 14         | 0.7          | -                          | -              | 100                  |

<sup>(1)</sup> CEY: Mixture of sodium citrate, fructose, Streptomycin sulphate and Penciline were added to 100 ml distilled water.

<sup>(2)</sup> eMOLE: Mixture of eMOLE, fructose, Streptomycin sulphate and Penciline were mixed in 100 ml saline solution. Egg yolk (16 ml) and glycerol (8 ml) were added to 76 ml of the mixture in CEY and eMOLE.

### Semen cryopreservation:

The diluted semen was placed in a water bath (37°C), then gradually cooled in a refrigerator (5°C) for 4 h as a period of equilibration. Extended semen was kept in iced water bath before filling in straws to keep its temperature (5°C), then straws were filled with semen in 0.25 ml French straws. The equilibrated semen was transferred into processing container and located horizontally in static nitrogen vapor 4 cm on the surface of liquid nitrogen (LN) at -196°C for 10 min, then the straws were placed vertically in a metal canister and immersed completely in LN container for 5 weeks.

Frozen straws were withdrawn from the liquid nitrogen container and semen were thawed at 37°C for 30 seconds, then the semen samples were evaluated.

### Semen evaluation:

Semen after dilution, equilibration and thawing was evaluated for progressive sperm motility percentage under a phase contrast microscope (Penta View LCD Digital Microscope) with closed circuit television (Ewuola and Egbunike, 2010). The motility was examined after dilution, equilibration and thawing. Sperm livability percentage was evaluated using Eosin and Nigrosin Stain (Mughal *et al.*, 2013) by counting 100 spermatozoa per slide. Abnormal sperm percentage was assayed by staining smears with Eosin-Nigrosin according to Campell *et al.* (1956) by light microscope. All sperm cells with coiled or double tail, damaged mid-piece and damaged or detached head were considered abnormal (Ewuola and Egbunike, 2010).

For acrosomal Integrity, the method of Jankovicova *et al.* (2006) was used with slight modification. 0.2% trypan blue solution was prepared. An equal quantity of semen and trypan blue was placed and smeared on a pre-warmed slide and fixed for 2 minutes with fixative solution containing 86 mL of 1N HCL and 14 ml of 37% formaldehyde. The spermatozoa (100 in number) were evaluated for their normal apical ridge by using phase-contrast microscope. Intact acrosome were light colored while damaged acrosome were violet stained.

Plasma membrane integrity was assessed using hypo-osmotic swelling (HOS) assay (Khan and Ijaz, 2008). HOS solution was prepared, 50  $\mu\text{l}$  of semen mixed with 500  $\mu\text{l}$  of pre-warmed 37°C HOS solution and incubated 37°C for 45 minutes. One hundred spermatozoa were counted and the number of spermatozoa showing characteristic swelling of tail, an indicative of intact plasma membrane, was recorded. The PMI was examined after dilution, equilibration and thawing.

### Enzyme activity:

Enzyme activity of aspartate amino-transaminases (AST), alanine amino-transaminases (ALT) and lactic dehydrogenase (LDH) was determined (Young, 1990) in seminal plasma of semen in each extender type after thawing by commercial Kits (Salucea Netherlands) and spectrophotometer (JENWAY-6405 UV/Vis).

### Fertility trail:

Estrous synchronized 36 sexually mature buffalo cows were taken from Al-Gemmezah Research Station.

These cows were randomly divided into 3 similar groups, 12 cows in each. Buffalo cows were synchronized by i.m. injection of 3 ml Estrumate (PGF2 $\alpha$ -Essex Animal Health Friesoythe, Germany). Cows in heat after 48-72 h of synchronization were artificially inseminated with semen with each extender with or without antioxidant. Semen was thawed at a rate of 37 °C for 30 s using filled plastic AI gun close to the cervix by the same inseminator. Pregnancy was diagnosed on day 50 post-AI by rectal palpation.

**Statistical analysis:**

The collected data were statistically analyzed by ANOVA in factorial design (4 types of extender x 2 antioxidants) using SPSS (2013) program. The significant differences at least at P<0.05) were tested using Duncan Multiple Rang test (Duncan, 1955). Arcsine transformation before performing the analysis of variance was done for the percentage values, then means as percentages were recalculated from the transformed values.

**RESULTS**

**Characteristics of spermatozoa in buffalo semen after dilution:**

Data in Table (2) cleared that all characteristics of spermatozoa were significantly (P<0.05) better in semen diluted with eMOLE extender, poorer in semen diluted with eMOLEa, but did not differ in semen diluted with CEYm as compared to that diluted with CEY.

As affected by coQ10 addition, all sperm parameters were better in semen diluted with than without coQ10 addition. However, the interaction effect between type of extender and coQ10 on all sperm parameters was not significant (Table 2), reflecting the best characteristics in buffalo semen diluted with eMOLE with coQ10.

It is of interest to observe that all characteristics were significantly (P<0.05) lower in semen diluted with eMOLEa as compared to the same extender without antibiotics even the control extender (Table 2).

**Table 2. Characteristics of spermatozoa in post-diluted buffalo semen as affected by type of extender, coQ10 addition and their interaction.**

| Item                    | Characteristics (%) of spermatozoa |                    |                    |                    |                    |
|-------------------------|------------------------------------|--------------------|--------------------|--------------------|--------------------|
|                         | Motile                             | Live               | Abnormal           | Acrosome damage    | HOS-t              |
| Extender type (ET):     |                                    |                    |                    |                    |                    |
| CEY (control)           | 65.63 <sup>d</sup>                 | 64.63 <sup>d</sup> | 17.94 <sup>d</sup> | 17.50 <sup>d</sup> | 63.13 <sup>d</sup> |
| CEYm                    | 67.19 <sup>b</sup>                 | 65.44 <sup>b</sup> | 16.94 <sup>b</sup> | 16.75 <sup>b</sup> | 64.13 <sup>b</sup> |
| eMOLEa                  | 60.00 <sup>c</sup>                 | 59.81 <sup>c</sup> | 21.44 <sup>a</sup> | 20.19 <sup>a</sup> | 57.56 <sup>c</sup> |
| eMOLE                   | 72.19 <sup>a</sup>                 | 72.00 <sup>a</sup> | 14.25 <sup>c</sup> | 13.69 <sup>c</sup> | 70.31 <sup>a</sup> |
| SEM                     | 1.09                               | 1.23               | 0.57               | 0.57               | 0.96               |
| Antioxidant (A):        |                                    |                    |                    |                    |                    |
| Without coQ10           | 63.91                              | 62.75              | 19.09              | 18.50              | 61.31              |
| With coQ10 (30 $\mu$ M) | 68.59                              | 68.19              | 16.19              | 15.56              | 66.25              |
| SEM                     | 0.77                               | 0.87               | 0.39               | 0.40               | 0.68               |
| P-value                 | 0.000***                           | 0.000***           | 0.000***           | 0.000***           | 0.000***           |
| Interaction (ET x A):   |                                    |                    |                    |                    |                    |
| P-value                 | ns                                 | ns                 | ns                 | ns                 | ns                 |

Means in the same column for each factor with different superscripts differ significantly (P<0.05).

ns: Not significant. \*\*\* Significant at P<0.001.

**Characteristics of spermatozoa in buffalo semen after equilibration:**

Data in Table (3) revealed the same trend of differences in characteristics of spermatozoa among different extender types or in extender without or with

coQ10 in semen after equilibration as found in semen after dilution. However, percentages of motility, livability and HOS-t were lower, while percentages of abnormality and acrosome damage were higher in semen after equilibration than in that after dilution.

**Table 3. Characteristics of spermatozoa in post-equilibrated buffalo semen as affected by type of extender, coQ10 addition and their interaction.**

| Item                    | Characteristics (%) of spermatozoa |                    |                    |                    |                    |
|-------------------------|------------------------------------|--------------------|--------------------|--------------------|--------------------|
|                         | Motile                             | Live               | Abnormal           | Acrosome damage    | HOS-t              |
| Extender type (ET):     |                                    |                    |                    |                    |                    |
| CEY (control)           | 60.94 <sup>d</sup>                 | 61.44 <sup>d</sup> | 20.81 <sup>d</sup> | 19.63 <sup>d</sup> | 59.25 <sup>d</sup> |
| CEYm                    | 61.44 <sup>b</sup>                 | 61.25 <sup>b</sup> | 20.06 <sup>b</sup> | 19.50 <sup>b</sup> | 59.75 <sup>b</sup> |
| eMOLEa                  | 48.75 <sup>c</sup>                 | 50.06 <sup>c</sup> | 25.94 <sup>a</sup> | 24.94 <sup>a</sup> | 47.63 <sup>c</sup> |
| eMOLE                   | 69.38 <sup>a</sup>                 | 69.06 <sup>a</sup> | 15.69 <sup>c</sup> | 15.44 <sup>c</sup> | 66.94 <sup>a</sup> |
| SEM                     | 1.14                               | 1.12               | 0.64               | 0.57               | 1.05               |
| Antioxidant (A):        |                                    |                    |                    |                    |                    |
| Without coQ10           | 58.75                              | 59.28              | 21.72              | 20.91              | 57.25              |
| With coQ10 (30 $\mu$ M) | 61.50                              | 61.63              | 19.53              | 18.84              | 59.53              |
| SEM                     | 0.81                               | 0.79               | 0.45               | 0.40               | 0.74               |
| P-value                 | 0.019*                             | 0.041*             | 0.001***           | 0.001***           | 0.034*             |
| Interaction (ET x A):   |                                    |                    |                    |                    |                    |
| P-value                 | ns                                 | ns                 | ns                 | ns                 | ns                 |

Means in the same column for each factor with different superscripts differ significantly (P<0.05).

NS: Not significant. \* Significant at P<0.05. \*\*\* Significant at P<0.001.

**Characteristics of spermatozoa in buffalo semen after thawing:**

Data in Table (4) showed similar differences in all characteristics among different extender types or in extender without or with coQ10 in semen after thawing as

found in those after dilution and equilibration. There was marked decrease in percentages of motility, livability and HOS-t and marked increase in percentages of abnormality and acrosome damage in semen after thawing as compared to in semen after dilution and equilibration.

**Table 4. Characteristics of spermatozoa in post-thawed buffalo semen as affected by type of extender, coQ10 addition and their interaction.**

| Item                  | Characteristics (%) of spermatozoa |                    |                    |                    |                    |
|-----------------------|------------------------------------|--------------------|--------------------|--------------------|--------------------|
|                       | Motile                             | Live               | Abnormal           | Acrosome damage    | HOS-t              |
| Extender type (ET):   |                                    |                    |                    |                    |                    |
| CEY (control)         | 44.67 <sup>b</sup>                 | 47.31 <sup>b</sup> | 28.56 <sup>b</sup> | 26.88 <sup>b</sup> | 44.94 <sup>b</sup> |
| CEYm                  | 46.25 <sup>b</sup>                 | 48.50 <sup>b</sup> | 28.06 <sup>b</sup> | 26.56 <sup>b</sup> | 45.56 <sup>b</sup> |
| eMOLEa                | 39.38 <sup>c</sup>                 | 41.75 <sup>c</sup> | 31.62 <sup>a</sup> | 29.44 <sup>a</sup> | 38.38 <sup>c</sup> |
| eMOLE                 | 56.25 <sup>a</sup>                 | 57.38 <sup>a</sup> | 21.69 <sup>c</sup> | 20.69 <sup>c</sup> | 58.38 <sup>a</sup> |
| SEM                   | 1.13                               | 1.28               | 0.58               | 0.62               | 0.88               |
| Antioxidant (A):      |                                    |                    |                    |                    |                    |
| Without coQ10         |                                    |                    |                    |                    |                    |
| With coQ10 (30 µM)    | 44.38                              | 46.37              | 28.81              | 26.84              | 44.38              |
| SEM                   | 48.91                              | 51.09              | 26.17              | 24.94              | 49.25              |
| SEM                   | 0.80                               | 0.90               | 0.41               | 0.44               | 0.62               |
| p-value               | 0.000***                           | 0.001***           | 0.000***           | 0.003**            | 0.000***           |
| Interaction (ET x A): |                                    |                    |                    |                    |                    |
| P-value               | ns                                 | ns                 | ns                 | ns                 | ns                 |

Means in the same column for each factor with different superscripts differ significantly (P<0.05).

NS: Not significant. \*\* Significant at P<0.01. \*\*\* Significant at P<0.001.

#### Enzyme activity in seminal plasma of semen after thawing:

Data in Table (5) showed the lowest activity of AST, ALT and LDH in seminal plasma of thawed semen diluted with eMOLE as compared to other types of extenders, but the differences were significant (P<0.05) only for ALT and LDH activities. However, the corresponding activities were the significantly (P<0.05) the highest in seminal plasma of thawed semen diluted with eMOLEa, reflecting the best sperm traits with eMOLE and opposite trends in eMOLEa.

On the other hand effect of coQ10 addition resulted in significant (P<0.05; P<0.01) reduction of all enzyme activities in seminal plasma as compared to those without coQ10. The interaction effect between type of extender and coQ10 on all enzyme activities was not significant (Table 5), reflecting the lowest enzyme activity in buffalo semen diluted with eMOLE with coQ10.

**Table 5. Enzyme activity of AST, ALT and LDH in seminal plasma of post-thawed buffalo semen as affected by type of extender, coQ10 addition and their interaction.**

| Item                  | Enzymatic Activity |                     |                     |
|-----------------------|--------------------|---------------------|---------------------|
|                       | AST                | ALT                 | LDH                 |
| Extender type (ET):   |                    |                     |                     |
| CEY (control)         | 29.40 <sup>b</sup> | 27.40 <sup>b</sup>  | 267.20 <sup>b</sup> |
| CEYm                  | 28.00 <sup>b</sup> | 23.90 <sup>bc</sup> | 240.40 <sup>c</sup> |
| eMOLEa                | 43.30 <sup>a</sup> | 35.10 <sup>d</sup>  | 288.60 <sup>a</sup> |
| eMOLE                 | 26.20 <sup>b</sup> | 21.20 <sup>e</sup>  | 224.20 <sup>c</sup> |
| ESM                   | 1.46               | 1.28                | 6.24                |
| Antioxidant (A):      |                    |                     |                     |
| Without coQ10         |                    |                     |                     |
| With coQ10 (30 µM)    | 33.75              | 28.40               | 263.45              |
| SEM                   | 29.70              | 25.40               | 246.75              |
| SEM                   | 1.03               | 0.90                | 4.41                |
| P-value               | 0.009**            | 0.025*              | 0.012*              |
| Interaction (ET x A): |                    |                     |                     |
| P-value               | ns                 | ns                  | ns                  |

Means in the same column for each factor with different superscripts differ significantly (P<0.05).

NS: Not significant. \*Significant at P<0.05. \*\* Significant at P<0.01.

#### Fertility trail:

Insemination of buffalo cows with thawed semen diluted with eMOLE+coQ10 resulted in the best fertility (conception rate of 91.66%), followed by that diluted with eMOLE without coQ10 (conception rate of 83.33%) and was the lowest for control extender, CEY (conception rate of 66.66%) as shown in Table (6).

**Table 6. Conception rate of buffalo cows artificially inseminated with frozen semen extended with CEY, and eMOLE with or without CoQ10.**

| Item                    | Conception rate |                     |                  |
|-------------------------|-----------------|---------------------|------------------|
|                         | Control (CEY)   | eMOLE without CoQ10 | eMOLE with CoQ10 |
| Inseminated animals (n) | 12              | 12                  | 12               |
| Conceived animals (n)   | 8               | 10                  | 11               |
| Rate of conception      | 66.66           | 83.33               | 91.66            |

## DISCUSSION

The current study aimed to evaluate the freezability of buffalo semen with eMOLE without or with antibiotic (eMOLEa) as new types of extenders, or as a replacement for the antibiotic components of citrate egg-yolk (CEYm) as compared to a conventional type of extenders (CEY). These four types of extenders were also evaluated without or with adding coQ10 at levels of 30 µM. In this study motility, livability, abnormality, acrosome damage and plasma membrane integrity (HOS-t) were evaluated in semen after dilution, equilibration and thawing. It is well reported that sperm viability decreases more than 50% during cryopreservation process due to exposing spermatozoa to chemical / toxic, osmotic, thermal, and mechanical stresses during the stages of dilution, cooling, equilibration, or freezing and thawing process (Andrabi, 2009). The marked reduction in viability, livability, membrane integrity and acrosome occurred through freezing processes, and subsequently sperm morphological abnormalities increase (Meyers, 2005; Pena *et al.*, 2009). During recent years, *Moringa oleifera* leave extract (MOLE) as aqueous, acetone and ethanolic extract possess antibacterial potential properties. Noteworthy that ethanolic

extract of the plant demonstrated higher activity than aqueous extract, in term of antibiotics, ciprofloxacin, cotrimoxazole and chloramphenicol contents (Doughari *et al.*, 2007). The recent results of El-Nagar (2017) on usage of MOLE as aqueous extract as an extender or as natural antioxidant for freezing Friesian bull semen encourage us to use MOLE as ethanolic extract or as a replacement of antibiotics in semen extender.

According to the obtained results, all sperm characteristic used in the current study were positively improved by using eMOLE or still to be as for control extender (CEY), when eMOLE was used as a replacement of antibiotic. These findings could be attributed to that MOLE has antioxidant and antimicrobial properties as compared CEY or the industrial antibiotics used in this conventional extender. Such results are in agreement with Sokunbi *et al.* (2015), who successfully used MOLE as a replacement of antibiotic in bull semen extender as well as El-Nagar (2017), who used MOLE as an extender or as an antioxidant. Addition of antibiotics to semen extender was one of the first major advances to significantly improve the fertility potential of artificial insemination (AI) in bovine (de Jarnette *et al.*, 2004). High percentage of normal sperm morphology is an indication of reduced microbial activities on the sperm cells (Sokunbi *et al.*, 2015). Enhancement of fertility potential in bovine following AI is critically depending on adding of antibiotics to semen extender (de Jarnette *et al.*, 2004). The present results are in agreement with current international standards of Certified Semen Services (CSS, 2011) regarding the antibiotic components of semen extenders and their recommendation for alternatives to streptomycin and penicillin (S&P) in extender for buffalo bull semen preservation. For many years, S&P considered the traditional antibiotic combination added to the diluents of buffalo bull semen. However, some of the microorganisms, which were previously sensitive to S&P, may have become resistant to these antibiotics. (Andrabi *et al.*, 2001; Hasan *et al.* 2001; Akhter *et al.*, 2007).

Some authors put in evidence that none of these antibiotics was tested, in particular, S&P, which were completely effective against bacterial growth in buffalo semen (Hasan *et al.*, 2001; Akhter *et al.*, 2008). An alternative combination of antibiotics is needed in semen extenders. According to Peixoto *et al.* (2011), ethanolic and aqueous MOLE contain several compounds with wide-spectrum anti-bacterial activity, which inhibit gram-positive and negative bacteria growth. Based on the present results, replacement of traditional antibiotic (S&P) with eMOLE proved to be efficient in eliminating bacterial growth and maintain semen characteristic after dilution, equilibration and thawing. Furthermore, MOLE is considered as a potent antioxidant due to its phenolic compounds, carotenoids, alkaloids, and proanthocyanidins, leading to acting as free radical terminators, reduces the destructive effects of oxidative stress, and decreases the need of antioxidant enzymes to counteract the increased free radicals (Pourmorad *et al.*, 2006; Arabshahi *et al.*, 2007).

Concerning the effect of coQ10 on animal reproduction, it was reported that the deficiency in CoQ10 may be the cause of asthenozoospermia in some male

(Lewin *et al.*, 1997), and incubation of sperm cells with CoQ10 improved the pattern of bull sperm cells motility (Ibrahim *et al.*, 2011; Thakur *et al.*, 2013). This also proved in our study, whereas all extenders enhanced sperm parameters with coQ10 as compared to without addition, indicating antioxidant effect of coQ10 in buffalo semen extenders. This role was indicated in *in vitro* maturation medium of rabbit oocytes (Abdel-Khalek *et al.*, 2016). Moreover, several authors reported that the fierce ROS effect on mobility and acrosomal damage of sperm cells could be diminished by CoQ10, especially in thawed semen (Saeed *et al.*, 2016; Talevi *et al.*, 2013).

The fertility rate is considered to be the best parameter to assess the quality of frozen-thawed semen (Vale, 1997). The final aim of buffalo bull sperm cryopreservation is the production of fertilized eggs after AI. A successful AI requires that a significant number of viable, fertile sperm cells are delivered at the site of fertilization in appropriate time (El-Sharawy *et al.*, 2012). The recorded higher conception rate (91.6%) achieved by cows inseminated by semen extended with eMOLE with coQ10 in the current study is satisfactory and mainly attributed to improving characteristics of spermatozoa in thawed semen, as well as lower activity of AST, ALT and LDH enzymes. Similarly, El-Nagar (2017) found the highest conception rate of Friesian cows artificially inseminated with semen extended with MOLE as compared to Tris egg-yolk. Such results were supported by the theory that, good quality of semen was characterized by lower AST, ALT and LDH of enzyme activity (Taha *et al.*, 2000; Borah *et al.*, 2015, El-Nagar, 2017). Generally, reducing activity of enzyme in seminal plasma of thawed semen is an indicator of membrane integrity, and subsequently sperm motility (El-Harairy *et al.*, 2011)

## CONCLUSION

Based on the foregoing results, eMOLE as a new extender, is promising for the extension and preservation of buffalo semen. Also, adding 4 ml of eMOLE could be a good substitute for the antibiotic component of CEY as a conventional semen extender. Moreover, supplementing semen extenders with CoQ10 as an antioxidant at a level of 30  $\mu$ M is able to enhance sperm function and fertility of cryopreserved buffalo semen.

Further studies are required to evaluate different concentration of Moringa in extenders as antibiotics or antioxidants addition for improvement the thawed semen quality and fertility of buffalo bull semen. We need a new generation of semen extenders free from chemical, commercial antibiotics and based on Nature extracts according to guild lines of Certified Semen Services (CSS). Also, more studies are required to better understanding the properties of Moringa for enhancement of fertility characteristics of buffalo bull semen quality.

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## تجميد وتقييم جودة السائل المنوي لطلائق الجاموس باستخدام مخفف المورنجا الجديد ومضاد الأكسدة كوايزيم كيو 10

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الخصوية المنخفضة لطلائق الجاموس باستخدام السائل المنوي المجمد ترجع إلى الضرر الحادث للحيوانات المنوية نتيجة تكوين بللورات ثلجية أثناء عملية التجميد والإسالة. هذه الدراسة أجريت لتقييم السائل المنوي المجمد لطلائق الجاموس باستخدام أربع أنواع من المخففات مع أو بدون إضافة كوايزيم كيو 10 بمستوي 30 مللي مول كمضاد للأكسدة (مخفف السترات ككترول، مخفف السترات مع استبدال المضاد الحيوي التقليدي بالمستخلص الكحولي لأوراق المورنجا، مخفف المستخلص الكحولي لأوراق المورنجا مع أو بدون استبدال المضاد الحيوي التقليدي كمخفف جديد). خصائص السائل المنوي وتشمل النسبة المئوية للحركة الفردية والحيوانات المنوية الحية والشاذة وذات الأكروسوم غير السليم والمتلوية الذيل قيمت في السائل المنوي بعد التخفيف وفترة الموازنة والإسالة. تم تجميع السائل المنوي من 4 طلائق جاموس ناضجة جنسيا (عمر 4-5 سنوات ووزن يتراوح ما بين 400 إلى 450 كيلو جرام) مرتين أسبوعيا لمدة 4 أسابيع متتالية. القننات ذات الحركة الجماعية أكبر من أو يساوي 70% تم خلطها وتخفيفها باستخدام 4 مخففات مختلفة تم تقسيمها إلى 8 أجزاء متساوية على درجة حرارة 37 درجة مئوية نصف العينات تم إضافة لها 30 مللي مول كوايزيم كيو 10 والنصف الآخر بدون إضافة. أظهرت النتائج أن كل خصائص السائل المنوي (النسبة المئوية للحركة الفردية والحيوانات المنوية الحية والشاذة وذات الأكروسوم غير السليم والمتلوية الذيل) بعد التخفيف وفترة الموازنة والإسالة تحسنت معنوياً (0.05) والنشاط الإنزيمي (AST, ALT, LDH) في بلازما السائل المنوي بعد الإسالة إنخفض بصورة معنوية (0.05) في مخفف المستخلص الكحولي لأوراق المورنجا كمخفف جديد. كل خصائص الحيوان المنوي والنشاط الإنزيمي أبقيا باستبدال المضاد الحيوي بـ 4 مللي من المستخلص الكحولي لأوراق المورنجا مستخلص. كل خصائص الحيوان المنوي تحسنت معنوياً (0.05) والنشاط الإنزيمي أبقى بإضافة 30 مللي مول من كوايزيم كيو 10 مقارنة بالمخففات التي بدون إضافة الكوايزيم كيو 10. معدل الخصوية في إناث الجاموس الملقح بالسائل المنوي المجمد بمخفف (المستخلص الكحولي لأوراق المورنجا مع إضافة الكوايزيم كيو 10) كان أعلى بصورة معنوية (0.05) 91.6%، ثم يليه مخفف (المستخلص الكحولي لأوراق المورنجا بدون إضافة الكوايزيم كيو 10) 83.3% وكانت أقل قيمة لمخفف السترات ككترول (66.6%). يستخلص من هذه النتائج أن المستخلص الكحولي لأوراق المورنجا كمخفف جديد يعتبر مخفف واعد لتخفيف وحفظ السائل المنوي لطلائق الجاموس. وأيضاً إضافة 4 مللي من المستخلص الكحولي لأوراق المورنجا يعتبر بديل جيد للمضادات الحيوية كأحد المكونات التقليدية (الكيميائية) المستخدمة في مخفف السترات الشائع. علاوة على ذلك، إن إضافة الكوايزيم كيو 10 كمضاد أكسدة بمستوي 30 مللي مول قادر على تحسين وظائف الحيوان المنوي وخصوية السائل المنوي المجمد لطلائق الجاموس.