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## Effect of epidermal growth factor on buffalo frozen spermatozoa biometry and metabolic activity

Mohamed MM Kandiel<sup>1\*</sup>, Ahmed RM El-Khawagah<sup>1</sup>, Karima Gh Mahmoud<sup>2</sup><sup>1</sup>Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, Egypt<sup>2</sup>Department of Animal Reproduction and Artificial Insemination, National Research Centre, Dokki, Giza, Egypt

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## ABSTRACT

**Objective:** To assess the effects of epidermal growth factor (EGF) on the Egyptian buffalo bull frozen semen, EGF was incorporated at 0 (control), 50, 100, 200 and 400 ng/mL of extender (Bioxcell®). **Methods:** Semen features, spermatozoa biometry, total liberated amounts of enzymes (aspartate transaminase, alanine aminotransferase, lactate dehydrogenase, acid phosphatase and alkaline phosphatase) and lipid peroxidation markers (thiobarbituric acid reactive substances, malondialdehyde, glutathione peroxidase, nitric oxide, catalase (CAT) and superoxide dismutase (SOD)) were determined in the spermatozoa-free extracellular extender.

**Results:** Spermatozoa membrane integrity significantly ( $P < 0.05$ ) increased, but DNA integrity decreased with EGF 200 ng/mL. Spermatozoa head (dimensions, area and perimeter), but not shape, as well as acrosome and midpiece measures substantially differed with regard to EGF. Principle piece length and volume markedly decreased (at 100 and 200 ng/mL), while total tail/flagellum length increased (at 50 ng/mL) after EGF supplementation. EGF 50 ng/mL was associated with the decline of nitric oxide levels and catalase enzyme activity, but EGF 100 ng/mL significantly decreased the total liberated amounts of enzymes (aspartate transaminase, lactate dehydrogenase, acid phosphatase and alkaline phosphatase) as well as lipid peroxidation markers (thiobarbituric acid reactive substances and malondialdehyde). **Conclusion:** EGF *in vitro* supplementation would affect the semen characteristics of buffalo bull with 100 ng/mL counteracted the freezing mediated oxidative stress indicated with the lowest enzymes leakage and lipid peroxidation.

## 1. Introduction

Efforts to improve the preservation of bull semen are focused on the modification of extenders[1], as well as on the implementation of various bio-active ingredients to maintain the activity and fertilizing capacity, and preserve the spermatozoa membrane integrity[2].

Epidermal growth factor (EGF), a polypeptide cytokine comprising 53 amino acid residues, was originally isolated and purified from the submandibular glands[3]. In males, EGF is produced by the Leydig (the principal source)

and germ cells in the testes on attainment of the maturity, and may modulate spermatogenesis as an autocrine and/or paracrine factor[4].

Few data are available addressing the effect of EGF *in vitro* on semen characteristics. The published data showed that EGF effects on the functions of ejaculated spermatozoa are dependent on EGF concentration and animal species. Lax *et al.*[5] verified the effect of EGF in inducing the occurrence of the acrosomal reaction in bovine spermatozoa in a dose-dependent manner. Špaleková *et al.*[6] noted that the effect of EGF on ram spermatozoa motility parameters was relied on its level and exposure time. In boar, EGF at 10 and 100 ng/mL significantly improved the parameters of spermatozoa movement without affecting acrosome status, membrane integrity or motility either in intact or acrosome reaction-induced spermatozoa[7].

Cryopreservation encourages widespread biophysical and

\*Corresponding author: Mohamed MM Kandiel, Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, 13736, Toukh, Qalubia, Egypt.  
Tel: (+2013) 2461–411  
Fax: (+2013) 2460–640  
E-mail: moahmed.kandil@fvmt.bu.edu.eg; kandiel75@hotmail.com

biochemical alterations in the membrane of spermatozoa due to its contents of various unsaturated fatty acids that ultimately decrease the fertility potential of the cells[8]. Cooling and thawing of spermatozoa resulted in the generation of superoxide radicals and nitric oxide[9]. In goats, Chauhan *et al.*[10] found a positive correlation between the leakage of spermatozoa enzymes (glutamic oxaloacetic transaminase, glutamate pyruvate transaminase, hyaluronoglucosaminidase, and acid and alkaline phosphatases) and acrosomal damage during dilution, cooling and freezing.

To the authors' knowledge, there are no previous studies entailed the impact of EGF on bull spermatozoa *in vitro*. Therefore, the current study aimed to assess the alternation in Egyptian buffalo bull frozen semen activity, spermatozoa biometry, DNA integrity, spermatozoa enzymes leakage and lipid peroxidation.

## 2. Material and methods

This study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the The Committee on Graduate Studies and Research, Faculty of Veterinary Medicine, Benha University. Informed written consent was obtained from 2016.

### 2.1. Semen collection, processing and evaluation

Three proven fertile Egyptian buffalo bulls, kept in the Faculty Educational Farm, Benha University, aged between 3.5 and 6.5 years, were used during the period from January to April, 2016. Two ejaculates were collected from each bull once weekly with the bovine artificial vagina (Ref. 005417, IMV, France) adjusted at  $(45.0 \pm 2.0)^\circ\text{C}$  for twelve consecutive weeks.

After semen sampling, spermatozoa progressive motility (at  $400 \times$ ), concentration (by Neubauer haemocytometer) and normality were verified prior to semen processing. Samples possessed the minimal macroscopic and microscopic good quality standards (concentration  $\geq 800 \times 10^6/\text{mL}$ , and motility as well as normality  $\geq 70\%$ ) were pooled and placed in a warm water bath (at  $37^\circ\text{C}$ ) for cryo-processing.

Semen samples were split into aliquots each of 1.0 mL, diluted with Bioxcell® extender (IMV, France), according to manufacturer's instructions, and supplemented with EGF (E1257, Sigma-Aldrich, Germany) at different concentrations in triplicates: 0 (control), 50, 100, 200 and 400 ng/mL. Extended semen ( $50 \pm 5 \times 10^6$  spermatozoa/mL) was slowly cooled (within 1.0–1.5 h) to  $5^\circ\text{C}$ , packed into 0.5 mL polyvinyl straws (Minitub, Germany) and equilibrated for (3–5) h. Freezing was accomplished at  $\sim 4$  cm above the liquid nitrogen level for 10 min.

At 24 h post-storage in  $\text{N}_2$ , Straws ( $n=4/\text{trial}/\text{treatment}$ ) were evaluated hourly post-thawing in a water bath at  $\sim 37^\circ\text{C}$  for 30 s. The viability index was calculated according to Milovanov[11] to be equal

to half of the post-thaw motility in addition to the summation of recorded motility at 1h, 2h and 3 h post-thawing.

Spermatozoa plasma membrane integrity was determined by HOS assay (HOS solution consisted of sodium citrate 0.735 g and fructose 1.351 g, dissolved in 100 mL distilled water) at  $37^\circ\text{C}$  for 60 min and the percentage of swollen and/or curled spermatozoa were recorded as HOS positive[12]. Spermatozoa morphology was assessed by Eosin-Nigrosin stain according to Andrabi *et al.*[13].

### 2.2. Evaluation of sperm DNA integrity by acridine orange stain

Frozen semen was washed with 5 mL of phosphate buffered saline, centrifuged ( $700 \times g$ ) and the spermatozoa pellets were re-suspended in 0.5 mL of PBS. A small aliquot (50  $\mu\text{L}$ ) of the spermatozoa suspension was glass smeared ( $n=\text{three slides/sample}$ ), air dried and fixed overnight in Carnoy's solution (3 parts Methanol/1 part Acetic acid) according to Liu *et al.*[14]. Once rinsed and air dried, the slides were stained for 5 min with freshly prepared acridine orange (AO) stain (comprised of 1% AO in distilled water (10.0 mL) added to a mixture of 0.1 M citric acid (40.0 mL) and 0.3 M  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  (2.5 mL). Dried slides were examined with a fluorescent microscope (Leitz, Germany; excitation of 450–490 nm). Spermatozoa with intact chromatin or DNA content showed green fluorescence. Those with an abnormal DNA content emitted fluorescence in a spectrum varying from yellow-green to red.

### 2.3. Spermatozoa biometry assessment

Spermatozoa images were randomly selected and photographed from Eosin-Nigrosin stained slides with the use of a Euromax microscope (Holland). Image J software (National Institutes of Health, USA) was used for the assessment of spermatozoa biometry parameters. The software was standardized against a decimal scale. Fifty normal spermatozoa were captured and evaluated per slide ( $n=4$  slides/treatment/trial). The units for measurement variables were micrometers ( $\mu\text{m}$ ) and the ratios were without units. Spermatozoa morphology was quantified in terms of the following morphological features.

#### 2.3.1. Head measures

The length (L), width (W), base width (B), acrosomal cap length and width were measured. The head area ( $\mu\text{m}^2$ ), Perimeter (P), Ellipticity (e), Elongation (El), Head shape (HS), Shape factor-1 (Rugosity), Shape factor-2, Shape factor-3 (Regularity) were calculated according to Van Duijn[15] as follows:

$$\text{Area (A)} = 1.05 - 0.081 \times B^2 + 0.64 W \times L$$

$$\text{Perimeter} = [3 \times (L+W) - ((3L+W)(L+3W))]$$

$$\text{Ellipticity} = L/W$$

$$\text{Elongation} = (L - W) \times (L + W)$$

$$\text{Head shape} = W/L$$

$$\text{Shape factor-1 (Sf1)} = 4 \pi A/P^2$$

$$\text{Shape factor-2 (Sf2)} = \text{Sf1} \times (L/W)$$

$$\text{Shape factor-3 (Sf3)} = [\pi (L \times W)/4]/A$$

### 2.3.2. Tail measures

The midpiece width [proximal and distal ( $\mu\text{m}$ )] and length ( $\mu\text{m}$ ), principal piece length ( $\mu\text{m}$ ), terminal piece length ( $\mu\text{m}$ ), and flagellum length ( $\mu\text{m}$ ) were assessed. The midpiece, principal piece and total flagellum volumes ( $\mu\text{m}^3$ ) were calculated according to Ros-Santaella *et al.*[16].

$$\text{Midpiece volume } (\mu\text{m}^3) = [\pi \times L/3] \times (R^2 + r^2 + R \times r)$$

Where L is the length of the midpiece. R is the half proximal midpiece width. r is the half distal midpiece width.

$$\text{Principal piece volume } (\mu\text{m}^3) = (\pi \times R^2 \times L)/3$$

Where R is the half midpiece width (*i.e.* Proximal or distal) and L is the length of the flagellum of the principle piece

$$\text{Total flagellum volume } (\mu\text{m}^3) = (\pi \times R^2 \times L)/3$$

Where R is the half midpiece width (*i.e.* Proximal or distal) and L is the length of the flagellum of the principle plus terminal piece

### 2.4. Extracellular enzymatic activity measurement

Frozen thawed semen samples were centrifuged at 1 000 g for 20 min. The supernatant fluid was collected and kept at  $-20^\circ\text{C}$  until analysis. The activity of AST and ALT enzymes was determined with QCA kits (Amposta, Spain). The activity of ACP, ALP and LDH enzymes was assayed with Stanbio kits (Texas, USA) spectrophotometrically.

### 2.5. Semen lipid peroxidation measurement

Thiobarbituric acid reactive substances (TBARS) were estimated with TBARS Assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to Armstrong *et al.*[17]. Malondialdehyde (MDA) production was measured according to Placer *et al.*[18]. Glutathione peroxidase (GPX) activity was determined by GPX commercial kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to Paglia

*et al.*[19]. Superoxide dismutase (SOD) activity was determined by SOD Assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to Marklund *et al.*[20].

The Catalase (CAT) activity was measured according to the method of Goth[21]. Briefly, 0.3 mL of frozen-thawed semen was incubated in 1.7 mL of substrate (65  $\mu\text{M}$  hydrogen peroxide in 50 mM PBS, pH 7.0) at  $37.5^\circ\text{C}$  for 60 s. The enzymatic reaction was terminated with 1.0 mL of 32.4 mM ammonium molybdate. The hydrogen peroxide was measured spectrophotometrically at 405 nm against control. The value of CAT activity was expressed as units (U) per milliliter (mL).

Nitric oxide (NO) concentration was assessed by monitoring the concentration of stable oxidation products of NO metabolites ( $\text{NO}_2^-/\text{NO}_3^-$ ). Nitrite was determined using the Griess reaction by adding 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric and incubation at  $37^\circ\text{C}$  in the dark acid for 10 min. The absorbance was recorded at 540 nm with a spectrophotometer. Nitrate was detected after reduction to nitrite using nitrate reductase as described elsewhere[22].

### 2.6. Statistical analysis

The data were analyzed and presented as mean  $\pm$  SE with one-way analysis of variance (ANOVA) using SPSS (Ver. 16). Multiple comparisons of the means were done with Dunnett's test. *P* value was set at  $<0.05$  to define statistical significance.

## 3. Results

### 3.1. Semen characteristics and spermatozoa biometry

Plasma membrane ( $P<0.05$ ) and DNA ( $P=0.07$ ) integrity of frozen-thawed spermatozoa clearly differed in association with EGF supplementation. There was a decrease in membrane integrity with 50 ng/mL, and DNA integrity with 50 and 200 ng/mL of EGF in respect to control. Other semen features (spermatozoa post-thawing motility, normality and viability index) differed numerically with the addition of EGF (Table 1).

Investigation of spermatozoa head biometry revealed considerable differences with EGF, with special emphasis to head length ( $P<0.001$ ) and width ( $P<0.05$ ), area ( $P<0.001$ ) and perimeter ( $P<0.001$ ) as well as acrosomal length ( $P<0.001$ ) and width ( $P<0.005$ ) as demonstrated in Table 2. The highest measures were recorded with EGF 50 ng/mL. In the meantime, EGF 100 ng/mL was associated with the lowest above mentioned measures.

Spermatozoa tail biometry showed substantial differences with EGF-exposure level, with special concern to the mid-piece (proximal end width, length and volume), the principal piece (length and volume) and consequently the total tail length and volume (Table 3). There was a marked ( $P<0.001$ ) decrease in midpiece proximal end width at

high levels of EGF (100–400 ng/mL), and principle piece length and volume at EGF 100 and 200 ng/mL. Nevertheless, midpiece length was significantly ( $P<0.001$ ) longer at EGF 50–400 levels, and the total tail/flagellum length was the longest ( $P<0.005$ ) at EGF 50 ng/mL.

### 3.2. Biochemical assay

#### 3.2.1. Extracellular enzymatic activity

An assessment of extracellular enzymatic activity in extended semen revealed substantial differences after inclusion of EGF in buffalo semen extender (Table 4). A noticeable decrease in the extracellular leaked AST ( $P<0.01$ ), ALP ( $P<0.05$ ), ACP ( $P<0.05$ ) and LDH ( $P<0.01$ ) had been found with inclusions of EGF at 100 ng/mL level.

#### 3.2.2. Lipid peroxidation

An assessment of lipid peroxidation indicators revealed clear differences in response to the enclosure of EGF in buffalo semen extender (Table 4). A significant ( $P<0.01$ ) decrease in TBARS and

MDA at 100 ng/mL, CAT and NO at 50 and 200 ng/mL, and SOD at 400 ng/mL of EGF. Nevertheless, SOD increased markedly with EGF 50 ng/mL.

### 4. Discussion

EGF has been detected in the male reproductive system and seminal plasma. It may contribute in male reproductive physiology by motivating the meiotic phase of spermatogenesis[23] and male fertility[24]. The current study highlighted the imminent impact of EGF *in vitro* on semen characteristics, lipid peroxidation and extra-cellular enzymes' activity in the cryopreserved buffalo semen.

In the present data, EGF supplementation in buffalo semen extender significantly altered plasma membrane and DNA integrity, though it did not influence spermatozoa motility, morphology and viability index. Furthermore, spermatozoa heads (head length and width, acrosomal length and width, spermatozoa head area and perimeter) as well as tails (midpiece width and length, principle

**Table 1**

The effect of epidermal growth factor on buffalo frozen semen characteristics.

Group	Plasma membrane integrity (%)	DNA integrity (%)	Post-thawing motility (%)	Sperm normality (%)	Head Abnormality (%)	Tail Abnormality (%)	Sperm Viability Index
Control	70.25±3.92	92.67±4.33	38.75±7.74	84.75±3.54	1.00±0.58	14.25±3.01	53.63±14.28
EGF 50	60.75±2.63 <sup>*</sup>	83.25±1.38 <sup>*</sup>	52.50±4.79	75.00±4.49	1.50±0.87	23.50±5.24	56.75±6.37
EGF100	65.50±1.26	85.50±2.22	50.00±4.08	81.00±2.16	2.25±0.25	16.75±2.32	55.00±9.74
EGF200	69.75±0.63	83.33±2.60	50.00±8.17	81.25±1.11	0.75±0.48	18.00±1.47	62.50±12.22
EGF400	72.25±1.55	89.67±0.88	36.25±2.39	84.75±2.18	0.75±0.48	14.50±2.06	39.88±6.33
F value	3.963 <sup>ns</sup>	2.757 <sup>ns</sup>	1.605 <sup>ns</sup>	1.833 <sup>ns</sup>	1.266 <sup>ns</sup>	1.458 <sup>ns</sup>	0.661 <sup>ns</sup>
P value	<0.05	0.07	0.23	0.18	0.33	0.26	0.63

Data are presented as mean±SE. <sup>\*</sup> $P<0.05$  with the control group. <sup>ns</sup>F value is not significant  $P>0.05$ .

**Table 2**

The effect of epidermal growth factor on the buffalo sperm head biometry.

Group	Sperm Head Length (μm)	Sperm Head width (μm)	Sperm Head base (μm)	Acrosome length (μm)	Acrosome width (μm)	Sperm Head Area (μm <sup>2</sup> )	Sperm Head Perimeter (μm)	Ellipticity	Elongation	Head shape	Shape factor-1	Shape factor-2	Shape factor-3
Control	8.33±0.10	4.48±0.05	1.62±0.05	5.23±0.08	4.34±0.06	29.66±0.50	41.18±0.38	1.87±0.03	0.299±0.007	0.543±0.008	0.219±0.002	0.408±0.004	0.989±0.002
EGF 50	8.38±0.09	4.56±0.06	1.64±0.07	5.17±0.09	4.37±0.08	30.37±0.55	41.55±0.39	1.85±0.03	0.294±0.007	0.548±0.008	0.220±0.001	0.405±0.004	0.990±0.003
EGF 100	7.70±0.12 <sup>****</sup>	4.23±0.07 <sup>*</sup>	1.52±0.06	4.58±0.11 <sup>****</sup>	4.01±0.07 <sup>**</sup>	25.88±0.60 <sup>****</sup>	38.31±0.47 <sup>****</sup>	1.83±0.04	0.292±0.01	0.553±0.012	0.221±0.002	0.403±0.006	0.989±0.003
EGF 200	7.98±0.08 <sup>*</sup>	4.35±0.06	1.54±0.06	4.77±0.08 <sup>****</sup>	4.13±0.06	27.57±0.50 <sup>*</sup>	39.60±0.36 <sup>*</sup>	1.84±0.03	0.297±0.01	0.548±0.009	0.220±0.002	0.405±0.004	0.991±0.003
EGF 400	8.04±0.12	4.44±0.13	1.45±0.05	4.86±0.09 <sup>**</sup>	4.24±0.05	27.79±0.51 <sup>*</sup>	40.11±0.38	1.87±0.05	0.305±0.008	0.571±0.037	0.217±0.002	0.402±0.008	0.995±0.003
F value	6.449 <sup>ns</sup>	2.481 <sup>ns</sup>	1.611 <sup>ns</sup>	8.973 <sup>ns</sup>	4.129 <sup>ns</sup>	9.836 <sup>ns</sup>	9.105 <sup>ns</sup>	0.901 <sup>ns</sup>	0.187 <sup>ns</sup>	0.458 <sup>ns</sup>	0.421 <sup>ns</sup>	0.901 <sup>ns</sup>	0.205 <sup>ns</sup>
P value	<0.001	<0.050	0.172	<0.001	<0.005	<0.001	<0.001	0.945	0.767	0.794	0.464	0.936	0.665

Data are presented as mean±SE. <sup>\*</sup> $P<0.05$ , <sup>\*\*</sup> $P<0.01$ , <sup>\*\*\*\*</sup> $P<0.0001$  with the control group. <sup>ns</sup>F value is not significant  $P>0.05$ .

**Table 3**

The effect of epidermal growth factor on buffalo sperm tail biometry.

Group	Midpiece width, proximal (μm)	Midpiece width, distal (μm)	Midpiece length (μm)	Principle piece length (μm)	Terminal piece length (μm)	Total Flagellum length (μm)	Midpiece volume (μm <sup>3</sup> )	Principal piece volume (μm <sup>3</sup> )	Total flagellum volume (μm <sup>3</sup> )
Control	1.05±0.03	0.77±0.02	10.49±0.25	22.70±0.47	5.34±0.20	38.42±0.56	13.92±0.82	6.88±0.45	11.69±0.81
EGF50	1.04±0.03	0.85±0.14	12.14±0.35 <sup>**</sup>	23.06±0.47	6.03±0.33	41.22±0.60 <sup>****</sup>	19.26±0.90	6.94±0.45	12.53±0.81
EGF100	0.93±0.04 <sup>*</sup>	0.70±0.03	12.53±0.55 <sup>**</sup>	20.17±0.60 <sup>**</sup>	5.640±0.21	38.35±0.87	13.28±1.08	4.83±0.38 <sup>****</sup>	9.14±0.71
EGF200	0.85±0.04 <sup>****</sup>	0.75±0.17	12.26±0.43 <sup>**</sup>	20.78±0.48 <sup>**</sup>	5.17±0.17	38.22±0.73	13.48±0.01	4.32±0.40 <sup>****</sup>	7.88±0.71 <sup>****</sup>
EGF 400	0.87±0.03 <sup>****</sup>	0.61±0.02	12.12±0.41 <sup>**</sup>	22.65±0.46	5.49±0.22	40.26±0.68	10.68±0.83	4.65±0.31 <sup>****</sup>	8.29±0.54 <sup>**</sup>
F value	8.245 <sup>ns</sup>	0.71 <sup>ns</sup>	5.486 <sup>ns</sup>	5.872 <sup>ns</sup>	1.906 <sup>ns</sup>	4.496 <sup>ns</sup>	1.606 <sup>ns</sup>	9.253 <sup>ns</sup>	7.591 <sup>ns</sup>
P value	<0.001	0.586	<0.001	<0.001	0.110	<0.005	0.173	<0.001	<0.001

Data are presented as mean±SE. <sup>\*</sup> $P<0.05$ , <sup>\*\*</sup> $P<0.01$ , <sup>\*\*\*\*</sup> $P<0.0001$  with the control group. <sup>ns</sup>F value is not significant  $P>0.05$ .

**Table 4**

Effect of epidermal growth factor on buffalo semen extra-cellular enzymatic activity (U/109 spermatozoa) and lipid peroxidation activity.

Group	AST	ALT	ALP	ACP	LDH	TBARS (nmol/mL)	MDA (nmol/mL)	GPX (U/mL)	NO (μmol/mL)	CAT (U/mL)	SOD (U/mL)
Control	69.10±6.57	25.58±1.94	803.67±15.90	1.68±0.37	83.71±7.47	3.11±0.44	27.26±4.62	4.11±1.19	36.64±2.74	34.05±2.59	12.09±1.34
EGF 50	60.09±4.89	21.02±1.80	876.33±74.83	2.53±0.21**	64.76±6.19*	2.76±0.14	42.67±1.98***	2.56±0.43	23.91±2.42*	20.28±1.48*	24.41±2.95**
EGF 100	34.99±0.69*	24.05±0.96	714.67±35.71	0.98±0.01**	42.30±0.84**	1.08±0.02**	17.92±0.35***	4.31±0.23	36.99±3.29	38.41±4.71	17.70±1.02
EGF 200	53.12±5.27	24.76±2.97	838.67±54.11	1.06±0.05*	62.88±6.36**	1.72±0.12**	29.87±4.21**	3.64±0.31	21.72±5.59*	20.86±1.99*	21.95±1.93*
EGF 400	56.41±5.40	22.01±1.04	965.00±31.43*	1.89±0.34	37.43±3.63***	2.20±0.30*	38.81±3.22*	2.88±0.28	31.27±2.54	37.49±3.66	13.24±1.75
F value	6.334 <sup>ns</sup>	1.021 <sup>ns</sup>	3.846 <sup>ns</sup>	6.771 <sup>ns</sup>	11.775 <sup>ns</sup>	10.122 <sup>ns</sup>	8.957 <sup>ns</sup>	1.582 <sup>ns</sup>	4.043 <sup>ns</sup>	8.287 <sup>ns</sup>	7.772 <sup>ns</sup>
P value	<0.01	0.44	<0.05	<0.01	<0.01	<0.01	<0.01	0.25	<0.05	<0.01	<0.01

Data are presented as mean±SE. AST: Aspartate transaminase, ALT: Alanine aminotransferase, ACP: Acid phosphatase, ALP: Alkaline phosphatase and LDH: Lactate dehydrogenase. TBARS: Thiobarbituric acid reactive substances, MDA: Malondialdehyde, GPX: Glutathione peroxidase enzyme, NO: Nitric oxide, CAT: Catalase enzyme, SOD: Superoxide dismutase enzyme. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  with the control group. <sup>ns</sup>F value is not significant  $P>0.05$ .

piece length and volume, and total tail length) were clearly differed with EGF levels as compared with control. The increase in midpiece volume may be decoded to greater swimming velocities and thus results in an advantage in spermatozoa competition[25]. These results suggested that EGF may have a role in spermatozoa morphological attributes. Former studies localized EGF receptors at higher extent in the acrosome region than to the post-acrosome and the flagellum[7]. Although no former studies verified the effect of EGF on frozen semen characteristics, fresh ram semen stored with EGF at 4 °C showed an enhanced motility, viability and membrane integrity[26]. EGF increased ram spermatozoa motility at all doses tested, especially when EGF was added to the spermatozoa after 2-day of storage. The increase in midpiece volume may be translated to greater swimming velocities and thus lead to an advantage in spermatozoa competition and consequently improve its fertilizing capacity[25]. EGF decreased the proportion of spermatozoa with damaged plasma membrane and apoptotic spermatozoa when given at 200 or 400 ng/mL[26]. Low concentration (0.1–10 nM) of EGF had no effect on the percent of human spermatozoa motility, but higher concentrations ( $\geq 25$  nM) significantly affected various spermatozoa motility characteristics, particularly velocity and amplitude of lateral head movement[27]. It is supposed that EGF effects are mediated via Ras or Rho components of ERK pathway, which are localized in the flagellum of bovine[28] and hamster[29] spermatozoa, and are involved in spermatozoa motility regulation. EGF at higher doses lowered the number of spermatozoa with PNA-labeled membrane, *i.e.* this growth factor can act as a membrane stabilizer[26].

Damage occurring to spermatozoa during the freezing-thawing procedures mostly affect spermatozoa's membranes (plasma and mitochondria) and in the worst case, the nucleus[30]. These injuries are most likely due to the formation of extra- and intracellular ice crystals and increasing solution concentration[31]. Cryopreservation of spermatozoa is associated with reactive oxygen species production and lipid peroxidation. This could harm spermatozoa membrane[32], causing spermatozoa pathology (ATP depletion) and insufficient axonemal protein phosphorylation leading to loss of

motility and viability[33]. Based on recent literature, cellular cryo-damage in reproductive cells has been extensively characterized in terms of changes in the cell structure, whereas biochemical variations have been fairly explored[32]. Investigation of the influence of EGF on spermatozoa biochemical features in terms of an extra-cellular enzymes' activity and lipid peroxidation markers showed considerable differences after inclusion of EGF in the buffalo semen extender, indicating that the EGF was able to inhibit the propagation of the peroxidative chain reaction. A noticeable decrease in AST, ALP, ACP and LDH, TBARS and MDA was noticed in extender supplemented with EGF at a level of 100 ng/mL. Nevertheless, CAT and NO activities dropped upon EGF supplementation at the level of 50 ng/mL and 200 ng/mL. SOD levels markedly ( $P<0.01$ ) reduced with EGF 400 ng/mL, but elevated with EGF 50 ng/mL. The antioxidant system in the cell is comprised of reduced glutathione, glutathione peroxidase (CAT and SOD)[34]. Natural antioxidant system and synthetic phenolic antioxidant butylated hydroxytoluene have been described as a defense functioning mechanism against lipid peroxidation in semen[35]. Supplementation with these antioxidants prior to the freezing procedures may be endorsed to improve spermatozoa the cryopreservation technique in the goat breeding industry[36].

In conclusion, EGF implemented in the extender of buffalo frozen semen affected the semen features and spermatozoa biometry. The supplementation of EGF at 50 ng/mL was associated with elongation of spermatozoa and an increase of the mid-piece size and/or volume, which is advantageous for better motility velocities and consequently its fertilizing capacity. EGF at the level of 100 ng/mL might have cryoprotective properties through its antioxidant activity as well as maintenance of the stability of the spermatozoa plasma membranes' lipid contents as indicated by the decrease in enzymes leakage and lipid peroxidation.

### Conflict of interest statement

The authors declare that they have no conflict of interest.



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