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# Effect of Propolis Ethanolic Extract Supplementation to Ram Semen Extenders on Sperm Characteristics, Lipid Peroxidation and some Enzymatic Activities in Seminal Plasma in Chilled Semen

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## ABSTRACT

This study designed to examine effects of adding different concentrations of propolis ethanolic extract (PEE) as antioxidant to ram semen extenders; tris egg yolk (TEY) or tris-soybean lecithin (TSBL) on sperm characteristics, lipid peroxidation and enzymatic activities in seminal plasma of ram semen preserved at 5°C for 48 hours. Semen was collected from five adult Rahmani rams using an artificial vagina twice/week for 5 weeks. Pooled semen was extended with TEY or TSBL supplemented with different concentrations from PEE (0, 0.1, 0.5 and 1.0 mg/ml). Polyphenols and flavonoid compounds in propolis ethanolic extract were identified. In each treatment, percentage of progressive motility, livability, and abnormality were determined. Also, percentages of sperm membrane integrity or chromatin damage were determined. Concentrations of total antioxidant (TAO) and malondialdehyde (MDA), and activity of acid phosphatase (ACP), lactate dehydrogenase (LDH), and asprtate (AST) and alanine (ALT) transaminase, were determined. Results showed that the most effective polyphenolic compounds in the prolopolis ethanolic extract was the chlorogenic acids, catechein, protocatchuic and pyrogallol (120.623, 53.006, 23.907 and 11.048 mg/100g extract, respectively). Luteo.6-arbinose 8-glucose was the predominant identified flavonoid component in propolis extract (5268.78ppm), followed by acacetin, hisperidin, apig.6-rhamnose8-glucose, apig.6-glucose8-rhamnose, kamp.3-(2-p-comaroyl) glucose and luteo.6-glucose 8-arbinose with concentrations (647.53, 561.09, 366.47, 150.46, 144.51 and 109.42 ppm, respectively. The effect of propolis ethanolic extract supplementation to ram semen extenders was significant ($P<0.05$) only on the overall percentages of sperm progressive motility and sperm chromatin damage while, sperm livability, abnormality and membrane integrity were not affected significantly ($P<0.05$). Concentration of TAC was numerically ($P= 0.82$) higher in TEY extender with different concentrations of PEE than TSBL. However, MDA was lower ($P<0.05$) in free TEY, followed by TSBL with PEE. Activity of LDH, AST and ALT tended to be lower in seminal plasma of semen extended with TSBL than TEY. These results showed beneficial effects of soybean lecithin and propolis ethanolic extract in tris based extender on progressive motility and chromatin integrity of spermatozoa up to 48 h of preservation at 5°C. Also, lipid peroxidation and enzyme activity were lower in TSBL than TEY. In addition, soybean lecithin can effectively replace egg yolk as a protective additive with propolis ethanolic extract for preservation extender without any detrimental effects on post-chilling semen quality in rams.

**Keywords**: Sheep, semen, egg yolk, soybean lecithin, propolis, preservation, sperm function.

## INTRODUCTION

The use of chilled semen considered a cheap solution to the decline in fertility of frozen semen and is more effective (Sri *et al.,* 2012) without the need for liquid nitrogen as compared to frozen semen (Gadea *et al.,* 2005). Research has been directed to ameliorate the preservation of ram semen by modification extender ingredients using addition of various components to preserve sperm characteristics throughout chilling-stored periods. Therefore, extender compositions play a key role in semen storage, because they provide the nutrients, controlled the cold shock, and antibacterial and antioxidant agents. Currently, two different kinds of semen extenders are in styles animal source (such as egg yolk) and plant source (such as soybean lecithin) based extenders, both are commercially available (Forouzanfar *et al.,* 2010).

Egg yolk (EY) is an essential component of semen extender that acts as a non-penetrating cryoprotectant, that prevents membrane damage (Anand *et al.,* 2017), and regulates the efflux of integral protein, phospholipids and cholesterol and subsequently protects the plasma membrane against temperature-related injury (Ferreira *et al.,* 2014). Although the addition of egg yolk changes the compos ion of extender, nevertheless it is recommended because of the excellent protection on sperm cells (Celeghini *et al.,* 2008). Also, its wide availability (Sugulle *et al.,* 2006), beneficial effects on sperm viability as a protectant of the plasma membrane and acrosome against cold shock during chilling or cryopreservation (Amirat *et al.,* 2004). The phospholipids, cholesterol and low density lipoproteins of egg yolk specifically have been identified as the protective components (Anton *et al.,* 2006). The action of EY protection may be attributed to phospholipids, cholesterol and high-density lipoproteins (HDL) and low density lipoproteins (LDL) content which confer effective preservation to the sperm against cold shock and the lipid-phase transition effect during the freeze–thaw process (Bergeron *et al.*, 2004; Anand *et al.,* 2017). In this respect, EY in Tris (hydroxymethylaminometahne) extenders achieved less sperm damage during preservation (Khumran *et al.,* 2017). Egg yolk contains some effective antioxidants like phosvitin and α-tocopherol, which have a possibility to reduce chain reactive oxidation and lipid peroxidation of the sperm membrane, (Alcay *et al.,* 2015). Although the favorable effects of egg yolk in extender, but used egg yolk facing many protests mainly attributed to hygiene concerns and risk of bacterial contaminations (Aires *et al.,* 2003). In addition, egg yolk contains substances may increase viscosity in the extender and disturb respiration of spermatozoa which may lead to reduction their motility (Sharafi *et al.,* 2009; Forouzanfar *et al.,* 2010).

Lecithin is a valuable plant origin to prevent sperm cold shock, because it consists of glycerol, two fatty acids, phosphate group and choline which play an important role in protecting sperm cell membrane. The previous facts has been developed and utilized to preserve spermatozoa of human (Jeyendran *et al.,* 2008), domestic cat (Vick *et al.,* 2010), stallion (Papa *et al.,* 2011), ram (Khalifa and Abdel-Hafez, 2014), buffalo (Akhter *et al.,* 2012), goat (Chelucci *et al.,* 2015) and bovine bull (El-Sisy *et al.,* 2016).

Sperm dysfunction, including lipid peroxidation (LPO), sperm DNA damage and loss of motility resulted

from uncontrolled production of reactive oxygen species (ROS) (De Lamirande *et al.,* 1997). Wherefore, the controlled of ROS are essential for normal fertilization, acrosomal reactions, capacitation and maturation of sperm physiology. However, the supplementation of anti-oxidants is well known method to enhance sperm characteristics during preservation of sperm cells (Ball *et al.,* 2001).

Propolis is a resinous nature material contains approximately 300 component, including polyphenols, phenolic aldehydes, coumarins, steroids, amino acids, sesquiterpene quinines and inorganic compounds (de Sousa *et al.,* 2011). Propolis has composed of wax 30%, resin and vegetable balsam 50%, essential and aromatic oils 10%, pollen 5%, and other substances (Tayeb and Sulaiman, 2014). Recently, the modern science has asserted that propolis has several effects and beneficial pharmacological such as antioxidative (Miguel *et al.,* 2014), antimicrobial (Akca *et al.,* 2016), antifungal (Sariguzel *et al.,* 2015), antivirus (Schnitzler *et al.,* 2010), antiinflammatory (Cavendish *et al.,* 2015). On the other hand, Capucho *et al.* (2012) found that propolis contains some minerals (Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe), some vitamins (B1, B2, B6, C and E) and a number of fatty acids. In accordance with previous studies, enrichment of rabbit semen tris-based extender with 0.24-0.32 mg of propolis ethanolic extract (PEE)/ml tris-extender maintain the sperm characteristics in good condition all over 72 h of chilling (El-Seadawy *et al.,* 2017). In addition, Olczyk *et al.* (2017) confirmed that propolis could reduce production of ROS and free radical (as superoxide anion $O^{-2}$, hydrogen peroxide $H_2O_2$ and hydroxyl radical $OH^-$).

Therefore, the present study was aimed to evaluate the effect of propolis ethanolic extract supplementation to ram semen extenders (containing egg yolk or soybean lecithin) on sperm characteristics, lipid peroxidation and some enzymes activity in seminal plasma in chilled semen at 5ºC for 48 hours.

## MATERIALS AND METHODS

The present study was conducted at Physiology and Biotechnology Lab, Animal Production Department, Faculty of Agriculture, Mansoura University, Egypt, in cooperation with the Experimental Research and Breeding Station in El-Serw; Damietta Governorate, belonging to the Animal Production Research, Agricultural Research Center, Ministry of Agriculture, Egypt.

**Extract preparation:**

Propolis was collected from colonies of honeybees located in Dakahlia Governorate, Egypt, in the summer of 2016. Propolis was kept in the dark at -20 °C until its processing. It was ground into a fine powder under liquid nitrogen. A known quantity of the propolis was extracted with absolute ethanol (1:10 *w/v*). Finally, the extract was lyophilized, weighed and stored at -20°C until required.

**Propolis analysis:**

Propolis extract was analyzed to identify polyphenols and flavonoid compounds according to the method described by Goupy *et al.* (1999) and Mattila *et al.* (2000) , at high performance liquid chromatography (HPLC) laboratory, Food Industries Institute, Agricultural Research Center, Giza, Egypt.

**Semen collection:**

Five sexually mature Rahmani rams of about 3.0 years old and 70-75 kg live body weight were used as semen donors. They were healthy and clinically free of external and internal parasites. All rams were housed in a yard with a concrete floor, roofing with asbestos and could more freely in enclosed area. They were fed concentrate feed mixture and roughage according to NRC (2007) to meet maintenance requirements to supply good semen quality and quantity. The Salt blocks were available continuously and fresh water was given freely throughout the experimental period.

Semen ejaculates were collected using an artificial vagina (two ejaculates weekly for five weeks/ram). Sperm characteristics of each ejaculate were evaluated for volume, progressive motility, livability, abnormality of spermatozoa and sperm cell concentration (by hemocytometer). Only ejaculates semen volume (≥ 0.8 ml), progressive motility (≥75%), live sperm (≥85%), abnormal sperm (≤15%) and sperm cell concentration ($\geq 2.9 \times 10^9$/ml) were pooled, extended and used in experimental proceeding.

**Preparation of semen extender:**

Semen was extended with tris egg yolk (TEY) or tris soybean lecithin (TSBL) Which consists of 2.442g/dl tris (AppliChem, Germany), 1.340g/dl citric acid (AppliChem, Germany), 0.145g/dl sodium citrate (Sigma-Aldrich, USA), 0.750g/dl fructose (Oxford, India), 100 IU/ml penicillin and 100 µg/ml streptomycin and 20% egg yolk or 1% soybean lecithin (LabM, MC041, UK).

**Experimental design:**

Pooled ram semen was extended with TEY or TSBL extenders at a rate of 1 semen: 5 extender and supplemented with propolis ethanolic extract at different concentrations (0, 0.1, 0.5, 1.0 mg/ml extender). Extended semen was preserved in refrigerator at 5°C for 48 h and evaluated at 4, 24 and 48 h.

**Semen evaluation:**

The assessment of the progressive motility visually scaled from 0 - 100 % using a phase contrast microscope (DM 500, Leica, Switzerland) supplied with a warm stage set at 37 °C. Sperm livability (%) was determined in a smear from semen stained by a mixture of 1.67% eosin and 10% nigrosin for estimating live and dead sperm according to (Moskovtsev and Librach, 2013). Sperm Abnormalities of head, mid-piece and tail were counted during the examination of live/dead sperm percentage (Menon *et al.,* 2011).

Positive resistance to hypo-osmotic swelling test (HOST) was used to evaluate the functional intact sperm plasma membrane, based on swollen tails. The hypo-osmotic solution consisted of 7.35 g sodium citrate and 13.51 g fructose dissolved in 1000 ml of distilled water. Concisely, 500 µl of hypo-osmotic solution was mixed with 50 µl of extended semen and was incubated at 37°C for up to one hour. Then a drop of well mixed semen was placed on a glass slide and covered with a cover slip. At least 200 spermatozoa were counted in different fields using microscopic examination (× 400) to evaluate percentage of coiled tail in sample.

Toluidine blue staining was performed as previously described by (Erenpreiss *et al.* 2004).

Spermatozoa stained as green to light blue were considered to have normal chromatin, while those stained dark blue to violet were considered to have damaged chromatin.

**Biochemical analysis in seminal plasma:**

Seminal plasma was separated from extended semen preserved at 5°C for 48 h and stored at -20°C until the assay of concentration of total antioxidant, TAC (Koracevic *et al.* 2001), malondialdehyde, MDA (Ohkawa *et al.* 1979), activity of acid phosphatase, ACP (Belfield and Goldberg, 1971), lactate dehydrogenase (Bais and Philcox, 1994), asprtate transaminase (AST) and alanine transaminase, ALT (Reitman and Frankel, 1957) using commercial kit (Biodiagnostic, Egypt) and spectro-photometer (SPECTRO UV-VIS AUTO, UV-2602, Labomed, USA).

**Statistical analysis**

The General Linear Model procedures of (SAS 2004), GLM analysis of variance (ANOVA) was used for statistical analysis of data. For sperm characteristics, two ways design (8 treatments x 3 times) was used. However, one way design was used for testing the effect of different concentrations of PEE on biochemical analysis in seminal plasma. Duncan multiple range test was used to test the differences among treatment means (Duncan 1955).

## RESULTS

**Analysis of polyphenolic and flavonoid compounds in propolis extract:**

Qualitative and quantitative analysis of polyphenolic and flavonoid compounds in proplis extract performed using high performance liquid chromatography (HPLC) procedure. Most effective polyphenolic compounds in the PEE was the chlorogenic acids, catechein, protocatchuic and pyrogallol, being 120.6, 53.0, 23.9 and 11.0 mg/100 g extract, respectively. However, luteo.6-arbinose 8–glucose was the predominant identified of flavonoids component in PEE, being 5268.78 ppm, followed by acacetin, hisperidin, apig.6- rhamnose 8-glucose, apig.6- glucose 8-rhamnose, kamp.3-(2-p-comaroyl) glucose and luteo. 6-glucose 8-arbinose, being 647.5, 561.1, 366.5, 150.5, 144.5 and 109.4 ppm, respectively (Table 1).

**Table 1. Polyphenolic and flavonoid compounds in propolis ethanolic extract using HPLC technique.**

| Phenolic compounds | (mg/100g) | Flavonoids | (ppm) |
|---|---|---|---|
| Pyrogallol | 11.05 | Luteo.6-arbinose 8 -glucose | 5268.78 |
| Gallic | 0.50 | Luteo.6- glucose 8-arbinose | 109.42 |
| 4-Amino-benzoic | 0.36 | Luteo.6-arbinose 8 -galactose | - |
| Protocatchuic | 23.91 | Apig.6- rhamnose8- glucose | 366.47 |
| Catechein | 53.01 | Apig.6- glucose8- rhamnose | 150.46 |
| Catechol | 3.48 | Narengin | 69.86 |
| Chlorogenic | 120.62 | Luteolin | - |
| Epicatechein | 0.86 | Luteo.7-glucose | - |
| P-OH-benzoic | 6.60 | Hisperidin | 561.09 |
| Caffeine | 1.95 | Rutin | 94.66 |
| Caffeic | 1.43 | Quercetin-3-O -Glucoside | 19.00 |
| Vanillic | 1.36 | Rosmarinic | 1.24 |
| P-coumaric | 0.71 | Apig. 7-O- neohespiroside | 11.25 |
| Ferulic | 0.87 | Apig.7- glucose | 96.98 |
| Iso-ferulic | 2.15 | Kaemp.3,7-dirhamoside | - |
| Oleuropin | - | Quercetrin | 64.19 |
| Reversetrol | - | Quercetin | 45.64 |
| e-vanillic | 13.48 | Kamp.3-(2-p-comaroyl) glucose | 144.51 |
| Ellagic | 1.36 | Naringenin | 13.23 |
| Alpha-coumaric | 0.25 | Hespertin | 41.68 |
| Benzoic | 4.97 | Kaempferol | 8.54 |
| 3,4,5-methoxy-cinnamic | 0.14 | Rhamnetin | 11.54 |
| Coumarin | 0.21 | Apigenin | 57.31 |
| Salycilic | 3.63 | Acacetin | 647.53 |
| Cinnamic | 0.49 | | |

**Sperm characteristics:**

**Effect of propolis ethanolic extract:**

Overall percentages of progressive motility significantly ($P<0.05$) increased by adding 0.5 or 1.0 mg PEE to TEY or adding 1.0 mg PEE to TSBL, being the highest for TSBL (81.0%) as compared to free TEY or TSBL (75.7 and 74.0%), respectively. Also, adding 0.1 mg PEE to TEY and 0.1, 0.5 or 1.0 mg PEE to TSBL significantly ($P<0.05$) decreased chromatin damage of spermatozoa, being the lowest for both types of extenders supplemented with 0.1 mg PEE (4.4%) as compared to free TEY or TSBL (6.5 and 6.3%), respectively. On the other hand, overall percentage of livability, abnormality and membrane integrity of spermatozoa was not affected by PEE addition to both types of extenders (Table 2).

**Effect of storage period:**

All sperm characteristics in preserved semen were affected significantly ($P<0.001$) by storage time. The results showed significantly deleterious effect on all sperm characteristics by advancing storage time (Table 3).

**Effect of interaction between propolis ethanolic extract and storage period:**

All sperm characteristics were not affected significantly by the interaction between treatment and storage time. This effect cleared marked reduction in the percentages of motility, livability and membrane integrity of spermatozoa, which was associated with increase of abnormality and chromatin damage percentages with advancing storage period in all types of extenders. Semen extended using TSBL supplemented with 1.0 mg PEE showed the best sperm characteristics after 48 hours of storage at cool temperature (Figures 1-5).

**Table 2. Effect of propolis ethanolic extract supplementation to ram semen extenders on sperm characteristics of chilled semen.**

| Type of Extender | Sperm characteristics (%) | | | | |
|---|---|---|---|---|---|
| | Progressive motility | Livability | Abnormality | Membrane integrity | Chromatin Damage |
| TEY | 74.0± 2.2$^{c}$ | 66.1±1.9 | 21.9±1.2 | 43.0±2.4 | 6.5±0.7$^{a}$ |
| TEY+0.1 mg PEE | 76.3± 2.2$^{bc}$ | 68.7±2.2 | 21.7±1.2 | 42.131.8 | 4.4±0.4$^{c}$ |
| TEY+0.5 mg PEE | 78.3± 2.2$^{ab}$ | 69.5±2.4 | 20.7±1.3 | 40.3±1.9 | 6.7±0.6$^{a}$ |
| TEY+1.0 mg PEE | 79.3±2.3$^{ab}$ | 66.7±2.5 | 20.4±1.6 | 43.9±2.3 | 6.3±0.4$^{a}$ |
| TSBL | 75.6±2.5$^{bc}$ | 66.4±2.3 | 22.7±1.1 | 42.5±1.9 | 6.3±0.5$^{a}$ |
| TSBL+0.1 mg PEE | 79.0±2.4$^{ab}$ | 68.5±2.7 | 22.1±1.6 | 41.7±1.8 | 4.4±0.5$^{c}$ |
| TSBL+0.5 mg PEE | 78.3±2.1$^{ab}$ | 66.2±2.6 | 23.8±2.0 | 39.5±1.7 | 5.3±0.5$^{b}$ |
| TSBL+1.0 mg PEE | 81.0±2.0$^{a}$ | 67.8±2.9 | 22.8±1.8 | 42.1±1.5 | 5.1±0.5$^{bc}$ |

**a, b and c: Means ±SEM denoted within the same column with different superscripts are significantly different at P<0.05.**
**TEY= Tris egg yolk. TSBL= Tris soya bean lecithin.**

**Table 3. Effect of storage ram semen at cool temperature for different periods on sperm characteristics.**

| Storage time (h) | Sperm characteristics (%) | | | | |
|---|---|---|---|---|---|
| | Progressive motility | Livability | Abnormality | Membrane integrity | Chromatin Damage |
| 4 | 85.5±0.7$^{a}$ | 76.4±0.7$^{a}$ | 16.4±0.5$^{c}$ | 49.3±0.9$^{a}$ | 3.5±0.2$^{c}$ |
| 24 | 79.1±0.7$^{b}$ | 67.9±0.9$^{b}$ | 21. 8±0.5$^{b}$ | 40.8±0.7$^{b}$ | 5.7±0.2$^{b}$ |
| 48 | 68.6±1.1$^{c}$ | 58.0±0.9$^{c}$ | 27.8±0.6$^{a}$ | 35.6±0.6$^{c}$ | 7.7±0.3$^{a}$ |

**a, b and c: Means ±SEM denoted within the same column with different superscripts are significantly different at P <0.001.**

**Antioxidant capacity and enzyme activity in seminal plasma:**

Effect of PEE was significant on concentration of malondialdehyde (MDA), and activity of acid phosphatase and lactate dehydrogenase (LDH) while, concentration of total antioxidants capacity (TAC), and activity of AST and ALT in seminal plasma of stored semen were not affected by PEE addition after 48 h storage period (Table 4).

It is of interest to note that all PEE concentrations significantly (P<0.05) increased MDA concentration in TEY, while insignificantly decreased MDA in TSBL as compared to free extenders (Table 4).

**Table 4. Effect of different concentrations of propolis ethanolic extract supplementation to ram semen extenders on antioxidant capacity and enzyme activity in seminal plasma of ram semen stored for 48 h at cool temperature.**

| Type of extender | TAC (Mm/l) | MDA (nmol/ml) | ACP (IU/l) | LDH (U/ml) | AST (U/ml) | ALT (U/ml) |
|---|---|---|---|---|---|---|
| TEY | 2.7±0.1 | 16.6±3.0$^{c}$ | 55.3±1.4$^{ab}$ | 427.0±33.9$^{a}$ | 104.7±20.3 | 27.0±8.1 |
| TEY+0.1 mg PEE | 2.6±0.5 | 58.5±3.6$^{a}$ | 63.0±1.6$^{a}$ | 256.3±21.2$^{bc}$ | 50.0±7.0 | 32.7±9.3 |
| TEY+0.5 mg PEE | 2.7±0.1 | 30.3±1.9$^{b}$ | 63.3±4.0$^{a}$ | 311.7±35.1$^{b}$ | 83.0±28.0 | 30.7±5.7 |
| TEY+1.0 mg PEE | 3.0±0.1 | 53.8±6.3$^{a}$ | 63.0±1.4$^{a}$ | 249.0±13.7$^{bc}$ | 94.3±17.6 | 36.3±5.7 |
| TSBL | 2.4±0.3 | 28.3±4.4$^{bc}$ | 47.8±4.6$^{bc}$ | 307.6±11.7$^{b}$ | 94.0±00.0 | 30.7±5.7 |
| TSBL+0.1 mg PEE | 2.7±0.1 | 23.7±2.7$^{bc}$ | 44.2±4.1$^{bc}$ | 282.8±17.9$^{bc}$ | 94.0±00.0 | 25.0±0.0 |
| TSBL+0.5 mg PEE | 2.4±0.6 | 24.0±2.1$^{bc}$ | 41.6±3.4$^{c}$ | 279.1±15.3$^{bc}$ | 94.3±17.6 | 36.3±5.7 |
| TSBL+1.0 mg PEE | 2.4±0.1 | 23.6±4.1$^{bc}$ | 35.7±6.5$^{c}$ | 206.9±30.8$^{c}$ | 74.0±10.0 | 30.7±5.7 |

**a, b and c: Means ±SEM denoted within the same column with different superscripts are significantly different at P<0.05.**

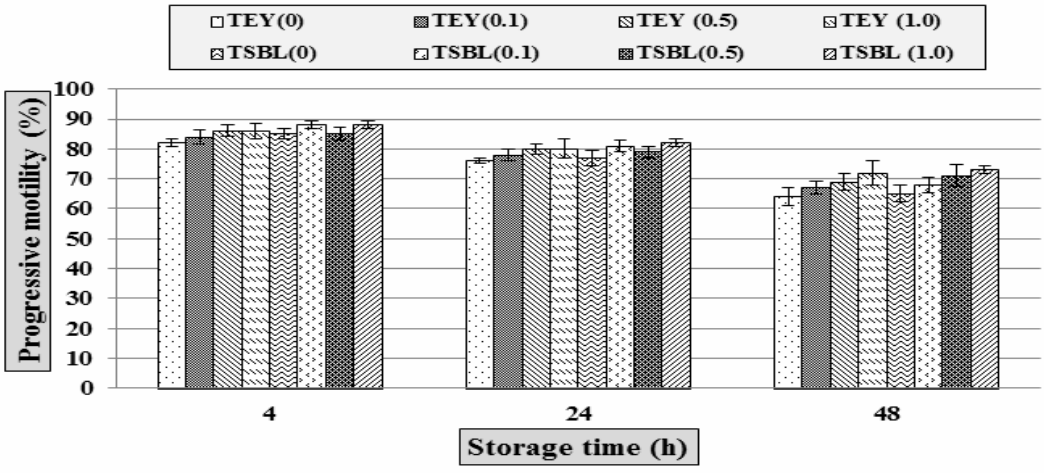


**Fig. 1.**

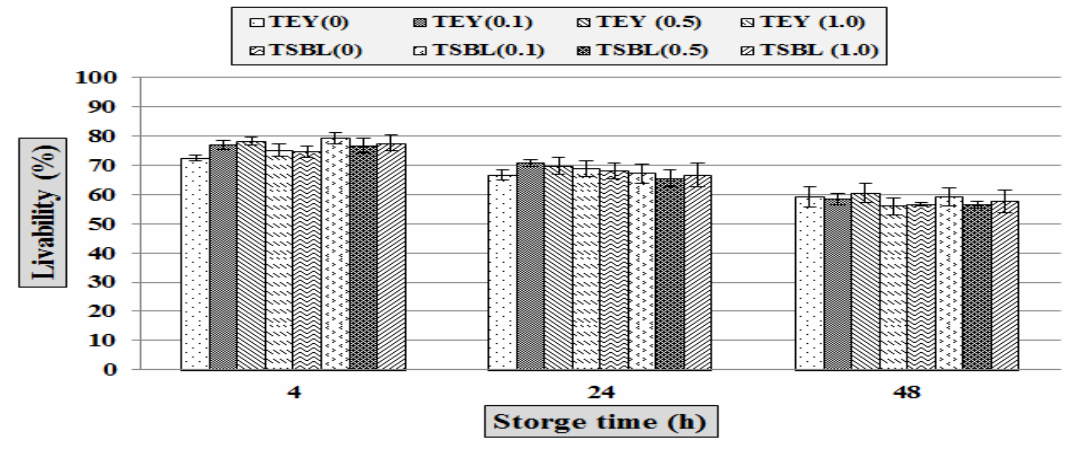


**Fig. 2.**

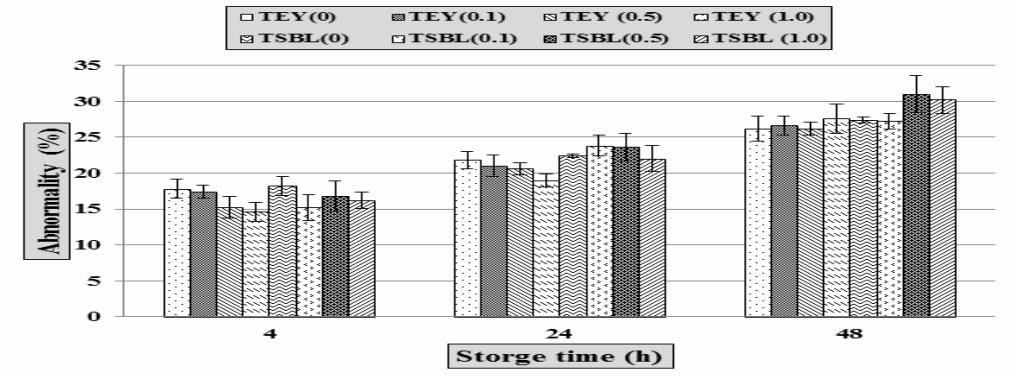


**Fig. 3.**

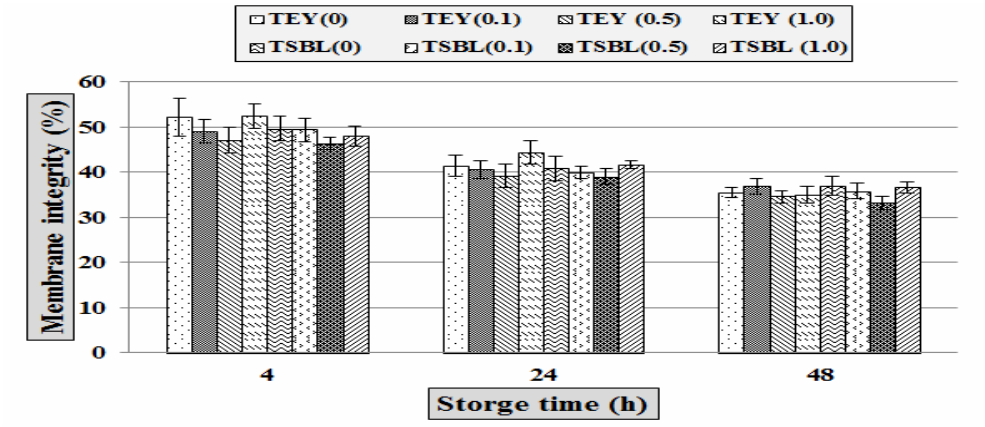


**Fig. 4.**

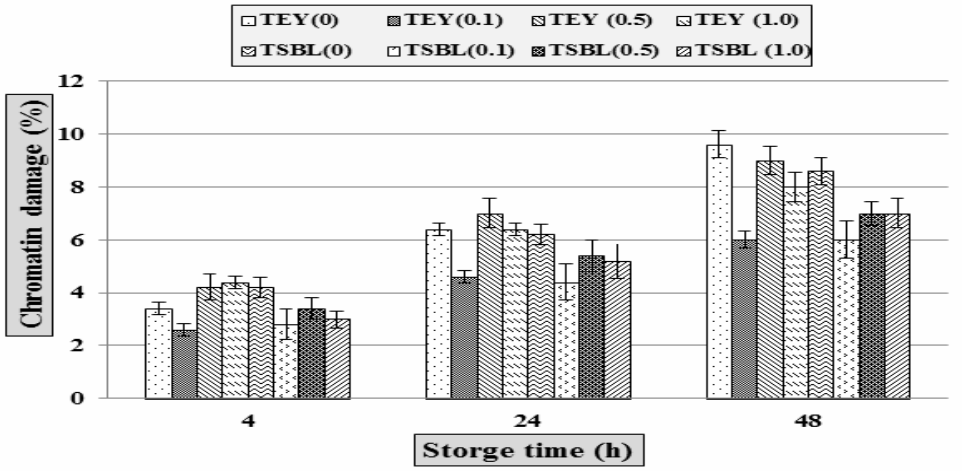


**Fig. 5.**

## DISCUSSION

Spermatozoa contain a high ratio of polyunsaturated fatty acid (PUFA) and a low ratio of cholesterol to phospholipids ratio which make sperm membrane morphology sensitive to excessive production of ROS with subsequent lipid peroxidation (Maxwell and Watson, 1996).

The present study aimed to evaluate the effect of adding different concentrations of propolis ethanolic extract (PEE) to ram semen extenders either TEY or TSBL on sperm characteristics, lipid peroxidation and some enzymatic activities in seminal plasma of semen stored for 48 h.

The current study showed that the progressive motility was improved significantly by increasing PEE concentration in TEY or TSBL extender. However, progressive motility was better in the TSBL than in TEY extender. Little information was available in relation to the effects of additive propolis extract to semen extender. According to Kasiotis *et al.* (2017), propolis removes the hydrogen peroxide and prevents the generation of hydroxyl radicals by the Diphenyl picrylhydrazyl (DPPH) and caffeic acid phenethyl reaction that acts as scavenger of lipid peroxyl radicals. The principal role of propolis to protect viable sperm during preservation is related to a basic biological ability of propolis in extender media. According to El-Seadawy *et al.* (2017), stated that PEE is strong antioxidant activity contained large amounts of antioxidative compounds, such as caffeic acid, ferulic acid, caffeic acid phenethyl and kaempferol and phenethyl caffeine which induced huge TAC concentration. In addition, some authors concluded that propolis extract could indicate the activation of antioxidant enzymes such as superoxide dismutase and catalase against free radicals (Rizk *et al.,* 2014; Santos, 2014; Zaghloul *et al.,* 2016). El-Seadawy *et al.* (2017) established that the addition of propolis ethanolic extract at 0.24-0.32 mg/ml tris-extender in rabbit semen extender indicated sperm motility up to 95.00, 88.33, 71.67 and 51.67% at chilling storage for 2, 24, 48 and 72 hours, respectively. The improvement of sperm motility when propolis extract was supplemented in extender types may be attributed to available of vitamins and/or minerals (Rizk *et al.,* 2014), maintained mitochondrial functionality (Santos, 2014), precluded free radicals containing (Öğretmen *et al.,* 2014), provided antioxidants (Zaghloul *et al.,* 2016) and scavenge of lipid peroxidation (Aslıhan and Seven, 2017).

Our results indicated that soybean lecithin produced comparable and best results in comparison with the egg yolk extender. In view of this, Emamverdi *et al.* (2013) found that lecithin which contains phosphatidyl choline and saturated fatty acids which maintains the structural stability of sperm cell membrane during preservation. Moreover, (Najafi *et al.,* 2014) recorded that extender contains lecithin as an egg yolk substitute has become available for the preservation and cryoprotection of animal spermatozoa. Layek *et al.* (2016) suggested that phospholipids from lecithin may integrate with sperm membrane to form a protective film against the lethal factors which resulted in most debris of spermatozoa. Lecithin has exogenous phospholipids could replaces some phospholipids of sperm membrane, refurbishing plasma membrane and improving tolerance against cold shock. This finding which came in agreement with that observed in some recently reports of (El-Sisy *et al.,* 2016), who ensured that lecithin interacts with and modifies sperm membrane phospholipids and proteins resulting in improved membrane flexibility that withstand injuries of sperm cell membrane. The lowest polyunsaturated fatty acids (PUFAs) in soybean lecithin might have the goodness effects on progressive sperm motility post-chilling. This notice was explained with study of Kaka *et al.* (2016), who reported that the most PUFAs in semen extenders reduced motility spermatozoa by make sperm susceptible to ROS production and lipid peroxidation under chilling condition.

Advancement of storage time for up to 48 hours in extender types can depressed significantly ($P<0.05$) the percentage of advanced sperm motility. These results are similar to that reported in stallion (Santos, 2014), bull (El-Sisy *et al.,* 2016), rabbit (El-Seadawy *et al.,* 2017) and ram (Khalifa and Khalil, 2016). Decreasing progressive motility attainted to gradual depletion of nutrients (Gibb and Aitken, 2016), enhance productive of free radical (Acharya *et al.,* 2016) and inhibit of fructolysis and respiration spermatozoa (Gündoğan *et al.,* 2003).

Our results showed that concentration of TAC was numerically ($P= 0.82$) higher in TEY extender compared with TSBL extender. This may be attributed to egg yolk which played an essential role to confer antioxidant activities to extension seminal plasma. This is manner with ensured results observed by (Ansari *et al.,* 2016), who indicated that EY had antioxidant activity by reducing levels of ROS, free radical and its protein has similar enzymatic capacity of superoxide dismutase and glutathione peroxidase. The non-significant among two types of extender may be related to amino acid presented in soy-lecithin extender (Sharafi *et al.,* 2015). It is an important scavenger of free which can support intercellular antioxidant system which has been a defense mechanism against ROS. The observed higher TAC concentration in treated extenders with propolis than control extender may be attributed to phenolic and flavonoid determined in propolis ethanolic extract (PEE) which has antioxidant activity.

Our results showed that MDA concentration was lower significantly in free TEY followed by TSBL extender supplemented with PEE. On the other hand, the enzymatic activity of ACP, LDH, AST and ALT tended to be lower in seminal plasma of semen extended with TSBL than TEY extender. It well known that estimation of melandialdehyde (MDA) in the seminal plasma is an end product of lipid peroxidation helps to assessing the extent of cellular damage; when cells membrane damage caused irreversible lose in essential metabolites and enzymes (Talluri *et al.,* 2017). Also, Losano *et al.* (2018) demonstrated that extender contains high PUFAs resulting in more susceptible spermatozoa membrane to ROS that leads to damages in membrane matrix and causes destruction of membrane structural and biochemical organs of sperm. High enzyme activity around spermatozoa in the extra medium is sign to injury sperm membrane regard cold shock as well as unsuitable concentration of extender ingredients (Sharafi *et al.,* 2015; Wang *et al.,* 2016).

## CONCLUSION

In conclusion, the present results suggested beneficial effects of soybean lecithin and propolis ethanolic extract in tris based extender on progressive motility and chromatin integrity of spermatozoa up to 48 h of preservation at 5°C. Moreover, a good positive indicator was observed that lower concentration of both of lipid peroxidation and enzyme activity using TSBL than TEY extender. In addition, soybean lecithin can effectively replace egg yolk as a protective additive with propolis

ethanolic extract for preservation extender without any detrimental effects on post-chilling semen quality in rams.

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## تأثير إضافة مستخلص البروبوليس الإيثانولى إلى مخففات السائل المنوى على صفات الحيوانات المنوية، أكسدة الدهون ونشاط بعض الإنزيمات فى بلازما السائل المنوى للكباش المحفوظ بالتبريد

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يعتبر البروبوليس (صمغ النحل) أحد منتجات نحل العسل والتى تمتلك خصائص مضادة للأكسدة. لذلك، إستهدف هذاالبحثدراسة تأثير إضافة مستويات مختلفة من مستخلص البروبوليس الإيثانولى كمضاد أكسدة إلى مخففات السائل المنوى للكباش على صفات الحيوانات المنوية، أكسدة الدهون ونشاط بعض الإنزيمات فى بلازما السائل المنوى لمنى الكباش والمحفوظ على درجة حرارة التبريد (5م°) لمدة 48 ساعة. إستخدم فى هذه الدراسة 5 كباش رحمانى ناضجة وتم جمع السائل المنوى منها بإستخدام المهبل الصناعى مرتين إسبوعياً لمدة 5 أسابيع. تم جمع السائل المنوى ثم خلطه معاً ثم تخفيفه بواسطة مخفف تريس صفار البيض أو مخفف تريس ليسيثين فول الصويا وتم إضافة مستخلص البروبوليس الإيثانولى لكلا المخففين بتركيزات مختلفة (صفر، 0.1، 0.5 و 1 مجم/مل). تم تحديد وتقدير مركبات البوليفينول والفلافونويد فى مستخلص البروبوليس. وتم تقدير الحركة التقدمية والحيوية والشواذ، وكذلك نسبة الحيوانات المنوية ذات الذيل الملفوف كمؤشر على سلامة الغشاء البلازمى للحيوانات المنوية وتم حساب ضرر الأكرسوم. كما تم قياس تركيز مضادات الأكسدة الكلى ومالون داي الدهيد ونشاط كل من إنزيم الفوسفات الحامضى واللاكتيك ديهيدروجينز وكذلك إنزيمات الكبد (GOT and GPT). تشير النتائج إلى أن معظم المكونات البولى فينولية الفعالة فى مستخلص البروبوليس الإيثانولى كانت عبارة عن حمض الكلوروجينيك، كاتشين، بروكاتشويك والبيروجالول بتركيزات (120.623، 53.006، 23.907 و 11.048 مجم/ 100 جم مستخلص) على التوالى. على الجانب الأخر أظهر تحليل الفلافونيدات أن مركب Luteo.6-arbinose 8 –glucose كان سائداً فى مستخلص البروبوليس بتركيز 5268.78 جزء فى المليون، يليه مركبات Acacetin، Hisperidin، Apig.6- rhamnose8- glucose، Apig.6- rhamnose8- glucose، Kamp.3- (2-p-comaroyl) glucose و Luteo.6- glucose 8-arbinose بتركيزات (647.53، 561.09، 366.47، 150.46، 144.51 و 109.42 جزء فى المليون) على التوالى. كما أدى إضافة مستخلص البروبوليس الإيثانولى بتركيزات مختلفة لمخففات السائل المنوى للكباش إلى ظهور تأثير معنوى إيجابى على كل من الحركة التقدمية للحيوان المنوى وكذلك إنخفاض ضرر الكروماتين، بينما لم تتأثر معنوياً صفات السائل المنوى الأخرى (الشواذ، الحيوية و سلامة الغشاء البلازمى). كان تركيز مضادات الأكسدة الكلية أعلى بصورة غير معنويه فى مخفف تريس صفار البيض المضاف إليه مستخلص البروبوليس بمستويات مختلفة مقارنة بمخفف تريس ليسيثين فول الصويا. أيضاً كان تركيز المالون داى ألدهيد منخفضاً فى بلازما السائل المنوى لمخفف تريس صفار البيض غير المحتوى على البروبوليس يليه السائل المنوى المخفف بمخفف تريس ليسيثين فول الصويا المضاف إليه البروبوليس بتركيزات مختلفة. كما كان نشاط إنزيم الفوسفات الحامضي واللاكتيك ديهيدروجينيز وكذلك إنزيمات الكبد منخفضة فى بلازما السائل المنوى للمنى المخفف بمخفف تريس ليسيثين فول الصويا مقارنةبمخفف ترس صفار البيض. تظهر النتائج الحالية تأثير إيجابى لمخفف تريس ليسيثين فول الصويا وكذلك مستخلص البروبوبوليس الإيثانولى على الحركة التقدمية للحيوان المنوى وكذلك قلة ضرر الكروماتين وذلك عند الحفظ على درجة حرارة التبريد (5م°) لمدة 48 ساعة. أيضا كانت أكسدة الدهون ونشاط الإنزيمات فى بلازم السائل المنوى أقل فى محفف تريس ليسيثين فول الصويا بالمقارنة بمخفف تريس صفار البيض. لذلك وبناءً على هذه النتائج يمكن إضافة البروبوليس لمخفف تريس ليسيثين فول الصويا بديلاً لمخفف تريس صفار البيض بمخفف بدون حدوث أى أضرار على جودة السائل المنوى المبرد للكباش الرحمانى.