Research Article

Role of Propolis Ethanolic Extract as an Antioxidant Supplement in Tris-Extender to Enhance Semen Quality of Egyptian Buffaloes during Cryopreservation

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Abstract | This study aimed to evaluate the effect of propolis supplementation on semen extender of Egyptian buffalo semen to improve sperm characteristics and antioxidant capacity of sperm medium after cryopreservation. Tris-egg yolk extender with different levels (0, 4, 8, 12, and 14 mg/ml) of propolis ethanolic extract (PEE) were used. Semen was collected once/week for 20 weeks from five buffalo bulls aged 3.5–5 years. The collected semen was pooled and distributed into five equal fractions, each fraction was diluted with Tris-extender at each level of PEE (PEE0, PEE1, PEE2, PEE3, and PEE4). Semen was gradually diluted at 37 °C with each extender at a rate of 1: 10, equilibrated for 4 hours at 4 °C, packaged in French straws, and frozen in liquid nitrogen at -196 °C. Evaluation of semen was achieved pre-freezing in equilibrated semen and post-thawing in semen thawed in a water bath at 37°C for 30 seconds. Results showed that PEE2 showed the highest ($P < 0.05$) positive impact on improving progressive motility, livability, abnormality, and acrosome integrity of spermatozoa in pre-freezing and post-thaw semen. All PEE-extenders increased ($P < 0.05$) viable sperm percentage, and level of total antioxidant capacity and superoxide dismutase activity, and decreased percentage of apoptosis, early apoptosis, necrosis, and malondialdehyde level; most favorable results were recorded with PEE2. The foregoing results indicated that supplementation of Tris-semen extender with propolis ethanolic extender at a level of 8 mg/ml increased the functional activity of spermatozoa, decreased sperm apoptosis, and necrosis, and improved the antioxidant status of sperm medium in cryopreserved semen of Egyptian buffaloes.

Keywords | Egyptian buffalo, Propolis extract, Cryopreservation, Semen variables, Lipid peroxidation

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INTRODUCTION

In the animal production sector, artificial insemination (AI) is an essential tool to improve the genetics and productivity of animals. Cryopreservation of semen is an important technique through artificial insemination to conserve and proliferate the superior genetic origins of animals therefore successful and effective cryopreservation of semen is needed for reproductive biotechnology. Cryopreservation of semen is a process including dilution, equilibration, freezing and thawing. In each stage, spermatozoa were negatively affected structurally and functionally (Bailey et al., 2003) because the cryopreserved sperm cells were exposed to mechanical and anisosmotic

stresses (Hammerstedt et al., 1990). Also, generation of reactive oxygen species (ROS) was increased as a result of oxidative stress causing deleterious effects on sperm function (Kasimanickam et al., 2006). Also, the insemination of contaminated semen reduced acrosome reaction and increased sperm agglutination, causing infection, inflammation, endotoxins, and then reproductive disorders (Pasing et al., 2013; Morrell, 2016). Therefore, a reduction of about 40-50% in sperm motility and viability was recorded following the cryopreservation due to these stressors (Mittal et al., 2019).

Propolis is produced naturally by honeybees. Propolis extract contains several compounds that have biologically pharmacological, antibacterial, antifungal, antioxidant, antimicrobial, antiprotozoal, and antifungal properties (Selem, 2012; Petruska et al., 2014). Generally, Egyptian brown propolis contains compounds like isoflavones, flavonoids, and fatty acids potent against oxidation, microbes, protozoa, and fungi, in addition to long-chain fatty acids (24.4% palmitic acid), flavonoids (13.7% 3-hydroxymethyl-1-Phenyl-1- Heptadecyn-3-Ol) and 10.15% flavones may be related to the biological action of PEE (Selem, 2012).

It was reported that propolis is used to increase male fertility (Bankova, 2005; Salatino et al., 2005) by improving sperm quality and maintaining enzymatic activities. In ram semen, propolis supplementation in the extender improved sperm viability and function (Mahmoud, 2017), semen quality, semen bacterial contamination (Khalifa et al., 2016), extracellular oxidative stress, and the production of endogenous free radicals in sperm medium (Kasimanickam et al., 2006). Propolis extract supplemented to ram semen extender improved sperm parameters (Mohamed and Zanouny, 2017). In boar semen, propolis, as a supplement in the extender, may protect sperm parameters, reduces malondialdehyde level, and provides minerals and vitamins to semen extenders, which keep high sperm quality (Akandi et al., 2015).

The poor fertility rate after the insemination of cryopreserved semen was reported in the reproduction of buffaloes (Mittal et al., 2019). Recently, many studies were conducted to evaluate the effect of various natural antioxidants supplementation to semen extenders on improving the fertility rate of cryopreserved buffalo semen. The present study hypothesized that the addition of propolis extract, as a natural antioxidant, to semen extender could improve the freezing of buffalo semen. To now, the appropriate levels of propolis supplementation in semen extender of Egyptian buffaloes were not studied as in other animal species. Therefore, the current study aimed to evaluate the effect of propolis supplementation, as a natural antioxidant, on semen extender of Egyptian buffalo semen to improve sperm characteristics and antioxidant capacity of sperm medium after cryopreservation.

MATERIALS AND METHODS

Preparation of propolis ethanolic extract (PEE)

Commercial Egyptian propolis was provided and grounded, then a lot of 200 g was dissolved in 500 ml ethanol at ambient temperature for 24 hours, then filtered by Whatman filter papers (No. 1) and the filtrate was dried under a vacuum at 40 °C.

Preparation of the experimental extenders

Tris-egg yolk extender with different levels of PEE (PEE0, PEE1, PEE2, PEE3, and PEE4) were used in this experiment with PEE at levels of 0, 4, 8, 12 and 14 mg/ml, respectively. Total of 3.025 g Tris (hydroxyl methyl amino methane), citric acid (1.675 g), glucose (0.75 g), lincomycin (25 mg), streptomycin (50 mg), glycerol (7 ml), chicken egg yolk (20 ml), and the determined level of PEE (0, 400, 800, 1200, or 1400 mg) was dissolved in bidistilled water up to 100 ml.

Semen collection and dilution

Five healthy and fertile buffalo bulls aged 3.5–5 years were used as semen donors in this experiment. All bulls were kept at the experimental station of animal production at Mahallet Mousa, Kafrelsheikh governorate, belonging to Animal Production Research Institute (APRI), Egypt. Bulls were kept under standard conditions of feeding and management. Semen was collected once a week for 20 weeks (100 ejaculates). An artificial vagina (Neustadt/Aisch, Müller, Nürnberg, Germany) pre-warmed to 42 °C was used for semen collection. Only ejaculates with progressive motility of ≥75% and sperm concentration of ≥ $0.7x10^9$ sperm/ml were pooled for freezing in this experiment.

After semen collection, the pooled fresh semen was distributed into five equal fractions, each fraction was diluted with Tris-extender at each level of PEE (PEE0, PEE1, PEE2, PEE3, and PEE4). Semen was gradually diluted at 37 °C with each extender at a rate of 1: 10, then the diluted semen was gradually cooled to 5 °C within 15 min and equilibrated for 4 hours at 4-5 °C in refrigerator (equilibration period). During the equilibration period, semen was transported into the laboratory in International Livestock Management Training Center, belonging to APRI. The equilibrated semen was packaged automatically in French straws (0.25 ml, IVM technologies, L' Aigle, France), then placed 4 cm above liquid nitrogen (LN) for 10 min and immersed in freeze in LN at -196 °C (Salisbury et al., 1978).

Sperm variables

Evaluation of semen was achieved pre-freezing in equilibrated semen and post-thawing in semen thawed in a water bath at 37 °C for 30 seconds. Percentages of progressive motility, livability, abnormality, and membrane integrity were determined.

Progressive sperm motility

Progressive motility of sperm cells in terms of spermatozoa number with forward movement in a long semi-arc pattern was counted by a phase-contrast microscope provided with a warm stage at 37°C (DM 500; Leica, Switzerland).

Sperm livability and abnormality

Sperm livability and abnormality were calculated in five fields according to Moskovtsev and Librach (2013) in a smear of semen-stained with 5% eosin and 10% nigrosine using a phase-contrast microscope at 400× magnification. The count of stained cells (dead spermatozoa) and unstained ones (live spermatozoa) was determined, then the percentage of live sperm was calculated. At the same time, the total count of spermatozoa having head, tail, and cytoplasmic droplet abnormalities, was determined for calculating the percentage of total morphological abnormalities according to Menon et al. (2011).

Acrosome integrity

The percentage of acrosome integrity (sperm cells with intact and attached acrosome) was calculated in a smear of semen stained by Giemsa under a light microscope at a magnification of 1000x using an oil immersion lens.

The percentage of normal acrosome was calculated for about 200 spermatozoa randomly selected from at least four microscopic fields. The acrosome was considered to be normal when the stain was clearly and evenly distributed over the spermatozoa anterior to equatorial segment.

Flow cytometric analysis

According to Chaveiro et al. (2007), semen samples were processed for Annexin V staining. In a 5 ml tube, 1 ml of sperm suspensions was well mixed with 2 ml binding buffer. In another 5 ml tube, sperm suspensions (100 µl) were mixed with 5 µl of annexin V (FITC label) and directly 5 µl PI (PE label), incubated for 15 minutes, in dark at room temperature, then suspended the sperm in 200 µl binding buffer. Flow cytometric analysis was performed on Accuri C6 Cytometer (BD Biosciences, San Jose, CA) using Accuri C6 software (Becton Dickinson) for acquisition and analysis (Masters and Harison, 2014). Platelet counting was done using the BD Accuri TM C6 flow cytometer. For the gated cells, the percentages of negative or positive to Annexin-V (A- / A+), PI (PI- / PI+), and double-positive cells were evaluated. Four categories of sperm cells were described by Pena et al. (2003) including (1) Viable: live without membrane dysfunction (A-/PI-), (2) Early Apoptotic: spermatozoa labeled with Annexin-V but not with PI (A+/PI-), (3) Apoptotic: dead spermatozoa with damaged permeable membranes (A+/PI+), and (4) Necrotic: dead sperm cells having complete lost membrane (A-/PI+)

Markers of antioxidant capacity and lipid peroxidation

The following biochemical parameters were measured in post-thawed sperm medium: Total antioxidant capacity (TAC, linearity up to 2 mM/l) as a marker of antioxidant capacity, malondialdehyde (MDA) as a marker of lipid peroxidation, and the activity of superoxide dismutase (SOD) as an antioxidant enzyme. All measurements were performed using a spectrophotometer (Spectro UV-VIS Auto, UV-2602, Labomed, Los Angeles, CA, USA) and commercial kits (Biodiagnostic, Giza, Egypt) according to the manufacturer's instructions.

Statistical analysis

The homogeneity of variance and normality of all numerical data distributions were checked using Levene's test and Shapiro-Wilk's test, respectively. Data were statistically analyzed by A completely randomized design (one-way ANOVA) using a software package (SAS, 2007) to study the effect of extenders with different PEE (PEE0-PEE4) on different parameters studied. The significant differences were separated by Duncan's test (Duncan, 1955) at a level of $P<0.05$. Data were presented as mean ± SEM. The statistical model was $Yij = \mu + Ei + eij$, where μ = Overall mean, Ei = Extender (PEE0-PEE4), and eij = Residual error. All percentage values were transformed by arcsine values before analysis.

RESULTS AND DISCUSSION

Pre-freezing sperm variables

Results shown in Table 1 revealed that sperm progressive motility, livability, and acrosome integrity percentages in pre-freezing buffalo semen significantly ($P < 0.05$) increased with PEE2 and PEE3, while the addition of PEE in PEE1 and PEE4 had no significant effects in comparison with free-extender. Abnormal sperm percentage was significantly ($P < 0.05$) improved by all extenders supplemented with PEE (PEE1-PEE4) as compared to the free-extender, being the best in PEE2, followed by PEE3, PEE1, and PEE4, respectively.

Post-thaw sperm variables

In post-thaw semen, all sperm variables studied including progressive motility, livability, abnormality, and acrosome integrity percentages were significantly ($P < 0.05$) improved

by all PEE levels, being the best in PEE2, followed by PEE3, PEE4, and PEE1, respectively (Table 2).

Flow cytometry sperm parameters

Data of sperm flow cytometry in post-thaw buffalo semen (Table 3) explored that all supplemented extenders significantly ($P < 0.05$) increased viable sperm percentage, and decreased the percentage of apoptotic sperm cells compared with free-extender. The percentage of early apoptotic spermatozoa was significantly ($P<0.05$) decreased by PEE1 and PEE2, while increased by PEE3 and PEE4 as compared to free-extender; most favorable results were recorded with PEE2. In comparison with free-extender, necrotic sperm percentage significantly ($P < 0.05$) decreased by PEE2 and increased by PEE4.

Antioxidant status of sperm medium

The effect of PEE addition in the extender of buffalo semen on TAC, lipid peroxidation biomarker (MDA), and enzyme activity of SOD in post-thawed sperm medium was significant (Table 4). All extenders supplemented with PEE significantly ($P < 0.05$) improved the level of TAC in the post-thaw sperm medium as compared to the free-extender. The level of MDA was significantly ($P < 0.05$) reduced, while the activity of SOD was significantly ($P < 0.05$) increased only by PEE1, PEE2, and PEE4.

Table 1: Sperm variables in pre-freezing buffalo semen as affected by PEE level.

Extender	Progressive motility (%)	Live sperm (%)	Abnormal sperm (%)	Acrosome integrity (%)
PEE0 (0 mg /ml)	74.64±0.36^{b}	78.54±0.88^{b}	11.85±0.28^{a}	83.47±0.42^{c}
PEE1 (4 mg /ml)	74.53±0.76^{b}	79.28±0.85^{ba}	10.17±0.25^{b}	83.93±1.28^{c}
PEE2 (8 mg /ml)	76.59±0.57^{a}	82.39±0.78^{a}	7.04±0.07^{d}	86.59±0.70^{a}
PEE3 (12 mg /ml)	76.01±1.96^{a}	81.21±0.46^{a}	8.01±0.13^{c}	85.51±1.04^{b}
PEE4 (14 mg /ml)	74.12±1.27^{b}	79.71±0.76^{ba}	10.43±0.59^{b}	83.70±0.56^{c}

Significant differences in the same column at $P < 0.05$.

Table 2: Sperm variables in post-thaw buffalo semen as affected by PEE level.

Extender	Progressive motility (%)	Live sperm (%)	Abnormal sperm (%)	Acrosome integrity (%)
PEE0 (0 mg /ml)	32.31±2.12^{d}	41.71±2.33^{c}	20.17± 2.31^{a}	45.39±2.28^{d}
PEE1 (4 mg /ml)	36.73±1.79^{c}	43.01±2.45^{b}	18.13±1.92^{c}	47.03±2.71^{c}
PEE2 (8 mg /ml)	43.37±1.82^{a}	53.09±1.79^{a}	14.03±0.44^{e}	56.77±1.19^{a}
PEE3 (12 mg /ml)	41.40±1.47^{b}	52.81±1.94^{a}	16.19±1.15^{d}	51.14±2.11^{b}
PEE4 (14 mg /ml)	35.34±1.83c	43.17±1.36^{b}	19.91±1.48^{b}	46.22±1.29^{c}

Significant differences in the same column at $P < 0.05$.

Table 3: Sperm flow cytometry characteristics in post-thaw buffalo semen as affected by PEE level.

Extender	Viable (%)	Early apoptosis (%)	Apoptosis (%)	Necrosis (%)
PEE0 (0 mg /ml)	25.52±1.81^{e}	11.13±1.21^{c}	47.86±2.81^{a}	15.61± 0.92^{c}
PEE1 (4 mg /ml)	32.88±1.61^{c}	10.94±0.88^{d}	42.53±1.34^{b}	13.81±0.61^{bc}
PEE2 (8 mg /ml)	46.11±1.15^{a}	8.09±0.76^{e}	32.27±1.81^{cd}	12.04±0.44^{b}
PEE3 (12 mg /ml)	40.50±0.49^{b}	12.28±1.15^{b}	33.34±1.47^{d}	13.09±1.29^{bc}
PEE4 (14 mg /ml)	28.38±0.83^{d}	15.27±0.79^{a}	38.28±1.63^{c}	18.97±0.89^{a}

Significant differences in the same column at $P < 0.05$.

Table 4: Antioxidant status of post-thaw buffalo semen as affected by PEE level.

Extender	TAC (mM/l)	MDA (nmol/ml)	SOD (U/mg)
PEE0 (0 mg /ml)	0.17±0.01^{d}	68.67±1.68^{a}	30.65±2.22^{d}
PEE1 (4 mg /ml)	0.33±0.03^{c}	56.15±0.97^{b}	43.65±5.18^{c}
PEE2 (8 mg /ml)	0.81±0.06^{ba}	34.23±1.35^{d}	59.16±1.16^{a}
PEE3 (12 mg /ml)	1.01±0.09^{b}	48.15±5.56^{c}	51.39±2.12^{b}
PEE4 (14 mg /ml)	1.14±0.06^{a}	67.04±1.70^{ab}	31.57±1.56^{d}

Significant differences in the same column at $P < 0.05$. TAC: Total antioxidant capacity. MDA: Malondialdehyde. SOD: Superoxide dismutase.

The most recent reports indicated the beneficial impacts of adding antioxidant extracts to semen extender for improving the freezing ability and fertilizing capacity of buffalo semen (Khalil et al., 2023). In our study, we evaluate the effect of PEE, as a promising natural antioxidant at different levels, added to Tris-extender of buffalo semen on sperm variables pre-freezing and post-thawing, and antioxidant status of post-thaw sperm medium.

Our results revealed that dilution of buffalo semen with Tris-extender supplemented with PEE (8 mg/ml) significantly improved progressive motility, livability, abnormality, and acrosome integrity of spermatozoa in pre-freezing and post-thaw semen. These findings were indicated by the highest recovery rate of motile, live, and acrosome integrity of sperm cells by Tris-extender (PEE2) supplemented with PEE at a level of 8 mg/ml (Figure 1).

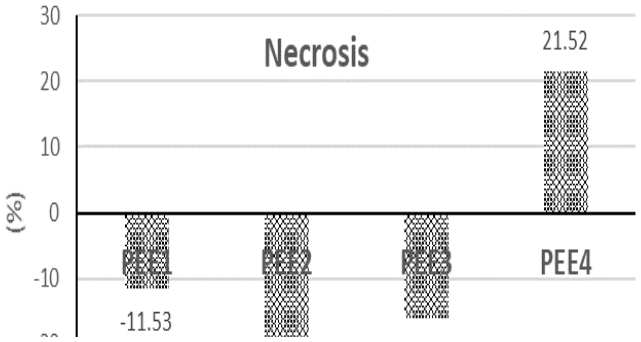

Figure 1: The recovery rate of progressive motility, livability, and acrosome integrity of sperm cells in post-thaw semen as compared to pre-freezing semen.

In harmony with our results, propolis extract in the extenders showed the highest ability to sustain the viability and motility of ram spermatozoa (Mohamed and Zanouny, 2017). Also, the percentage of live sperm with intact and detached acrosome was increased, while dead sperm with intact and detached acrosome was decreased by propolis addition in the extender of stored ram semen (Mohamed, 2017). Supplementation of semen extenders with 0.2% of propolis powder or glue increased sperm motility, livability, and normality of spermatozoa (Khalifa et al., 2016). In boar semen, propolis addition to a semen extender may protect sperm parameters such as motility and live sperm percentages (Akandi et al., 2015). In rats, propolis added to the semen extender increased sperm motility and total sperm output and decreased the count of dead and abnormal spermatozoa (Ciftci et al., 2012). Propolis can protect sperm cell membranes against ROS generation (Russo et al., 2006). Supplementation of semen dilution with propolis increased motility, improved cryoprotection, and maintained sperm membrane integrity (El-Battawy and Brannas, 2015). Similarly, honey and propolis solution, as bee products, improved the storage and freezing abilities of frozen in stallion semen (El-Sheshtawy et al., 2016).

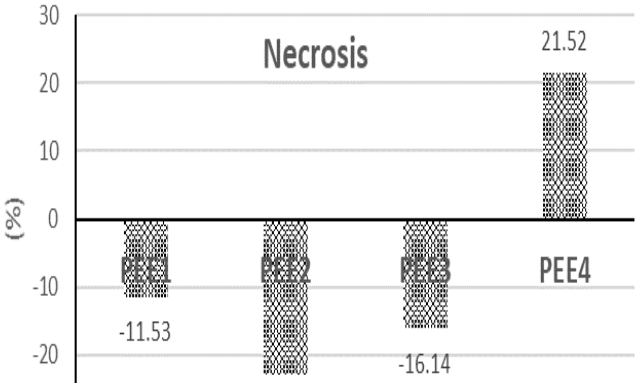

Figure 2: Rate of increase in viable spermatozoa (%) in PEE-extenders relative to PEE0.

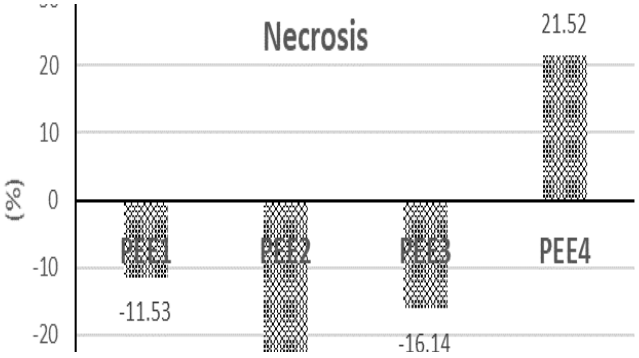

Figure 3: Reduction rate in sperm apoptosis (%) in PEE-extenders relative to PEE0.

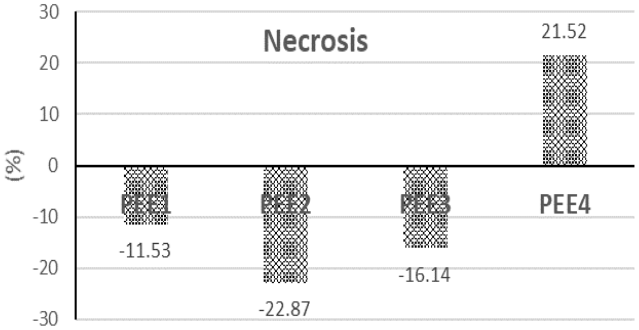

Figure 4: Rate of change (±) in sperm early apoptosis (%) in PEE-extenders relative to PEE0.

The sperm flow cytometry analysis in our study indicated positive impacts of all PEE levels on viability (Figure 2), apoptosis (Figure 3), early apoptotic (Figure 4), and necrosis (Figure 5) in post-thaw semen. Among all supplemented extenders, PEE2 (8 mg/ml) showed the best results. In accordance these results, Akandi et al. (2015) found that propolis supplementation with a semen extender improves sperm viability and DNA integrity. In addition, extenders containing propolis were reported to lower the release of enzymes such as ALT, AST, ALP, and LDH significantly into the sperm medium (Mohamed and Zanouny, 2017; Mohamed, 2017) which may reflect the high sperm membrane integrity during incubation and storage (Zeidan et al., 2004).

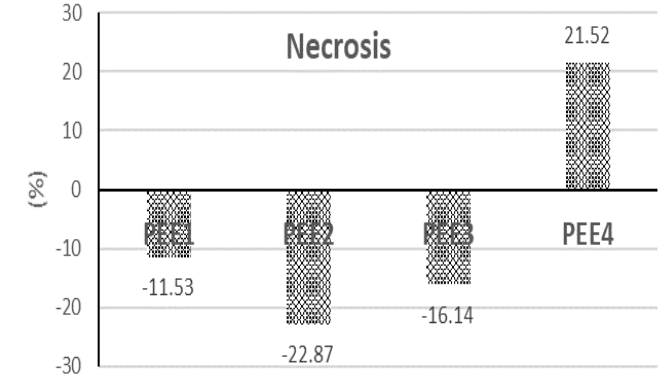

Figure 5: Rate of change (±) in sperm necrosis in PEE-extenders relative to PEE0.

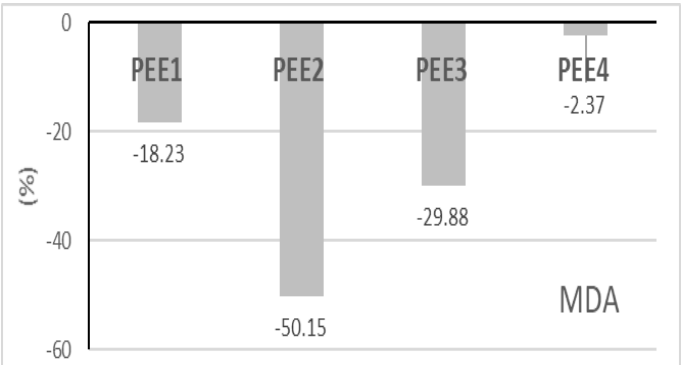

Figure 6: Rate of increase in TAC level in various extenders with PEE relative to PEE0.

Improving sperm characteristics in pre-freezing and post-thaw semen was explained by several authors. In this context, Mohamed and Zanouny (2017) indicated the beneficial effects of propolis in semen extensions to its activity as a natural antibiotic. The drastic decline in sperm characteristics is associated with a gradual reduction in nutrients such as potassium, sodium, and protein needed for sperm metabolism (Olurode and Ajala, 2016). Propolis in sperm medium keeps high-quality viable spermatozoa because it acts as antimicrobial resistance, vitamin and mineral supplement to semen extender, and decreases lipid peroxidation level (Akandi et al., 2015). Moreover, several authors found that propolis activity as a free radical scavenger protects sperm cell membranes against impaired effects by decreasing the formation of barbituric acid reactive substances (Russo et al., 2006). As such we found that extender with 8 mg PEE/ml (PEE2) improved TAC (Figure 6), SOD (Figure 7), and MDA (Figure 8). Similar to these results, Akandi et al. (2015) showed a decrease in MDA level by propolis addition to boar semen extender. They added that propolis may provide minerals and vitamins to the extender to increase sperm quality and viability after preservation. Mammalian tissues contain many antioxidant enzymes (catalase, SOD, glutathione peroxidase, glutathione-Stransferase, and reduced glutathione) which are responsible for scavenging ROS and protecting the cells against stressors. On the other hand, propolis as a natural compound contributes to the detoxification process from ROS (Jasprica et al., 2007; Yousef and Salama, 2009). The obtained results herein regarding the antioxidant status of sperm medium indicated the protection ability of propolis against the free radicals which attack sperm membranes by decreasing the production of their barbituric acid reactive substances (Russo et al., 2006).

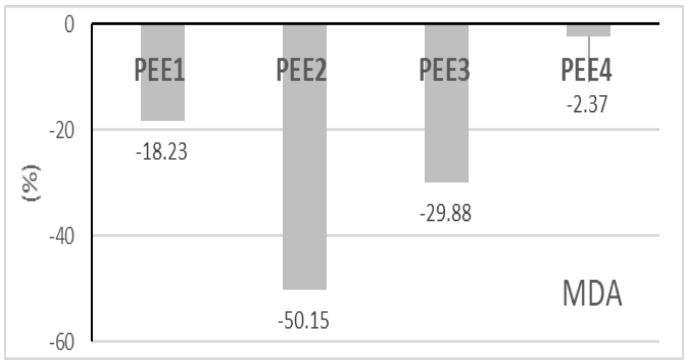

Figure 7: Rate of change (±) in SOD activity in various extenders with PEE relative to PEE0.

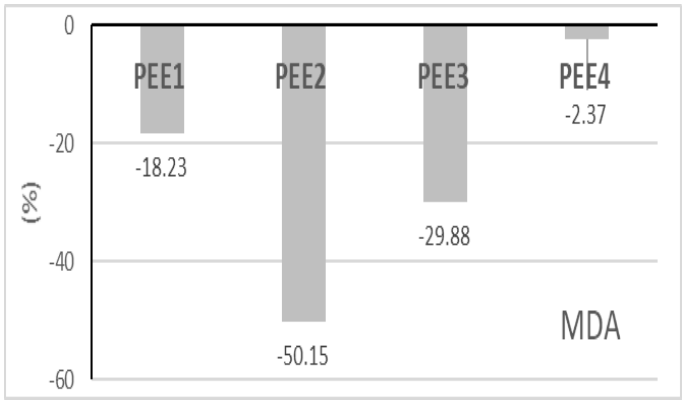

Figure 8: Reduction rate in MDA level in various extenders with PEE relative to PEE0.

The scavenging free radical activity of propolis for protection against lipid peroxidation was reported by Moraes et al. (2014). Flavonoid compounds in propolis like quercetin, flavones, isoflavones, flavonones, anthocyanins, catechins, and is catechins (Alves and Kubota 2013) are capable of scavenging free radicals and reducing lipid peroxidation (Moraes et al., 2014).

CONCUSSIONS AND RECOMMENDATIONS

The foregoing results indicated that supplementation of Tris-semen extender with propolis ethanolic extender at a level of 8 mg/ml improved the physical sperm characteristics and, decreased sperm apoptosis and necrosis, and improved the antioxidant status of sperm medium in cryopreserved semen of Egyptian buffaloes.

NOVELTY STATEMENT

Authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. There has been no significant financial support for this work that could have influenced its outcome. As Corresponding Author, I confirm that the manuscript has been read and approved for publication by all the named authors.

AUTHOR'S CONTRIBUTION

Conceptualization: AEA-K, AMS. Data curation: WMN, EZE, HSA-S. Formal analysis: AEA-K, WMN, AMS. Investigation: AEA-K, EZE, HSA-S. Methodology: EZE, HSA-S, AMS. Resources: AEA-K, WMN. Validation: HSA-S, AMS. Writing-original draft: AEA-K. Writing-review and editing: AEA-K, WMN. Approved the final manuscript (all authors).

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Institutional review board statement
The study was conducted based on the recommended NIH Guide for the care and use of laboratory animals by the Faculty of Veterinary Medicine Ethics Committee, Kafrelsheik University, Egypt. All precautions were followed to diminish animal suffering during the experiment.

Ethical approval
The Institutional Animal Care and Animal Ethics Committee approved the experiment, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. All precautions were followed to diminish animal suffering during the experiment (KFS2020-3).

Consent to participate
Not applicable

Consent to publish
Not applicable

Data availability statement
The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Conflict of interests
We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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