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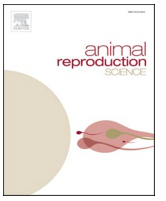

Protective effect of epidermal growth factor on cryopreservation of dromedary camel epididymal spermatozoa: Evidence from *in vitro* and *in silico* studies

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ABSTRACT

Epidermal growth factor (EGF) plays a crucial role in maintaining male reproductive capacity in mammals, however, its protective effects on cryopreserved dromedary camel epididymal spermatozoa have not been thoroughly investigated. This study aims to investigate the potential protective role of EGF on cryopreserved camel epididymal spermatozoa, supported by evidence from a molecular docking study. We assessed sperm motility, kinematics parameters, oxidative stress, ultrastructural changes, apoptosis, and molecular docking markers in camel epididymal spermatozoa following cryopreservation. Camel epididymal spermatozoa (n = 30 pairs of testes) were collected from local slaughterhouses. The epididymal spermatozoa were diluted with a freezing medium (SHOTOR extender) supplemented with different concentrations of EGF; 0 (EGF0), 50 (EGF50), 100 (EGF100), 200 (EGF200), and 400 (EGF400) ng/mL in SHOTOR extender and cryopreserved using a standard protocol. All EGF groups showed significant improvements in sperm progressive motility, viability, and sperm membrane function after equilibration at 5 °C for 24 hours. Regarding frozen-thawed samples, sperm progressive motility and some kinematic parameters (DAP, VSL, VCL and AHL) were significantly higher in the EFG400 group compared to the other groups ($P < 0.01$). A significant increase in the percentage of live/acrosome-intact sperm was observed, accompanied by a significant decrease in malondialdehyde levels in all EGF groups ($P < 0.05$). Both the EGF200 and EGF400 groups showed significantly higher sperm viability and significantly lower percentages of apoptotic and necrotic sperm compared to the other groups ($P < 0.05$). EGF supplementation preserved the ultrastructural integrity and cryotolerance of epididymal camel spermatozoa. The docking analysis indicated that EGF exhibited higher binding affinity with apoptosis sperm markers, including caspase-3 and bcl-2-associated X (Bax) proteins, with binding energies of −502.0 and −621.0 kcal/mol,

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respectively. In conclusion, the addition of EGF to SHOTOR extender was found to have beneficial effects on sperm motility, kinematics parameters, sperm viability, acrosome integrity, sperm ultrastructural features, and reduced oxidative stress and apoptosis-like changes in cryopreserved epididymal camel spermatozoa.

1. Introduction

Camels play a vital role in ensuring global food security due to their ability to thrive in arid environments and utilize natural pastures (Al-Jassim and Sejian, 2015; Boudalia et al., 2023). They provide valuable products such as milk, meat, and beneficial molecules for therapeutic purposes (Burger, 2016; Saadeldin and Cho, 2021). The global camel population has declined significantly in recent decades due to various factors, including globalization, industrialization, and changes in economic and social structures. One challenge associated with camel breeding is their seasonal reproductive cycle, which limits their breeding capacity (Alim et al., 2019). To enhance the reproductive capacity of camels, assisted reproductive technologies such as *in vitro* embryo production, (Saadeldin and Cho, 2021; Wani, 2021), and sperm cryopreservation (Purohit et al., 2023) have been implemented in camel farms. Sperm cryopreservation is a key tool for maintaining reproductive sustainability in camel farms and conserving genetic biodiversity in wild species (Deen et al., 2003). Conversely, the cryopreservation process can have detrimental impacts on sperm function and quality after thawing in various domestic animals (Abdelnour et al., 2023a; Khalil et al., 2023a,b; Tamargo et al., 2024) including camels (Desantis et al., 2021). Consequently, the identification of potent antioxidants to improve sperm cryotolerance in camels is of utmost importance. Previous studies have demonstrated the efficacy of L-carnitine (Abdelnour et al., 2022) and propolis-loaded nanoliposomes (Abdelnour et al., 2023b) in maintaining sperm structure and function during cryopreservation in farm animals.

Epidermal growth factor (EGF) is a small protein comprising 53 amino acids (6 kDa), which is ubiquitously present in various biological fluids, including seminal plasma, blood plasma, saliva, urine, and milk. The multifaceted roles of EGF in regenerative medicine (Špaleková et al., 2011) and mammalian reproduction (Elson et al., 1984; Oliva-Hernández and Pérez-Gutiérrez, 2008) have been extensively studied. EGF has been shown to regulate the capacitation process and acrosome reaction in human spermatozoa (Furuya et al., 1993), as well as in mice, and bovines (Oliva-Hernández and Pérez-Gutiérrez, 2008). Moreover, EGF supplementation has been reported to enhance sperm motility in chilled ram semen (Špaleková et al., 2011), and improve embryo production rates following *in vitro* fertilization in buffalo when added to maturation and fertilization media (Ahmed et al., 2023). A recent study has demonstrated that growth factors can significantly improve human sperm quality by attenuating oxidative stress, enhancing mitochondrial activity, and reducing DNA fragmentation (Ghasemian Nafchi et al., 2023; Ribas-Maynou et al., 2024). EGF, produced by various testicular cells including spermatocytes, Leydig cells, and spermatids, plays an essential role in modulating spermatogenesis in males (Yan et al., 1998). EGF is involved in the regulation of the acrosomal reaction, sperm capacitation, and motility traits of spermatozoa through the activation of tyrosine kinase (Furuya et al., 1993; Naz and Kaplan, 1993) and protein kinase C (Lax et al., 1994). The receptor for EGF has been identified in the acrosomal region of mammalian spermatozoa (Daniel et al., 2010). The addition of EGF (>25 nM) to human semen extenders has been shown to improve sperm fertilizing capacity, as assessed by the zona-free hamster oocyte test (Naz and Kaplan, 1993). Furthermore, EGF may enhance sperm function by modulating calcium interactions with sperm, thereby facilitating ovum penetration (Costello et al., 2009). Consequently, we hypothesized that the addition of EGF to the camel freezing medium would enhance sperm viability and its ability to withstand freezing. The main objective of this study was to investigate the effects of adding EGF to the extender before freezing on the post-thaw quality of epididymal camel sperm, including kinematic parameters, acrosome integrity, oxidative biomarkers, apoptosis-related changes, and sperm ultrastructure. Additionally, we performed molecular docking analysis to investigate the interaction between EGF and apoptosis markers, including Bcl-2-associated X protein (Bax) and caspase-3, on the sperm membrane.

2. Material and methods

This study was conducted during the rutting season (January – April 2023) at the Physiology and Biotechnology Laboratory, Faculty of Agriculture, Mansoura University. Camel testes were obtained from a local abattoir (Kerdasa abattoir, Giza). As the camels were sourced from a slaughterhouse, ethical approval was not required under Egyptian regulations (Guide for the Care and Use of Laboratory Animals, 2016) (Fahmy and Gaafar, 2016). Epidermal Growth Factor (EGF, E9644) was purchased by Sigma-Aldrich Company.

2.1. Camel epididymal sperm collection

Sixty testes, along with their attached epididymides, were obtained from healthy, mature dromedary camels (aged 7–12 years) during the breeding season (January to April) from the Kerdasa abattoir in Giza. The specimens were collected over five times, with approximately 12 testes collected each time (Shahin et al., 2020; Desantis et al., 2021). The camel testes were obtained within 30 minutes of slaughter. The testes were transported to the Physiology and Biotechnology Laboratory in phosphate-buffered saline (PBS) supplemented with 0.06 mg/ mL of penicillin and 0.05 mg/mL of streptomycin at 4 °C within 5 hours. In the laboratory, each testis was cleaned with sterile warm saline (37 °C), and the epididymis was detached (Shahin et al., 2020). The epididymides were then washed three times with warm saline and once with ethyl alcohol (70 %). Several incisions were made in the tail and body of the

epididymis using a sterilized scalpel to collect the spermatozoa. The epididymis was rinsed 3–4 times with a warm extender (37°C, without additives) using a sterile disposable syringe (20 gauge). The samples were immediately assessed for sperm progressive motility, and those with a motility rate of ≥ 60 % were pooled. The sperm concentration was then adjusted to 80 × 10^6 sperm/mL. A total of 5 pools were processed and evaluated.

2.2. Preparation of the extender and experimental design

Based on the protocol of Niasari-Naslaji et al. (2006), the SHOTOR extender was composed of a buffer solution [2.6 g Tris (AppliChem, Germany), 1.2 g glucose (Sigma-Aldrich, USA), 1.35 g citric acid (AppliChem, Germany), 0.9 g fructose (Sigma-Aldrich, USA), 1000 µg/mL streptomycin and 1000 IU/mL penicillin dissolved in deionized water (100 mL)]. The freezing extender had an osmolality of 330 mOsm/kg and a pH of 6.9. The SHOTOR extender was prepared by mixing 74 mL of buffer, 6 mL of glycerol, and 20 mL of egg yolk, enriched with different concentrations of epidermal growth factor (EGF): 0 (EGF0), 50 (EGF50), 100 (EGF100), 200 (EGF200) and 400 (EGF400) ng/mL following the protocol for bull (Alkhawagah et al., 2022). The extended epididymal spermatozoa were cooled to 5 °C for 24 hours for equilibration before being loaded into 0.25 mL straws, placed 4 cm above the liquid nitrogen vapor for 10 minutes and plunged into the liquid nitrogen (-196 °C). The samples were stored in liquid nitrogen until thawing at 37 °C in a water bath for 30 seconds, following the method described by Shahin et al. (2020). The experimental design of this study is summarized in Fig. 1.

2.3. Sperm evaluation

2.3.1. Sperm progressive motility (SPM)

The progressive motility (SPM) of equilibrated and frozen-thawed epididymal spermatozoa was estimated using a phase-contrast microscope (Leica DM 500, Germany) equipped with a hot stage set to 37 °C.

2.3.2. Sperm abnormalities and viability

A 10 µL sample of extended epididymal spermatozoa was placed on a glass slide and mixed with 5 % eosin/10 % nigrosin staining following the method described by Shahin et al. (2021). The percentage of live spermatozoa (unstained) in each sample was determined by examining 200 spermatozoa under high magnification (×400) using a light microscope (DM 500, Leica, Switzerland). The morphological abnormalities of spermatozoa, including defects in the head, tail, and the presence of cytoplasmic droplets were calculated during the examination of 200 live/dead spermatozoa.

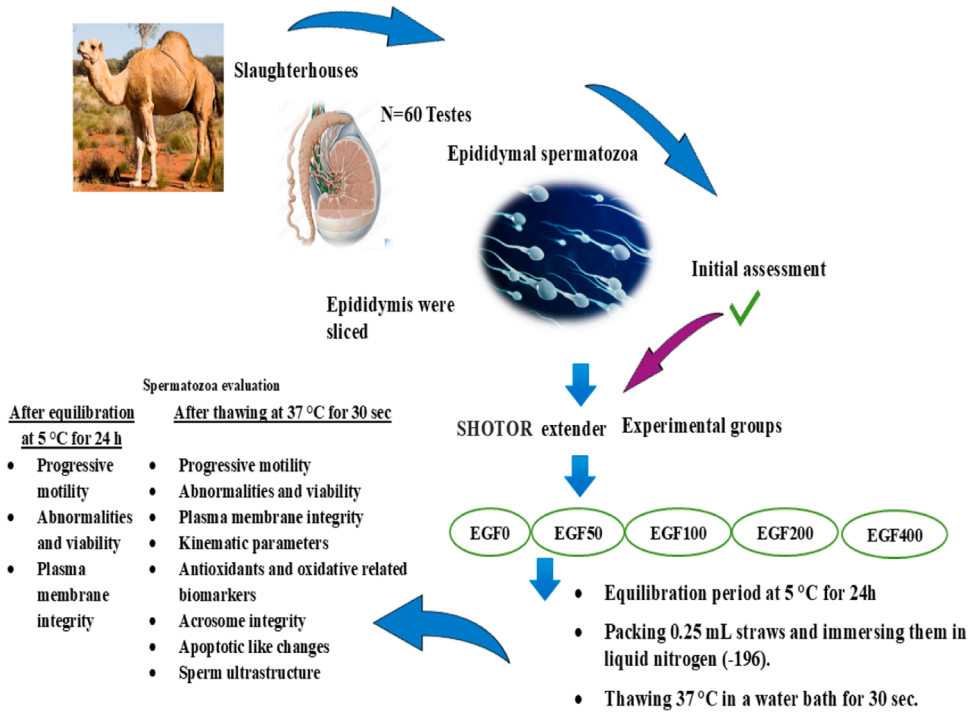

Fig. 1. Schematic diagram illustrates experimental design.

2.3.3. Assay of sperm plasma membrane function

The Hypo-Osmotic Swelling Test (HOST) was performed to assess the state of spermatozoa plasma membrane as described by El-Bahrawy et al. (2017). The sperm membrane function was assessed after equilibration at 5°C for 24 hours and in frozen/thawed of samples. The composition of the HOST solution was detailed in our previous works (Shahin et al., 2021; Abdelnour et al., 2023a). A 10 μL mixture of extended epididymal spermatozoa and HOST solution was placed on a preheated slide and covered with a coverslip. A total of 200 sperm cells from each sample were assessed under phase-contrast microscopy. Sperm showing the characteristic swelling of the tail were classified as HOST positive and considered as having a functional plasma membrane.

2.4. Evaluation of sperm kinematic parameters

The kinematic characteristics of frozen-thawed epididymal spermatozoa were analyzed using a computer-assisted semen analysis (CASA; Sperm Vision; Ref: 12520/5000; Minitube Hauptstrae 41, 84184 Tiefenbach, Germany). A phase-contrast microscope with a 20 × negative contrast objective was used, attached to an Olympus BX microscope (Hamburg, Germany) and a rapid scan digital camera for video capture. Videos were analyzed at 60 frames per second under × 4 dark-field illumination at 37°C. Approximately 1500 spermatozoa per treatment were analyzed using CASA (Dessouki et al., 2022). The motion parameters of approximately 1500 sperm cells were recorded to measure distances such as average path length (DAP), curved line distance (DCL), and straight-line distance (DSL). Additionally, velocities (μm/s) such as curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) were measured, along with beat cross frequency (BCF, Hz) and amplitude of lateral head displacement (ALH, μm). Other derived parameters, including linearity (LIN = VSL/VCL), wobble (WOB = VAP/VCL), and straightness (STR = VSL/VAP), were also calculated.

2.5. Assay of antioxidants and oxidative related biomarkers

Frozen–thawed epididymal spermatozoa were centrifuged at 4430 x g for 10 minutes at 4°C.

Then the extender was collected and stored at −20°C pending analysis. The total antioxidant capacity (TAC), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and nitric oxide (NO) were assessed in the extender of frozen–thawed epididymal spermatozoa using methods described by Koracevic et al. (2001), Ohkawa et al. (1979), Bilodeau et al. (2001) and Aebi (1984), respectively. Measurements were performed using a spectrophotometer (UV-2602; Labomed, USA) and commercially available kits (Biodiagnostic, Giza, Egypt) according to the manufacturer's instructions.

2.6. Evaluation of acrosome integrity

The acrosome integrity status was assessed using the Giemsa staining method outlined in the procedure of Brackett and Oliphant (1975). Frozen-thawed epididymal spermatozoa was mixed with 0.2 % trypan blue and incubated at 37°C for 10 minutes in a water bath. The samples were then extended with modified Brackett and Oliphant medium and centrifuged at 700 g for 6 minutes. The epididymal spermatozoa was repeatedly suspended in the medium to achieve clear or light liquid consistency. The sperm suspension was placed on a glass slide and covered with a second glass slide. The slides were quickly dried on a 40°C heating stage and then stained with Giemsa stock solution. After staining, the slides were rinsed under a stream of distilled water and air-dried.

Following the method described by Brackett and Oliphant (1975),100 sperm cells from each group were assessed for acrosome integrity as follows: a. Viable sperm with an intact acrosome. b. Non-viable sperm with an intact acrosome, c. Non-viable sperm with a reacted acrosome d. Viable sperm with exocytosed acrosome.

2.7. Evaluation of sperm apoptotic like changes

The staining of Annexin V was performed to assess apoptotic-like changes by Flow Cytometry. Extended epididymal spermatozoa was centrifuged, and the separated sperm cells were suspended in 2 mL of binding buffer and thoroughly mixed. Subsequently, 100 μL of the suspension was mixed with propidium iodide (PI, 5 μL; phycoerythrin label) and Annexin V (5 μL; fluorescein isothiocyanate label), incubated in a dark environment for at least 15 minutes, and then suspended in 200 μL of binding buffer. The results were acquired and analyzed using an Accuri™ C6 Cytometer as outlined by Masters and Harrison (2014). The labeling patterns in the Annexin (AN)/PI analysis were categorized as follows: viable with phosphatidylserine (PS) translocated (AN+/PI−), viable (AN−/PI−), dead and PS translocated (AN+/PI+), and dead late necrotic sperm (AN−/PI+).

2.8. Sperm ultrastructure analysis

Sperm ultrastructural analysis was conducted using Transmission Electron Microscopy (TEM), as described in detail in our previous studies (Abdelnour et al., 2022; Khalil et al., 2023b). In brief, frozen-thawed epididymal spermatozoa samples were incubated with 3 % glutaraldehyde at 4°C overnight. The specimens were then centrifuged at room temperature for five minutes and washed with PBS twice. Subsequently, the samples were exposed to gradual ethanol levels (50 %, 60 %, 70 %, 90 % and 100 %) for 15 minutes three times, embedded in Epon resin, and ultrathin-sectioned (60–80 nm) for TEM. The epididymal spermatozoa samples were dried, and the ultrathin sections were examined via a JEOL 2100 TEM (JEOL, Tokyo, Japan) at 80 kV to assess sperm ultra-morphology, including the plasma membrane, acrosome status, and mid-piece in 100 sperm cells per group. Finally, image analysis and acquisition were

performed using Soft Imaging Viewer software (Gatan Microscopy Suite Software, version 2.11.1404.0) and Digital Micrograph.

2.9. Molecular docking trial

The three-dimensional (3D) structures of human EGF and camel Bax were retrieved from UniProt database (https://www.uniprot.org/), while the 3D structure of camel caspase-3 was generated from their amino acid sequence by Phyre2 protein modeling software (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index). Molecular docking of human EGF (3D) with camel Bax (3D) and camel caspase-3 (3D) was performed using the ClusPro server (https://cluspro.bu.edu/home.php). The interactions between EGF and Bax, as well as EGF and caspase 3, were analyzed and visualized by PDBsum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html).

2.10. Statistical analysis

All numerical data were checked for homogeneity of variance using Levene's test and for normality of distribution using the Shapiro–Wilk test. The data were analyzed using the SAS program (version 2007, Cary, NC, USA) with one-way ANOVA followed by Tukey's honest significant difference test. Data are presented as mean ± SE. The mathematical model used to analyze all variables was Yij = μ + TRTi + eij, where Yij = observations, μ = overall mean, TRT = effect of the EGF levels (0, 50, 100, 200, and 400 ng/mL), and eij = random error. Statistical significance was considered when the *P*-value was less than 0.05.

3. Results

3.1. Effects of EGF on epididymal camel spermatozoa variables after equilibration (5°C for 24 h)

All EGF-equilibrated groups (except EGF50) showed significantly higher percentages of progressive sperm motility ($P < 0.001$) compared to the control group (Table 1). The EGF400 group exhibited the highest values for sperm viability and membrane function compared to the other EGF groups ($P < 0.001$). Furthermore, all groups equilibrated with EGF exhibited higher percentages of sperm with membrane function compared to the EGF0 group ($P < 0.001$). Moreover, there was no significant effect of EGF on the percentages of cytoplasmic droplets or abnormal sperm morphology ($P = 0.64$, 0.92, respectively).

3.2. Effects of EGF on frozen-thawed epididymal camel spermatozoa

After thawing, all samples cryopreserved with EGF exhibited significantly higher percentages of progressive sperm motility, sperm viability, and sperm with membrane function compared to EGF0 group (Table 2, $P < 0.0001$). The presence of cytoplasmic droplets and abnormalities in the spermatozoa remained unchanged by EGF supplementation to the SHOTOR-extender (50–400 ng/mL; $P > 0.05$). The most significant improvement in membrane function of epididymal camel spermatozoa was observed with the EGF400 group.

3.3. Effects of EGF on kinematic parameters of frozen-thawed epididymal camel spermatozoa

As shown in Table 3, the values of PM, DAP, DCL, DSL, VAP, and VSL were significantly higher in the EGF400 group compared to the other treatments ($P < 0.0001$). All EGF-treated groups exhibited a significant improvement in ALH ($P < 0.0001$) compared to the EGF0 group. Both the EGF200 and EGF100 groups had higher values for PM, DAP, VAP, VCL, and VSL compared to the EGF0 group. The EGF50 group showed the highest values of STR compared to the other groups. BCF was significantly enhanced in the EGF200 and EGF400 groups compared to the other groups ($P < 0.0001$). The value of DSL was not significantly affected by EGF supplementation, except in the EGF400 group. No statistically significant changes were observed among all groups for LIN and WOB ($P > 0.05$).

Table 1
Effect of supplementing SHOTOR extender with different concentrations of epidermal growth factor (EGF; 0, 50, 100, 200 and 400 ng/mL) on camel epididymal sperm parameters after equilibration at 5°C for 24 hours.

Extender[1]	Sperm attributes (%)				
	Progressive motility	Viability	Membrane function	Cytoplasmic droplet	Abnormality
EGF0	73.0 ± 1.22^{b}	75.8 ± 1.07^{c}	75.0 ± 1.82^{c}	42.8 ± 2.85	9.2 ± 1.36
EGF50	75.0 ± 1.58^{b}	78.8 ± 1.69^{bc}	77.0 ± 1.64^{ab}	41.0 ± 2.10	8.4 ± 0.81
EGF100	82.0 ± 1.22^{a}	83.2 ± 0.80^{ab}	81.4 ± 2.25^{ab}	42.4 ± 1.44	8.0 ± 1.10
EGF200	82.0 ± 1.22^{a}	83.4 ± 1.03^{ab}	80.8 ± 2.62^{ab}	42.2 ± 2.08	8.0 ± 1.00
EGF400	83.0 ± 1.22^{a}	85.4 ± 1.21^{a}	84.0 ± 1.30^{a}	38.4 ± 2.48	8.4 ± 0.68
P value	< 0.0001	< 0.0001	0.03	0.64	0.92

[1]The SHOTOR extender was supplemented with various concentrations of epidermal growth factor (EGF); 0 (EGF0), 50 (EGF50), 100 (EGF100), 200 (EGF200) and 400 (EGF400) ng/mL, respectively. Data presented as mean ± SE, n = 5 replicates. $^{a\text{-}c}$ Different letters in each column indicate significant differences between the groups ($P < 0.05$).

Table 2
Effect of supplementing SHOTOR extender with different concentrations of epidermal growth factor (EGF; 0, 50, 100, 200 and 400 ng/mL) on camel frozen-thawed epididymal sperm parameters.

Extender[1]	Sperm characteristics (%)				
	Progressive motility	Viability	Membrane function	Cytoplasmic droplet	Abnormality
EGF0	37.0 ± 1.22[b]	38.6 ± 1.33[b]	34.4 ± 1.03[c]	42.0 ± 2.47	12.8 ± 0.66
EGF50	45.0 ± 1.58[a]	47.6 ± 1.63[a]	43.4 ± 1.36[b]	40.4 ± 1.12	11.2 ± 0.86
EGF100	48.0 ± 1.22[a]	51.6 ± 1.83[a]	46.8 ± 1.66[ab]	36.0 ± 2.07	11.8 ± 0.86
EGF200	47.0 ± 1.22[a]	49.0 ± 2.17[a]	45.6 ± 2.25[ab]	37.8 ± 1.46	12.0 ± 0.71
EGF400	51.0 ± 1.87[a]	53.2 ± 1.46[a]	51.2 ± 1.39[a]	36.4 ± 1.40	11.4 ± 0.81
P value	< 0.0001	< 0.0001	< 0.0001	0.11	0.65

[1]The SHOTOR extender was supplemented with various concentrations of epidermal growth factor (EGF); 0 (EGF0), 50 (EGF50), 100 (EGF100), 200 (EGF200) and 400 (EGF400) ng/mL, respectively. Data presented as mean ± SE, n = 5 replicates. [a-c] Different letters in each column indicate significant differences between the groups ($P < 0.05$).

Table 3
Impacts of supplementing SHOTOR extender with different concentrations of epidermal growth factor (EGF; 0, 50, 100, 200 and 400 ng/mL) on kinematic parameters of camel frozen-thawed epididymal sperm.

Item[1]	Extender[2]					
	EGF0	EGF50	EGF100	EGF200	EGF400	P value
PM (%)	42.0 ± 3.28[c]	43.6 ± 1.77[c]	48.8 ± 1.87[b]	49.8 ± 2.02[b]	54.2 ± 1.74[a]	0.004
DAP (μm)	18.1 ± 0.35[c]	18.1 ± 0.56[c]	21.2 ± 0.57[b]	21.6 ± 0.66[b]	25.0 ± 0.64[a]	< .0001
DCL (μm)	28.7 ± 0.72[d]	29.3 ± 0.89 [cd]	33.2 ± 0.81b[c]	35.1 ± 1.12[b]	39.8 ± 1.32[a]	< .0001
DSL (μm)	12.9 ± 0.46[b]	13.3 ± 0.48[b]	14.5 ± 0.55[b]	14.9 ± 0.37[b]	17.5 ± 0.48[a]	< .0001
VAP (μm/sec)	41.2 ± 0.87[c]	41.9 ± 1.47[c]	48.3 ± 1.47[b]	49.9 ± 1.50[b]	57.4 ± 1.57[a]	< .0001
VCL (μm/sec)	64.6 ± 1.56[d]	67.4 ± 2.52 [cd]	75.3 ± 2.09[bc]	80.4 ± 2.53[b]	90.9 ± 3.13[a]	< .0001
VSL (μm/sec)	29.6 ± 1.23[c]	31.0 ± 1.20[bc]	33.2 ± 1.34[bc]	34.4 ± 0.89[b]	40.1 ± 1.10[a]	< .0001
STR (%)	71.2 ± 1.70[ab]	73.5 ± 1.34[a]	68.2 ± 1.22[b]	68.7 ± 0.99[ab]	69.3 ± 0.84[ab]	0.04
LIN (%)	45.2 ± 1.30	45.5 ± 1.18	43.7 ± 0.92	42.2 ± 0.79	43.7 ± 0.67	0.16
WOB (%)	63.5 ± 0.56	61.8 ± 0.79	63.7 ± 0.49	61.5 ± 0.76	62.7 ± 0.84	0.14
ALH (μm)	2.7 ± 0.07[c]	3.3 ± 0.07[a]	2.9 ± 0.09[bc]	3.1 ± 0.03[ab]	3.1 ± 0.11[ab]	< .0001
BCF (Hz)	24.3 ± 1.04[b]	22.0 ± 0.50[c]	24.9 ± 0.51[b]	26.4 ± 0.44[a]	27.5 ± 0.50[a]	< .0001

[1] DCL, distance curved line (μm); DSL, distance straight line (μm); VCL, velocity curved line (μm/sec); DAP, distance average path (μm); VAP, velocity average path (μm/sec); straightness (VSL/VAP); VSL, velocity straight line (μm/sec); LIN, linearity (VSL/VCL); STR, WOB, wobble (VAP/VCL); BCF, beat cross frequency (Hz) and ALH, amplitude of lateral head displacement (μm). [2]The SHOTOR extender was supplemented with various concentrations of epidermal growth factor (EGF); 0 (EGF0), 50 (EGF50), 100 (EGF100), 200 (EGF200) and 400 (EGF400) ng/mL, respectively. Data presented as mean ± SE, n = 5 replicates. [a-d] Different letters in each row indicate significant differences between the groups ($P < 0.05$).

3.4. Effects of EGF on acrosome status of frozen-thawed epididymal camel spermatozoa

As observed in Table 4, the supplementation of EGF show higher percentages of live sperm with intact acrosome ($P < 0.0001$). There was no statistically substantial difference among all treatments for live sperm with detached acrosome ($P = 0.49$) and dead sperm with intact acrosome ($P = 0.17$). The EGF400 group had the lowest values of dead sperm with detached acrosome compared to the other groups ($P < 0.01$). The EGF0 group showed similar results for dead sperm with detached acrosome compared to the EGF-treated groups (except for EGF400) ($P > 0.05$).

Table 4
Impacts of supplementing SHOTOR extender with different concentrations of epidermal growth factor (EGF; 0, 50, 100, 200 and 400 ng/mL) on the acrosome integrity of camel frozen-thawed epididymal sperm.

Extender[1]	Acrosome integrity (%)			
	Live sperm with intact acrosome	Live sperm with detached acrosome	Dead sperm with intact acrosome	Dead sperm with detached acrosome
EGF0	32.0 ± 1.52[b]	19.4 ± 1.63	27.2 ± 1.20	21.4 ± 1.72[a]
EGF50	43.0 ± 0.63[a]	17.8 ± 2.27	22.4 ± 1.66	16.8 ± 1.74[ab]
EGF100	43.6 ± 0.81[a]	17.6 ± 1.03	23.0 ± 1.58	15.8 ± 0.86[ab]
EGF200	42.8 ± 1.02[a]	14.8 ± 2.60	23.4 ± 1.96	19.0 ± 1.67[ab]
EGF400	45.8 ± 1.07[a]	19.8 ± 2.56	22.0 ± 1.30	12.4 ± 1.99[b]
P value	< 0.0001	0.49	0.17	0.01

[1]The SHOTOR extender was supplemented with various concentrations of epidermal growth factor (EGF); 0 (EGF0), 50 (EGF50), 100 (EGF100), 200 (EGF200) and 400 (EGF400) ng/mL, respectively. Data presented as mean ± SE, n = 5 replicates. [a,b] Different letters in each column indicate significant differences between the groups ($P < 0.05$).

3.5. Effects of EGF on total antioxidant capacity, oxidative and nitrosative biomarkers in frozen-thawed epididymal camel spermatozoa

Supplementation of extender with various concentrations of EGF (50–400 ng/mL) did not significantly impact on the TAC, H_2O_2, and NO levels in frozen-thawed epididymal camel spermatozoa (Table 5). All EGF-supplemented groups showed lower levels of MDA than the control one ($P < 0.0001$), with the lowest values exhibited in the EGF100 group (Table 5). TAC and NO levels exhibited a tendency to increase, while H_2O_2 levels showed a tendency to decrease across all EGF-supplemented groups. Conversely, these changes were not statistically significant ($P > 0.05$) when compared to the control group.

3.6. Effects of EGF on apoptosis-like changes of frozen-thawed epididymal camel sperm

As shown in Table 6, the EGF200 and EGF400 groups had significantly higher numbers of viable spermatozoa and significantly lower numbers of apoptotic spermatozoa ($P < 0.05$). The EGF100 group showed intermediate values for viable and apoptotic spermatozoa ($P < 0.05$). The EGF50 and EGF0 groups had similar results for apoptosis-like changes in the frozen-thawed epididymal camel sperm ($P > 0.05$). The EGF400 group had the lowest value of necrotic spermatozoa.

3.7. Sperm ultrastructure

The impact of SHOTOR-extender supplementation with EGF, at various concentrations, on the ultrastructure of camel frozen-thawed spermatozoa was examined (Fig. 2A-I). After thawing, signs of damage were observed in sperm extended without supplementation (Fig. 2 A, B). These sperm cells exhibited a rough or cracked surface, reacted acrosomes with loss of the plasma membrane, and lacked acrosomal content in the EGF100 group (Fig. 2D). Enriching the SHOTOR-extender with 50 ng/mL EGF (Fig. 2 C) or 100 ng/mL EGF (Fig. 2D, E) slightly improved the sperm ultrastructure. Furthermore, the addition of 200 ng/mL or 400 ng/mL of EGF preserved the sperm membrane structure, regular nucleus, mitochondria, homogeneously condensed chromatin bordered by an intact plasma membrane and stability of the acrosome. The EGF400 and EGF200 groups had regularly shaped mitochondria in the sperm (Fig. 2F-I).

3.8. Molecular docking analysis

To confirm the anti-apoptotic effects of EGF after cryopreservation of camel spermatozoa, molecular docking was performed with caspase-3 and bax. The molecular docking interaction of EGF with apoptosis markers, including bax and caspase-3, is presented in Fig. 3 A and 3B, respectively. The molecular docking scores are −502.0 and −621.0 kcal/mol, respectively. EGF interacts with bax through six hydrogen bonds and two salt bridges (Fig. 3 A). EGF also interacts with camel caspase-3 through three hydrogen bonds and three salt bridges (Fig. 3B). Overall, EGF shows promise as a molecule for future preparation of anti-apoptotic effects during the cryopreservation process in animals.

4. Discussion

Successful cryopreservation of gametes can significantly enhance the genetic diversity of animal populations and increase the profitability of farm animals. For instance, improving sperm cryopreservation techniques for male camels can advance reproductive technologies in this species. Additionally, preserving genetic diversity in camels is crucial for achieving sustained genetic improvement and accelerating rapid adaptation to evolving breeding patterns (Turri et al., 2013).

During the cryopreservation process, sperm function is often compromised by the excessive production of reactive oxygen species (ROS). The sperm membrane, which is the primary organelle exposed to adverse effects of cooling and freezing environments, requires protection during cryopreservation, particularly from ROS. Hence, it is essential to protect the membrane to improve cryoresistance and potential fertility after cryopreservation. To achieve this target, many antioxidants have been added to the freezing extender. We investigated the impact of varying concentrations of EGF (0, 50, 100, 200, and 400 ng/mL) on several aspects of camel frozen-thawed

Table 5
Effect of supplementing SHOTOR extender with different concentrations of epidermal growth factor (EGF; 0, 50, 100, 200 and 400 ng/mL) on total antioxidant capacity, oxidative and nitrosative biomarkers in camel frozen-thawed epididymal sperm.

Extender[1]	TAC (mM/L)	MDA (nmol/mL)	H_2O_2 (mM/L)	NO (μmol / L)
EGF0	1.04 ± 0.08	23.19 ± 0.91[a]	0.158 ± 0.011	25.07 ± 1.24
EGF50	1.26 ± 0.07	18.10 ± 1.04[b]	0.130 ± 0.013	27.22 ± 1.05
EGF100	1.28 ± 0.04	14.31 ± 0.83[c]	0.137 ± 0.011	27.15 ± 1.53
EGF200	1.28 ± 0.08	16.26 ± 0.71[bc]	0.134 ± 0.010	26.90 ± 0.90
EGF400	1.35 ± 0.14	15.65 ± 0.83[bc]	0.112 ± 0.007	27.08 ± 0.46
P value	0.16	< 0.0001	0.08	0.61

[1]The SHOTOR extender was supplemented with various concentrations of epidermal growth factor (EGF); 0 (EGF0), 50 (EGF50), 100 (EGF100), 200 (EGF200) and 400 (EGF400) ng/mL, respectively. Data presented as mean ± SE, n = 5 replicates. [a-d] Different letters in each column indicate significant differences between the groups ($P < 0.05$). NO = Nitric Oxide, MDA= Malondialdehyde, TAC= Total Antioxidant concentration, and H_2O_2 = Hydrogen Peroxide.

Table 6
Effect of supplementing SHOTOR extender with different concentrations of epidermal growth factor (EGF; 0, 50, 100, 200 and 400 ng/mL) on apoptosis-like changes of camel frozen-thawed epididymal sperm (Annexin V/PI assay).

Extender[1]	Apoptosis-like changes (%)			
	Viable (A−/PI−)	Early apoptotic (A+/PI−)	Apoptotic (A+/PI+)	Necrotic (A−/PI+)
EGF0	31.3 ± 0.90^{c}	18.4 ± 0.20	46.4 ± 1.13^{a}	3.9 ± 0.03^{a}
EGF50	36.3 ± 1.18^{c}	19.2 ± 0.55	40.3 ± 1.79^{a}	4.2 ± 0.07^{a}
EGF100	43.7 ± 1.58^{b}	19.9 ± 0.18	32.3 ± 1.67^{b}	4.1 ± 0.09^{a}
EGF200	53.7 ± 2.54^{a}	19.0 ± 0.85	23.7 ± 1.62^{c}	3.6 ± 0.10^{ab}
EGF400	59.5 ± 1.15^{a}	18.3 ± 1.00	19.4 ± 1.21^{c}	2.8 ± 0.47^{b}
P value	< .0001	0.47	< .0001	0.01

[1]The SHOTOR extender was enriched with various concentrations of epidermal growth factor (EGF); 0 (EGF0), 50 (EGF50), 100 (EGF100), 200 (EGF200) and 400 (EGF400) ng/mL, respectively. Data presented as mean ± SE, n = 3 replicates. [a-c] Different letters in each column indicate significant differences between the groups ($P < 0.05$).

epididymal sperm. This included sperm motility, kinematic parameters, acrosome integrity, oxidative biomarkers, apoptosis like changes, and sperm ultrastructure. The results demonstrated that the addition of EGF to SHOTOR extender significantly improved progressive motility, viability, and membrane function of epididymal camel spermatozoa after cooling the samples at 5 °C for 24 hours and after freezing/thawing. The addition of EGF (100–400 ng/mL) had more beneficial effects in progressive sperm motility after equilibration at 5°C for 24 hours than the low dose (50 ng/mL), while the EGF400 group produced the best results in majority of evaluated parameters.

The cauda region of the epididymis is a valuable resource for collecting spermatozoa from deceased wild animals including camels (Martinez-Pastor et al., 2005; Turri et al., 2013). The ability of sperm to move effectively is widely considered a crucial factor in determining the potential of ejaculated sperm to successfully fertilize an egg. In this study, the addition of EGF to the SHOTOR extender showed better percentages of sperm motility in the thawed samples compared to the EGF0 group. This molecule can lead to an increase in intracellular free calcium concentration, potentially regulating sperm motility (Oliva-Hernández and Pérez-Gutiérrez, 2008), and supporting the progressive motility of sperm as noticed in our research. Moreover, it has been reported that EGF (100–400 ng /mL) significantly improved ram sperm motility in cooled semen preserved at 5–7 °C for 48 hours (Špaleková et al., 2011). This effect is likely due to EGF's ability to activate signaling pathways involved in cytoskeletal dynamics and energy production within sperm (Daniel et al., 2010)

Epidermal growth factor can increase the MAP kinase activities during the early stages of maturation, enhancing sperm function such as sperm motility (Li et al., 2009). The addition of urea to the bull freezing extender decreased sperm motility, but supplementing the freezing medium with EGF (10 ng/mL) significantly restored this reduction in sperm motility (Kowsar et al., 2021). In a previous study, Medan et al. (2008) observed that the addition of catalase (at either 250 or 500 IU/mL) to the extender significantly improved sperm motility percentages and greatly reduced the percentages of dead spermatozoa, sperm anomalies and acrosomal damage in dromedary cooling semen. While there are no previous studies to compare our results regarding the potential effects of EGF on camel epididymal sperm motility, our findings suggest that EGF may have a positive impact on sperm motility. Alkhawagah et al. (2022) found significant improvements in total sperm motility and several velocity parameters of bull sperm after supplementation with EGF (200 and 400 ng/mL) during freezing.

The presence of EGF has been detected in the plasma membrane of sperm cells in various mammalian species, such as human (Damjanov et al., 1993), boar (Oliva-Hernández and Pérez-Gutiérrez, 2008), rabbit (Naz and Ahmad, 1992) and bull (Lax et al., 1994). The stability of the plasma membrane is essential for sperm function and fertilization capacity, as it protects the sperm contents from exposure to the external environment. The fertilization process does not occur when the plasma membrane of the sperm is physically injured. Hence, it is crucial to protect the integrity of the sperm membrane by fortifying the extender with antioxidants to ensure DNA and acrosome integrities for improving fertility competence. In this study, the percentages of sperm with membrane function were higher in frozen samples supplemented with EGF (50–400 ng/mL), while sperm morphology and cytoplasmic droplets remained unaffected. A meta-analysis conducted by Ghasemian Nafchi et al. (2022) indicated that GFs may play a protective role against oxidative damage. This protection is thought to occur through the activation of a specific cellular pathway known as the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which promotes the expression of antioxidant defenses within sperm cells. Additionally, GFs can improve mitochondrial biogenesis through the serine-threonine kinase protein kinase B (AKT), resulting in higher sperm progressive motility. In buffaloes, insulin-like growth factor 1 (IGF1) stabilizes proteins in the sperm membrane, reducing cryoinjury and preserving post-thaw sperm motility (Selvaraju et al., 2016). Several studies (Shahin et al., 2020; 2021) have demonstrated that adding antioxidants or nanoparticles to epididymal camel sperm can significantly improve sperm membrane function by reducing oxidative stress. Based on current evidence, it is suggested that EGF can enhance the stability of the sperm plasma membrane.

The evaluation of human sperm motion speed is significantly important as it serves as a critical factor for fertility, especially when the percentage of motile spermatozoa drops below 40 % (Vasan, 2011). Sperm kinematic variables in camels were previously assessed using the CASA analyzer (El-Bahrawy et al., 2017; Al-Bulushi et al., 2019). All kinematic parameters of frozen-thawed epididymal camel spermatozoa were higher in EGF400 group compared to other groups (except for STR, ALH and BCL; $P < 0.05$). The analysis of sperm motility considerations has revealed significant correlations between LIN, VCL, ALH, VAP, and fertilization rates based on *in vitro* human studies (Hirano et al., 2001; Aghazarian et al., 2021). Furthermore, VCL and VAP, are considered noteworthy indicators of

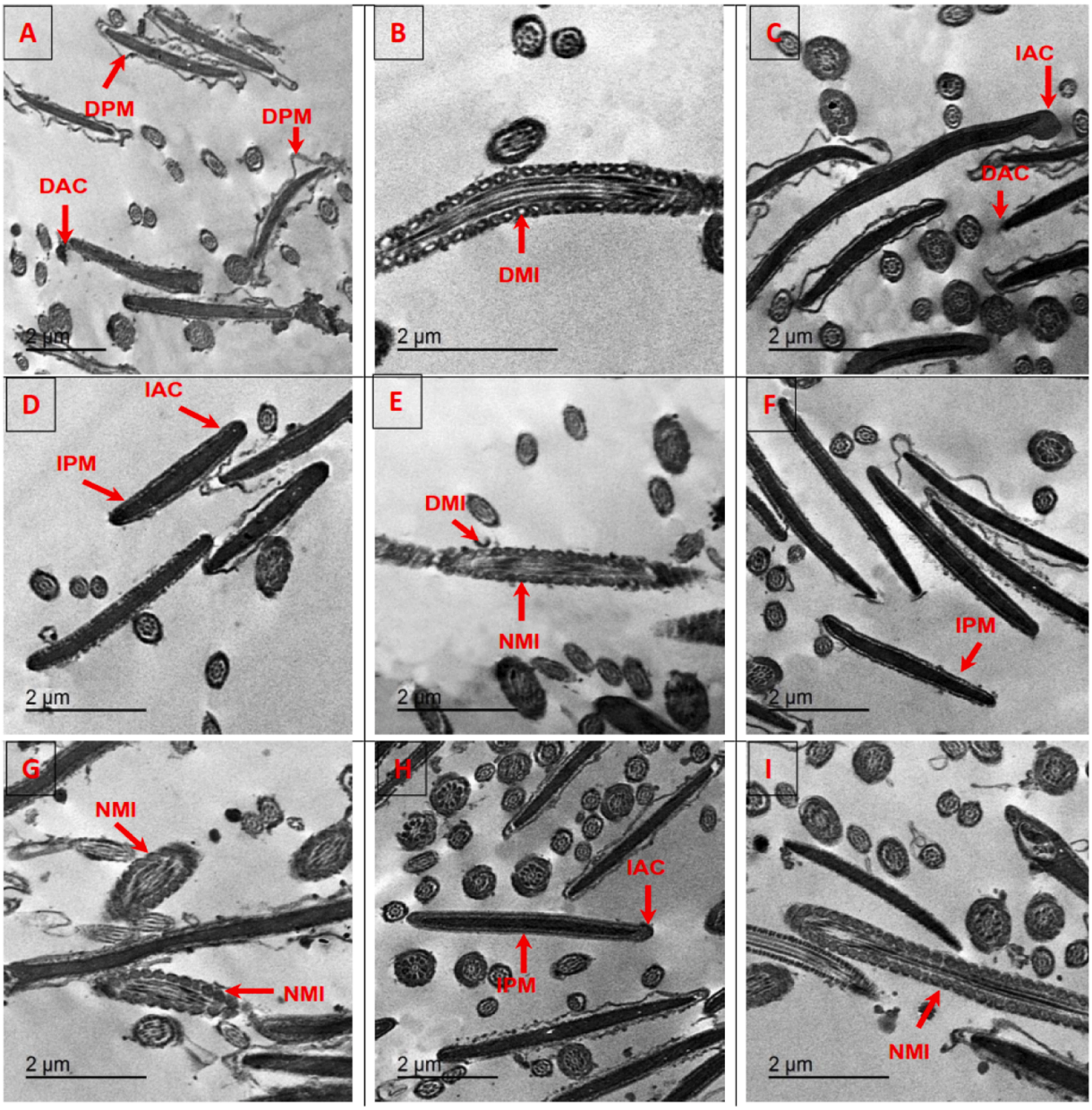

Fig. 2. (A-I). The impacts of supplementing SHOTOR-extender with epidermal growth factor (EGF) at various concentrations 0 (EGF0, Fig. 2 A, 2B), 50 (EGF50, Fig. 2 C), 100 (EGF100, Figs. 2D, 2E), 200 (EGF200, Fig. 2 F, 2 G) and 400 (EGF400, Figs. 2H, 2I) ng/mL on ultrastructural of frozen-thawed camel spermatozoa. Damaged plasma membrane (DPM), intact plasma membrane (IPM), normal mitochondria (NMI), damaged mitochondria (DMI), intact acrosomal (IAC), and damaged acrosomal (DAC).

fertilization potential in humans under *in vitro* conditions (Donnelly et al., 1998). The kinematic parameters of frozen-thawed epididymal camel spermatozoa were significantly improved by adding 400 ng/mL of EGF. These results are consistent with findings from previous studies on bull semen (Alkhawagah et al., 2022).

Epidermal growth factor activates receptor tyrosine kinases on the cell's surface, leading to cellular proliferation by accelerating tyrosine autophosphorylation (Mahony and Gwathmey, 1999). This process is crucial for sperm tail proteins to achieve hyperactive motility, enabling bull spermatozoa to penetrate the cumulus cells and zona pellucida layer of the oocyte (Somanath et al., 2004). Capacitation is a crucial step for sperm to fertilize an egg. Epidermal growth factor has been shown to inhibit the synthesis of protein kinase, an enzyme that plays a crucial role in accelerating the process of capacitation (Naz and Rajesh, 2004). Adding EGF at 400 ng/mL improved acrosome integrity in frozen sperm, leading to increased live sperm with intact acrosomes. In bulls, EGF (50–400 ng/mL) added to freezing extender did not affect acrosomal integrity compared to the control group (Alkhawagah et al.,

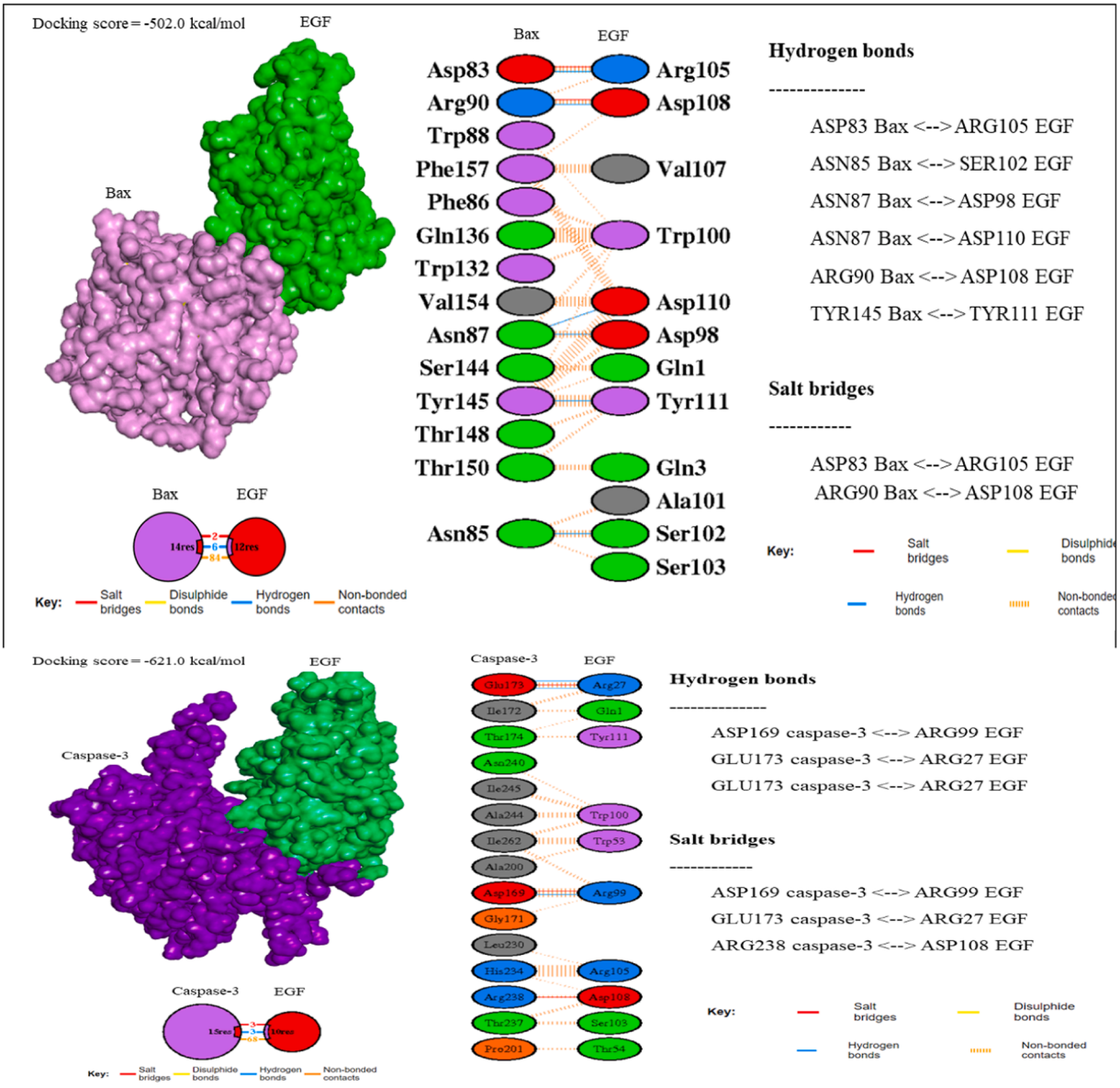

Fig. 3. (A, B). Molecular docking interaction of human's EGF against camel's Bax and camel's caspase 3. The molecular docking interaction of EGF with apoptosis markers, including bax (Fig. 3 A) and caspase-3 (Fig. 3B) were investigated. The molecular docking scores are −502.0 and −621.0 kcal/mol, respectively. EGF interacts with bax through six hydrogen bonds and two salt bridges (Fig. 3 A). EGF also interacts with camel caspase-3 through three hydrogen bonds and three salt bridges (Fig. 3B).

2022). In contrast to our present data, previous studies have shown that EGF (10 or 100 ng/mL) did not induce any changes in acrosome status, membrane function, or sperm motility when assessed after *in vitro* capacitation in boars (Oliva-Hernández and Pérez-Gutiérrez, 2008). In an earlier study, Naz and Kaplan (1993) clarified that EGF did not show any positive or negative effects on human sperm cell capacitation and/or acrosome reaction during incubation with EGF for 7–8 hours at 37 °C (in 5 % CO_2 and 95 % air mixture). This may be attributed to the limited variables investigated in this study. Contrary to our results, EGF can stimulate human sperm capacitation by activating the tyrosine kinase of the EGF receptor (Furuya et al., 1993). The EGF receptor has been found in many mammalian species, such as bovine (Kassab et al., 2007) and alpaca (Lama pacos) (He et al., 2009). Further research is necessary to determine the expression of growth factors in camel sperm membrane and other parts of sperm cells to optimize freezing protocols for different species and enhance reproductive outcomes.

Recent research indicates that EGF supplementation can mitigate oxidative stress and improve sperm quality. Alkhawagah et al. (2022) reported a significant decrease in MDA levels and a slight increase in TAC in EGF-treated frozen-thawed bull sperm. Similarly, Ghasemian Nafchi et al. (2023) demonstrated that plasma rich in growth factors (1 %) reduced MDA levels in teratozoospermic human

sperm compared to the control samples. Moreover, EGF's protective effects may be attributed to its activation of AKT, a signaling pathway involved in preventing oxidative stress-induced apoptosis (Wang et al., 2000). Our study aligns with these findings, showing a significant improvement in sperm viability and a reduction in apoptotic and necrotic sperm with 200 or 400 ng/mL EGF supplementation. The addition of anti- apoptotic molecules such as L-carnitine (Abdelnour et al., 2023a), thymoquinone nanoparticles (Khalil et al., 2023b) and quercetin (Abdelnour et al., 2023b) to the freezing extender has been shown to reduce OS production in cryopreserved semen of various animals, leading to a decrease in apoptotic sperm and improved sperm cryopreservation.

In bulls, Alkhawagah et al., (2022) reported that EGF did not have significant effects on the percentages of necrotic and apoptotic cells in frozen-thawed semen. In contrast to our findings, Miguel-Jiménez et al. (2023) found that EGF increased the NO in raw ram semen. These discrepancies may be due to species-specific differences in EGF expression and the inherent cryoresistance of epididymal sperm compared to ejaculated sperm (Santiago-Moreno et al., 2023).

According to a study by Oliva-Hernández and Pérez-Gutiérrez (2008), EGF has been found in the epididymis tissues and accessory glands of boars, indicating its reproductive functions outside the testis. It is widely accepted that a series of consecutive protein interactions occurring in various microenvironments within the epididymis are crucial for sperm maturation. This approach leads to enhance sperm motility and the ability to fertilize eggs in wild small ruminants (Santiago-Moreno et al., 2023). *In silico* modeling and molecular docking are valuable tools for improving our understanding of protein binding affinity and the mechanisms underlying binding stability. These systems allow us to discover the structural basis of protein-ligand interactions and gain insights into the functional roles of proteins in biological structures by revealing their three-dimensional structures (Batran et al., 2022). In the scope of reproductive biology, applying *in-silico* methodologies can significantly enhance our ability to study protein-ligand and protein-drug interactions, as well as the dynamics of protein-ligand complexes and supporting the interactions between antioxidant and the target such as sperm. In the current study, the molecular docking assessment revealed the binding affinity of human's EGF to either camel's Bax or camel's caspase 3, confirming the anti-apoptotic effect of EGF (Johnstone et al., 2005). Furthermore, this point needs more comprehensive molecular investigations. The *in-silico* approach can provide additional evidence on how EGF interacts with the structural components of sperm.

The freezing-thawing process of sperm has been associated with the excessive production of ROS, which can affect the ultrastructure features of sperm. In this study, we observed abnormal ultra-structures in epididymal camel spermatozoa after the cryopreservation process, including damaged acrosomes, swollen plasma membranes, and dysfunction in mitochondria and endoplasmic reticulum. To preserve the ultrastructure of cryopreserved sperm, various antioxidant supplements including metallic nanoparticles and vitamins were added to the extender for epididymal camel spermatozoa, as previously described by Shahin et al. (2020). These compounds play a crucial role in protecting sperm from cryo-damage by maintaining membrane integrity, motility, and overall functionality. Moreover, the addition of antioxidants to freezing extenders has been shown to improve and maintain sperm ultrastructure in various animal species (Abdelnour et al., 2022, 2023a; Khalil et al., 2023a,b). Our study shows that adding EGF to the extender enhanced the ultrastructural characteristics of thawed epididymal camel sperm. Investigating the proteomic and transcriptomic changes in camel sperm during cryopreservation is crucial for improving assisted reproductive technologies in camels.

5. Conclusion

In conclusion, the addition of EGF (100–400 ng/mL) to SHOTOR extender resulted in the best outcomes for progressive sperm motility after equilibration at 5°C for 24 hours. This study suggests that the addition of EGF to the SHOTOR extender reduces oxidative markers and enhances post-thaw sperm movement, progressive sperm motility, viability, membrane function, and acrosome integrity, improving the ability of epididymal camel sperm to withstand freezing. Specifically, a concentration of 400 ng/mL of EGF significantly improved frozen-thawed sperm viability, membrane function, and kinematic parameters of epididymal camel spermatozoa. Molecular docking assessment was conducted for the first time, revealing the binding affinity of EGF to either camel's Bax or camel's caspase 3 in the spermatozoa's plasma membrane, confirming the anti-apoptotic effect of EGF.

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CRediT authorship contribution statement

Sameh A. Abdelnour: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing — original draft, Writing — review & editing, Visualization. **Wael A. Khalil:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing — original draft, Writing—review & editing, Visualization. **Mahmoud A. E. Hassan:** Conceptualization, Methodology, Validation Software, Formal analysis, Investigation, Data curation, Writing — original draft, Visualization. **Ibrahim T. El-Ratel:** Methodology, Investigation, Data curation, Writing — original draft, Visualization. **Sherif Mohamed Dessouki:** Methodology, Software, Writing — original draft. **Mostafa A. El-Harairy:** Validation, Writing — review & editing. **Kandil A. A. Attia:** Conceptualization, Methodology, Investigation, Data curation, Writing — original draft, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data that support the findings of this study are available from the corresponding author, W.A.K. upon reasonable request.

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