Research Article

Effects of Propolis Ethanolic Extract Administration on Quality of Fresh and Cryopreserved Semen, Redox Status, and Sperm Flow Cytometry Parameters of Heat-Stressed Rabbit Bucks

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Abstract | Heat stress (HS) conditions adversely affect sperm function and quality traits of rabbit bucks. This study amid to investigate the efficacy of propolis ethanolic extract (PEE) oral administration on antioxidant capacity, reproductive hormones, immunity response, and quality and fertility of fresh and cryopreserved rabbit buck semen under HS conditions. A total of 40 APRI line rabbit bucks (7months old and 3.00±0.20 kg live body weight) were divided into four treatments (10/treatment). The 1st, 2nd, 3rd, and 4th treatments were orally administrated with 2 ml distilled water with 0,100, 200 and 300 mg of PEE/buck, respectively, for 40 days, as a treatment period, and then semen was collected twice/week for 12 weeks. Results revealed that all PEE levels decreased *libido* and abnormality of spermatozoa, while increased semen size and percentages of motility, acrosome status, integrity of membrane, total sperm output, sperm concentration, sperm mitochondrial potential, and initial semen fructose. All PEE levels increased serum follicle-stimulating hormone, luteinizing hormone, testosterone, immunoglobulin, and antibody titer, while reduced lysozyme. All PEE levels improved antioxidants capacity, while reduced malondialdehyde in blood serum and in seminal plasma. Advanced motility, membrane integrity, livability and sperm viability were improved, but sperm abnormality, apoptosis and necrosis were reduced in cryopreserved semen by all PEE levels. Total antioxidants capacity was increased, while malondialdehyde was decreased in post-thawed semen by all PEE levels. Conception rate of doe rabbits was improved by artificial insemination with fresh and thawed buck semen with all PEE levels, being the highest with PEE (300 mg/buck) and slightly higher for fresh than thawed semen. In conclusion, PEE oral administration (300 mg/buck) for 40 days may be used, as an effective tool, to improve antioxidants capacity, immunity, and quality and fertilizability of fresh and cryopreserved semen of rabbit bucks under HS conditions.

Keywords | Rabbits, Propolis, Semen quality, Flow cytometry, Fertility

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INTRODUCTION

In rabbits, maintaining semen characteristics, reproductive performance, and physiological homeostasis under heat stress (HS) is crucially needed in sub-tropical regions (El-Desoky et al., 2017). Hosny et al. (2020) and El-Ratel et al. (2021) showed that HS cues lead to negative effects on characteristics of semen and fertilizing ability, resulting in pronounced economic loss. HS alters testicular characteristics, reduces characteristics, count/ejaculate, normality, and defragmentation of buck spermatozoa (Abdulrashid and Juniper, 2016). The negative impacts of HS on animal fertilizing ability are partially attributed to accumulations of the free radical's production (Mizera et al., 2019). In rabbits, sperm plasma membrane contains poly-unsaturat-

ed fatty acids causing increasing lipid peroxidation due to reactive oxygen species (ROS) generation, as a result of displaying a high metabolic activity of spermatozoa (Attia et al., 2017). During the sperm cell maturation, spermatozoa lose most of endogenous antioxidants in their cytoplasm, as a protection of ROS, which reduced sperm motility and fertilizing ability (Russo et al., 2006). Accordingly, utilization of treatment of rabbits with natural antioxidants can be a tool to improve semen quality of heat-stressed bucks (Hosny et al., 2020).

Commercial propolis (bee glue) has useful pharmacological impacts (Cedikova et al., 2014). It contains some minerals (Fe, Na, Ca, I, Mg, K, Cu, Mn, and Zn), vitamins (E, B2, B6, B1, and C), various enzymes, antioxidants constituents (polyphenols and flavonoids), and fatty acids (El-Seadawy et al., 2017; Khafaji et al., 2019). Propolis is considered as a strong natural antioxidant because it contains flavonoid as a scavenger of ROS for protecting cellular membranes from lipid peroxidation (Yousef and Salama, 2009) by increasing antioxidant enzymes to attack ROS (Jasprica et al., 2007). Furthermore, propolis also has various beneficial biological activities such as antimicrobial, antifungal, antiviral, anti-inflammatory and immunostimulating properties (El-Harairy et al., 2018).

Regarding the impact of propolis on semen quality; it activates steroidogenesis, enhances fertility via improving spermatogenesis in term of increasing semen production with good quality, and improves enzymes of seminal plasma of male rats (Yousef and Salama, 2009; Gul Baykalir et al., 2016). In rabbits, propolis was reported to improve sperm output and weights of the testicles, seminal vesicles, and decreased sperm head and tail abnormalities percentage (Gabr, 2013; Hashem et al., 2013; Handayani and Gofur, 2019). Moreover, propolis extract treatment activated synthesis of steroid enzymes in the testis (Rizk et al., 2014). Propolis shows modulatory activity on cellular mitochondrial energy production (Handayani and Gofur, 2019). Also, the blood biochemical profiles and immune status of doe rabbits was affected positively by propolis treatment (Attia et al., 2019).

In addition, survival of buck's sperm post-cooling and thawing is limited (El-Seadawy et al., 2017). Freezing of semen is important process for artificial insemination (AI) of superior genetic animals (Abdelnour et al., 2020), because it impairs sperm cells, leading to sub-lethal effects on sperm lifespan (Mostek et al., 2017). Antioxidant treatments might be a method for protecting sperm from damage during freezing process of buck fertility (El-Seadawy et al., 2017; Abdelnour et al., 2020). Propolis ethanolic extract (PEE) supplementation in extender of rabbit semen maintains semen quality (El-Seadawy et al., 2017). According to the available literature, different levels of raw propolis were used to improve semen quality of rabbit bucks *in vivo* (Gabr, 2013) or cryopreserved buck semen diluted with extenders containing propolis *in vitro* (El-Seadawy et al., 2017). We hypothesis that, using PEE may be more benefit than raw propolis on production of good quality semen, which may improve freezability of spermatozoa from the deleterious effects of semen cryopreservation. Aim of this study was to evaluate the impact of oral treatment of PEE on oxidative stress, reproductive hormones (FSH, LH, and testosterone), and immunity response as well as quality and fertility of fresh and cryopreserved semen produced by heat-stressed rabbit bucks.

MATERIALS AND METHODS

The current study was conducted at a private commercial rabbit farm, Mansoura City, Dakahlia Governorate, Egypt, in co-operation with Poultry Production Department, Faculty of Agriculture, Damietta University. All procedures and experimental protocols were performed according to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 legislations on the protection of animals used for scientific purposes.

Animals and Climatic Conditions

Forty sexually mature APRI line (Abou Khadiga et al., 2010) bucks (7 months old and 3.00±0.20 kg live body weight) were individually housed in galvanized wire cages (40×50×35 cm) accommodated with feeders for a commercial pellet diet rations and automatic drinkers that provided fresh-water (*ad-libitum*). All bucks were kept under same feeding management and hygiene conditions.

Averages of ambient temperature (AT) and relative humidity (RH) in the rabbitry during the experimental period were recorded daily at 2 p.m. using a digital thermometer and hygrometer. Mean of AT was 32.45◦C and RH was 75.60%. The temperature–humidity index (THI) value was calculated (Marai et al., 2002). During the experimental period values of THI (31.08) indicated that all bucks were under very severe HS.

Experimental Design

The bucks (n=40) were equally divided into 4 experimental groups (ten/group). Oral administration of 2 ml distilled water was used for bucks in the 1st group (G1) as a control. Oral administration of distilled water (2 ml) with PEE at levels of 100, 200 and 300 mg/buck were given for bucks in G2, G3, G4, respectively, for 40 days (1st May to 9th Jun), as treatment period and from 10th Jun to 1st August, as semen collection period.

Preparation Of Pee

According to El-Seadawy et al. (2017), PEE was prepared by grounding 50 g crude propolis, then the powder of propolis (50 g) was extracted by 80% ethanol (500 ml), and stirred continuously by using shaking incubator (150 rpm) for 7 days in a dark room. The extracted propolis was exposed to filtration and evaporation at 40 °C and reduced pressure. The extract was reconstituted in 10 ml of dimethylsulfoxide and stored (-80 °C) till further use.

Sexual Desire And Semen Collection

Before the semen collection period, the experimental bucks were trained for collection of semen by an artificial vagina. Semen was collected morning (8 a.m) twice/wk by artificial vagina (40-41°C) using a teaser doe. Semen collection period was 12 successive weeks (9 wks for collection of fresh semen and another 3 wks for semen cryopreservation). On day of semen collection, sexual desire was determined by a stop-watch as an indication of *libido* or reaction time (El-Ratel et al., 2021).

Fresh Semen Evaluation

Total of 180 ejaculates were collected twice/wk for 9 successive wks from ten bucks in each for fresh semen evaluation (*in vivo* study), then averages of weekly semen parameter during the whole collection period for each rabbit were analyzed. Volume of net semen (VS, without gel mass) of each ejaculate with gel mass was measured by a graduated tube. pH value of semen was measured by a pH paper (Universalindikator pH 0 to 14 Merck, Merck KgaA, 64271 Darmstadt, Germany). Ejaculates were placed in a water bath at 37°C during the evaluation in the laboratory. During evaluation, semen was protected from exposing to heat shocks (low or high temperature) and direct light. Collector, daytime, and place were the same during the course of semen collection.

The percentage of advanced sperm motility in fresh semen was determined in 5 microscopic fields using a phase-contrast microscope (Leica DM 500, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) supplied with a hot stage (37°C) at 100× magnification with classifications of subjective assessments ranging from 0 to 100% (El-Ratel et al., 2021).

An eosin-nigrosine (1:5) stain was used for determining sperm livability for 200 sperm cells/field in 5 microscopic field per using phase contrast microscopy (400× magnification) to count live sperm cells (non-stained) or dead sperm cells (purple stained), then the percentage of live sperm was computed. In same slide of livability determination, sperm abnormality (head and tail) was determined. An improved Neubauer hemocytometer slide (GmbH +Co., Brandstwiete 4, 2000 Hamburg 11, Germany) was used for measuring sperm cell concentration (SCC) at a dilution rate of 1: 99 by saline solution (El-Ratel et al., 2021).

Acrosomal status was determined with naphthol yellow S and erythrosine B stains. Count of sperm cells with or without the acrosomal cap in a field of 200 spermatozoa were recorded and classified to sperm cells with intact or damaged acrosome, and then percentage of sperm cells with intact acrosome was calculated (El-Ratel et al., 2021). Hypo-osmotic swelling test (HOS-t) was used to determine integrity of sperm membranes. The hypo-osmotic solution consisted of fructose (9 g) and sodium citrate (4.9 g) in distilled water with osmolarity level of 100 mOsmol/kg. A mixture of semen (30 µl) and the hypo-osmotic solution (300 µl) was incubated in for 30 min in a water bath at 37°C, and then percentage of sperm cells with curled tail in 5 microscopic fields was computed (El-Ratel et al., 2021).

Mitochondrial potential percentage of spermatozoa was performed after the methods of Naseer et al. (2018). The concentration of semen initial fructose was determined immediately in fresh semen samples. Total count of sperm cells in each ejaculate outputs, as total sperm output/ejaculate (TSO) was also computed: TSO (10^6/ejaculate) = VS (ml) × SCC (×10^6/ml).

Redox Status And Immunity Assessments In Blood Serum

At the termination of the treatment period with PEE (40 days), blood samples were collected from 5 animals carefully chosen from each group. Blood samples were taken before feeding (8 a.m.) were harvested into clean un-heparinized and sterile tubes from the marginal ear-vein of the bucks after topical anaesthetized by Xylocaine 4% anaesthetic. Blood samples were centrifuged at 700 *g* for 20 min (T32c; Janetzki, Wallhausen, Germany), and the collected serum was stored at –20°C for further analysis.

Redox status, total antioxidant capacity (TAC), glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and malondialdehyde (MDA) in blood serum were assessed via commercial kits using a spectrophotometer (Shimadzu, Kyoto, Japan) according to manufacturer's instructions. Immunoglobulin's (IgA, IgG, and IgM) levels in blood serum were determined by using ELISA kits. Antibody titer and Lysozyme activity were also determined in blood serum according to Wegmann and Smithies (1966) and Schultz, (1987) respectively.

Reproductive Hormones In Blood Serum

Serum concentrations of testosterone (Cat# MBS704954), follicle-stimulating hormone (FSH, Cat# E-EL- RB2044) and luteinizing hormone (LH, Cat# E-EL- RB0832) were measured by using ELISA kits (Elabscience Biotechnolo-

gy Co., Ltd, www.elabs cience. com).

Seminal Plasma Of Fresh Semen

Antioxidants status including activities of TAC, GSH, GPx, and SOD, and lipid peroxidation marker (MDA level) starting from the 9th week of semen collection were determined in the seminal plasma stored at –20°C after centrifugation of each ejaculate at 700 *g* for 20 min.

Semen Cryopreservation

During the last three weeks of the semen collection period (12 weeks), 60 ejaculates were collected from all bucks (n=10) in each group (10 bucks x twice/week x 3 weeks) for semen cryopreservation. On each day of semen collection, ejaculates of each group without gel mass were pooled, diluted at a rate of 1:5 with tris-egg yolk-fructose diluent (Tris, 3.028 mg/dl; citric acid, 1.675 mg/dl; fructose 1.250 mg/dl; gentamycin 50 µg/ml; distilled water up to 100 ml) according to Abdelnour et al. (2020). Semen was packed in French straws (0.25 mL, IVM technologies, L' Aigle, France) after the dilution and equilibration (at 5 ◦C for 4 h), then offered at 4-5 cm overhead liquid nitrogen (LN) for 10 min, then kept in LN for 30 days at -196 ◦C.

Evaluation Of Post-Thawed Semen

Semen was evaluated after thawing straws (37◦C/30 s) in a water bath. Sperm variables in post-thawed semen including percentages of advanced motility, livability, abnormality, and curled tail (membrane integrity) of sperm cells were performed as done previously in fresh semen. According to Chaveiro et al. (2007), post-thawed semen samples were taken for Annexin V staining. About one ml of sperm suspensions was added to 2 ml binding in a tube (5 ml), then sperm suspensions (100 µL) were placed in another test tube containing 5 µl of annexin V (A) and directly 5 µl propidium iodide (PI) staining, and the final samples were incubated in dark conditions (at room temperature for 15 min). The incubated samples were suspended in a binding buffer (200 µl) to be ready for flowcytometric assessment on Accuri C6 (BD Biosciences, San Jose, CA, USA) Cytometer provided with Accuri C6 software (Becton Dickinson) for analysis and acquisition (Masters and Harrison, 2014). The platelet calculation was prepared by the BD Accuri TM C6 flow cytometer. Viable, apoptotic, and necrotic sperm cells percentages were classified according to Peña et al. (2003).

Antioxidant Capacity In Post-Thawed Sperm Medium

Samples of post-thawed semen were centrifuged for 20 min at 700 *g* for 20 min at 4 ◦C, and then the supernatant extender was separated and stored at –20 ◦C for assessing TAC by checking hydrogen peroxide decomposition using OxiSelect commercial kits (Cell Biolabs, USA, Najafiet al., 2020). Level of MDA in post-thawed sperm medium was measured by thiobarbituric acid reaction technique, (Feyzi et al., 2008). Protein carbonyls level was used as a protein oxidation marker in post-thawed sperm medium. Level of total protein carbonyls (POC) was measured by using a commercial protein carbonyl ELISA kit (MBS2600784; MyBioSource, Giza, Egypt) based on the manufacturer's procedure (Mostek et al., 2017).

Fertility Study

To evaluate the sperm fertility, total of 160 sexually mature multiparous rabbit does (APRI) were used. On day of AI, all doe rabbits were induced to ovulation by intramuscular injection of each female with 0.20 ml Receptal (Intervet equivalent B.V. Boxmeer Holland) according to El-Ratel et al .(2017). Does were distributed into four groups (n=20 in each) for AI by fresh diluted or cryopreserved semen at the end of the collection period. For AI with semen in fresh case, semen of each group was pooled, diluted at a rate of 1:5 with saline solution, and then 20 does were artificially inseminated with 1 ml diluted semen from each group. For AI with cryopreserved semen, 20 does were artificially inseminated with post-thawed semen (0.25 ml straw/doe in each group. The AI technique was applied using one use curved plastic pipette at 15 cm (Imporvet, SA, Barcelona, Spain). Doe rabbits were inseminated by inserting semen at a distance of at least 12 cm after bypassing the rim of the pelvis to indicate the proper insertion of the semen to the vagina (El-Ratel, 2017). Pregnancy was diagnosed by palpation of the abdomen 10-12 days after AI, then pregnancy rate was calculated. At birth, kindling rate and total litter size/doe were also calculated.

Statistical Analysis

A complete randomized design (one way-ANOVA) was made for analyzing the obtained data by using the SAS software (SAS, 2000). The statistical model as follows: $Y_{ij} = \mu + G_i + e_{ij}$, where Y_{ij} = observed values, μ = the general mean, G_i = the group effect, and e_{ij} = the random error. Before statistical analysis, percentage values were transported to arcsine values. The significant differences among means were tested by the multiple-range test at a level of $P< 0.05$ (Duncan, 1955). Pregnancy and kindling rates were statistically analyzed by Chi-square test.

RESULTS

Reaction Time and Semen Production

Results of Table 1 show that PEE administration at all levels reduced ($P<0.05$) reaction time and percentages of abnormality (head and tail), while increased ($P<0.05$) semen volume and advanced sperm motility, intact acrosome, membrane integrity, TSO per ejaculate, SCC concentration, mitochondrial potential sperm, and initial semen

Table 1: Effects of different levels of propolis ethanolic extract on sexual desire and semen quality of heat-stressed rabbit bucks.

Item	Control	Propolis ethanolic extract level/buck			P-value
		100 mg	200 mg	300 mg	
Reaction time (s)	24.92 ±0.83[a]	20.75±1.10[b]	16.42±0.88[c]	16.08 ±0.84[c]	0.0001
Net semen volume (ml)	0.74±0.03[c]	0.78±0.01[b]	0.86±0.02[a]	0.87±0.01[a]	0.0001
Semen pH value	7.12 ±0.02	7.14±0.02	7.10±0.03	7.13±0.05	0.7236
Advanced sperm motility (%)	71.67±1.78[c]	75.83±0.83[bc]	80.42±1.85[b]	85.83±1.61[a]	0.0001
Livability (%)	64.83 ±1.64[d]	77.42±1.25[c]	82.33±1.42[b]	87.25±1.05[a]	0.0001
Total abnormality (%)	22.33±0.90[a]	18.92±1.07[b]	15.08±0.97[c]	14.25±0.99[c]	0.0001
Head abnormality (%)	5.33±0.43[a]	4.08 ±0.36[b]	3.17±0.37[bc]	2.42±0.34[c]	0.0001
Tail abnormality (%)	17.00±0.86[a]	14.67±0.87[b]	11.92±0.96[b]	11.83±0.98[b]	0.0009
Intact acrosome (%)	72.50±0.99[c]	81.17±1.63[b]	82.67±1.12[b]	86.75±1.78[a]	0.0001
Membrane integrity (%)	26.08±1.16[c]	35.58±1.08[ab]	34.50±1.27[b]	38.42±1.21[a]	0.0001
SCC (×10⁶/ml)	218.080±3.16[c]	247.17±3.46[b]	280.17±1.66[a]	287.67±2.40[a]	0.0001
TSO/ejaculate (×10⁶)	161.48±3.39[d]	194.00±5.13[c]	239.57±3.77[b]	251.08± 3.45[a]	0.0001
Mitochondrial potential sperm (%)	40.17±0.98[c]	46.58±1.37[b]	51.42±0.98[a]	16.08 ±1.11[a]	0.0001
Initial semen fructose (mg/dl)	72.92±0.88[c]	77.83±1.28[b]	82.17±1.42[a]	51.92±1.56[a]	0.0001

SCC= sperm cell concentration/ml, TSO= total sperm output/ejaculate.

[a,b, c and d] Means in the same column and effect bearing different superscripts are significantly different (P≤0.05).

Table 2: Effect of different levels of propolis ethanolic extract on reproductive hormones in serum of heat-stressed rabbit bucks.

Hormonal profile	Control	Propolis ethanolic extract level/buck			P-value
		100 mg	200 mg	300 mg	
FSH (mIU/ml)	10.6 ±0.14[b]	12.51±0.10[a]	12.77±0.15[a]	12.75±0.09[a]	0.0001
LH (mIU/ml)	11.44±0.50[b]	13.30±0.08[a]	13.34±0.06[a]	13.41±0.10[a]	0.0001
Testosterone (ng/ml)	3.04±0.04[b]	3.55±0.06[a]	3.57±0.07[a]	3.60±0.05[a]	0.0001

FSH= Follicle-stimulating hormone, LH=Luteinizing Hormone

[a and b] Means in the same column and effect bearing different superscripts are significantly different (P≤0.05).

fructose as compared to the control. However, the impact of PEE administration on pH value of semen was not significant. The most positive effect of PEE administrations on enhancing the *libido* and semen quality of male rabbits under HS was at a level of 300 mg/buck.

Reproductive Hormones

According to the results of Table 2, all levels of PEE administrations increased (P<0.05) the reproductive hormonal profile of FSH, LH, and testosterone in blood serum compared with the control.

Immunity Response

Table 3 lists effects of different levels of PEE on serum immunoglobulin (IgA, IgG, and IgM), antibody titer, and lysozyme in heat-stressed rabbit bucks. PEE improved (P<0.05) concentrations of IgA, IgG, IgM and antibody titer, while serum lysozyme concentration decreased (P<0.05) in all treatments as compared to the control group. Generally, PEE treatment at 300 mg/ buck showed the most beneficial impacts (P<0.05) on all parameters of immunity response in heat- stressed rabbit bucks (Table 3).

Oxidative Capacity

The impacts of oral administration of PEE levels on oxidative capacity in serum and seminal plasma of bucks are listed in Table 4. It can be observed that the inclusion of all PEE levels increased (P<0.05) TAC, GSH and GPx, and SOD levels, while decreased (P<0.05) levels of MDA, as lipid peroxidation, in serum and in seminal plasma compared with the control.

Sperm Variables In Post-Thawed Semen

The influence of different levels of PEE on the advanced motility, membrane integrity, livability and abnormality percentages in post-thawed semen are presented in Table 5. In post-thawed semen, the percentages of advanced mo

Table 3: Effects of different levels of propolis ethanolic extract on immunity response of heat-stressed rabbit bucks.

Item	Control	Propolis ethanolic extract level/buck			P-value
		100 mg	200 mg	300 mg	
IgG (g/dl)	4.29±0.031[c]	4.85±0.044[b]	5.21±0.080[a]	5.24±0.044[a]	0.0001
IgM (g/dl)	1.21±0.026[b]	1.54±0.025[a]	1.60±0.056[a]	1.62±0.025[a]	0.0001
IgA (g/dl)	0.72±0.022[b]	0.88±0.027[a]	0.96±0.015[a]	0.94±0.033[a]	0.0001
Antibody titer	5.61±0.080[b]	6.59±0.093[a]	6.55±0.066[a]	6.56±0.039[a]	0.0001
Lysozyme (µg/ml)	92.56±1.775[a]	81.85±2.128[b]	80.73±2.033[b]	79.56±2.221[b]	0.0013

IgA= immunoglobulin A, IgG= immunoglobulin G, IgM= immunoglobulin M.
[a,band c] Means in the same column and effect bearing different superscripts are significantly different (P≤0.05).

Table 4: Effects of different levels of propolis ethanolic extract on antioxidants status and lipid peroxidation in blood serum and seminal plasma of heat-stressed rabbit bucks.

Item	Control	Propolis ethanolic extract level/buck			P-value
		100 mg	200 mg	300 mg	
Blood serum					
TAC (mmol/l)	1.25±0.020[c]	1.43±0.034[b]	1.56±0.023[a]	1.60±0.019[a]	0.0001
GSH (mg/dl)	21.82±1.093[b]	26.22±0.570[a]	26.31±1.278[a]	27.44±1.642[a]	0.0224
GPx (mg/dl)	5.94±0.052[b]	6.30±0.034[a]	6.28±0.041[a]	6.33±0.034[a]	0.0001
SOD (IU)	7.13±0.046[b]	7.81±0.080[a]	7.82±0.052[a]	8.11±0.191[a]	0.0001
MDA (nmol/ml)	16.06±0.729[a]	10.70±1.053[b]	10.41±1.128[b]	9.41±1.294[b]	0.0019
Seminal plasma of fresh semen					
TAC (mmol/l)	0.96±0.039[c]	1.17±0.047[b]	1.24±0.020[ab]	1.31±0.022[a]	0.0001
GSH (mg/dl)	15.84±0.642[b]	18.48±1.149[ab]	21.13±1.357[a]	21.24±0.984[a]	0.0071
GPx (mg/dl)	3.14±0.043[b]	3.51± 0.063[a]	3.66±0.034[a]	3.65±0.103[a]	0.0001
SOD (IU)	3.65±0.142[b]	4.64±0.118[a]	4.72±0.098[a]	4.90±0.061[a]	0.0001
MDA (nmol/ml)	11.92±0.198[b]	13.47±0.339[a]	13.54±0.402[a]	13.78±0.370[a]	0.0047

TAC= antioxidant capacity, GSH= glutathione, GPx= glutathione peroxidase, SOD= superoxide dismutase, MDA= malondialdehyde.
[a,b and c] Means in the same column and effect bearing different superscripts are significantly different (P≤0.05).

Table 5: Effects of different levels of propolis ethanolic extract on sperm variables and flow cytometry parameters of post-thawed sperm of heat-stressed bucks.

Item	Control	Propolis ethanolic extract level/buck			P-value
		100 mg	200 mg	300 mg	
Cryopreserved sperm variables					
Advanced motility (%)	41.50±1.675[c]	56.50±2.363[b]	59.50±2.522[ab]	64. 00±1.633[a]	0.0001
Membrane integrity (%)	35.40±1.579[b]	53.00±2.38[a]	57.00±1.699[a]	58.20±1.737[a]	0.0001
Livability (%)	43.80±1.526[c]	60.10±2.292[b]	60.90±3.507[b]	69.60±1.231[a]	0.0001
Abnormality (%)	16.70±1.758[a]	12.90±0.752[b]	10.70±0.789[b]	10.50±1.067[b]	0.0001
Sperm flow cytometry parameters in cryopreserved semen					
Viable (%)	32.10±1.509[c]	43.80±1.114[ab]	40.20±2.107[b]	46.40±1.033[a]	0.0001
Apoptosis (%)	24.50±1.7842[a]	18.40±0.909[b]	14.40±0.979[c]	13.20±1.083[c]	0.0001
Necrosis (%)	20.40±0.901[a]	16.10±1.016[b]	12.50±0.833[c]	11.70±1.0116[c]	0.0001

[a,b and c] Means in the same column and effect bearing different superscripts are significantly different (P≤0.05).

Table 6: Effects of different levels of propolis ethanolic extract on antioxidants capacity in post-thawed sperm medium of heat-stressed bucks.

Item	Control	Propolis ethanolic extract level/buck			P-value
		100 mg	200 mg	300 mg	
TAC (ng/ml)	12.89±0.344[b]	15.09±0.386[a]	15.56±0.218[a]	15.77± 0.124[a]	0.0001
MDA (nmol/ml(	66.50±1.733[a]	43.59±0.708[b]	38.57±2.543[c]	32.48± 0.844[d]	0.0001
Protein carbonyl (ng/ml)	2.93±0.126[a]	1.66±0.143[b]	1.42± 0.065[b]	1.40± 0.063[b]	0.0001

TAC= total antioxidant capacity, MDA= malondialdehyde

[a,b, c and d] Means in the same column and effect bearing different superscripts are significantly different (P≤0.05).

Table 7: Conception and kindling rates of rabbit does inseminated artificially by fresh or thawed semen of rabbit bucks treated with different levels of propolis ethanolic extract.

Item	Control	Propolis ethanolic extract level/buck			P-value
		100 mg	200 mg	300 mg	
Fresh semen					
Pregnancy rate	12/20 (60%)[b]	16/20 (80%)[a]	18/20 (90%) [a]	18/20 (90%)[a]	------
Kindling rate	10/12 (83.33%)	14/16 (87.5%)	17/18 (94.44%)	17/18 (94.44%)	------
Semen preservation					
Pregnancy rate	8/20 (40%)[c]	11/20 (55%)[b]	14/20 (70%) [a]	15/20 (75%)[a]	------
Kindling rate	5/8 (62.50)[b]	8/11 (72.72%)[ab]	11/14 (78.57%)[ab]	12/15 (80%)[a]	------

[a,b and c] Means in the same column and effect bearing different superscripts are significantly different (P≤0.05).

.tility, membrane integrity, livability and viable sperm were increased (P<0.05), while sperm abnormality, apoptosis, and necrosis percentages were reduced (P<0.05) by all levels of PEE. Administration of PEE (300 mg/buck) gives the best sperm variables in cryopreserved semen (Table 5)

Antioxidants Capacity Post-Thawed Sperm

TAC level in post-thawed sperm medium was increased (P<0.05), and MDA level was decreased (P<0.05) by all does of PEE as compared to the control (Table 6).

Fertility Trail

Effects of PEE levels on conception (CR) and kindling (KR) rates of does inseminated by fresh diluted and thawed semen are presented in Table 7. Results reveal that CR was improved (P<0.05) by AI with fresh and thawed semen administrated with all levels of PEE, being slightly higher for fresh than thawed semen. KR was not affected by fresh semen of bucks treated with PEE, but KR of does inseminated with thawed semen treated with PEE (300 mg/buck) increased (P<0.05) as compared with control and other levels of PEE.

DISCUSSION

The main target required from the reproductive performance of rabbit bucks is a good semen characteristics, and this is in need physiologically for ROS boundaries to avoid its role (El-Seadawy et al., 2017). Alleviation of HS deleterious effects on reproduction and physiological responses of rabbit bucks is major challenge in hot regions (El-Desoky et al., 2017). HS conditions increase ROS production while decreasing antioxidant status resulting in oxidative stress (Peña et al., 2019), which negatively affects functions of the testes, including reducing the production of testosterone and decreasing the quality and fertilizing ability of spermatozoa (El-Ratel et al., 2021).

Natural products treatment has increased sperm motility and fertility by counteracting the degrading effects of ROS, because they contain polyphenols, enzymes, vitamins, minerals, and other antioxidants that play a role in decreasing ROS (El-Seadawy et al., 2017; El-Ratel et al., 2021).

Experimental males suffered from very severe HS in the present study according to t THI value (31.08, Marai et al., 2002). According to the foregoing our results, the orally administration of PEE provision sustained semen quality, reproductive hormones, and immunity and antioxidants capacities in heat-stressed bucks, in terms of maintaining the function of spermatozoa and decreasing apoptotic sperm cells by decreasing oxidative stress (El-Kholy et al., 2021). Antioxidant supplementation enhanced the low *libido* of control heat-stressed bucks, which may be due destroying Leydig cell function and suppressing testosterone production (Rai et al., 2004). In our study, libido of bucks administrated with PEE was improved (Hashem et al., 2013) by reducing reaction time which was in association with increased testosterone level (El-Ratel, 2021). Simi-

larly, Capucho et al. (2012) reported positive impacts of dietary propolis on sexual desire of male rats.

In our study, PEE increased levels of FSH and LH which may be considered a sign of improved testosterone secretion. These findings may be attributed to phytoestrogens, as phenolic compounds, that metabolised in cell into enterolactone and enterodiol (Zingue et al., 2017). Phytoestrogens have influenced on hypothalamus and adeno-hypophesis that lead to production of LH and FSH to promote gonads to increase testosterone generation and the process of spermatogenesis (Pineda and Dooley, 2003). Propolis plays a vital role in fertility hormones like testosterone, FSH and LH hormones of Iraqi local roosters (Khafaji et al., 2019) and rats (ElMazoudy et al., 2011). It has the ability to increase mRNA expressions of major enzymes (17β- and 3β-hydroxysteroid dehydrogenases) which are responsible for androgenesis process in testes including (Khafaji et al., 2019). Propolis could increase LH and testosterone of male Albino rats (Shalaby and Saleh, 2011) due to the phenolic acid and flavonoids in propolis that prevents and deletes the harmful effects of ROS (El-Metwally et al., 2017).

Our results indicated that PEE administration increased quantity and quality of semen, including net semen volume, and percentages of sperm motility, vitality, normal morphology, acrosomal and membrane integrities, and mitochondrial potential of sperm cells as well as SCC and TSO, which were adversely affected by HS in rabbit bucks. These results are in agreement with Hashem et al. (2013) on buck semen quality. Several studies have reported that propolis treatment enhance sperm output and decrease abnormalities in head and tail of spermatozoa (Gabr, 2013; Handayani et al., 2019). Manipulations of fatty acids in diet reflect profiles of fatty acids of seminal cell membrane, changing the quality and function of sperm cells (Alizadeh et al., 2014). Thus, the enhancement of sperm quality shown in the current study can be associated with containing propolis fatty acids. Propolis has preventing ability to cell damage by improving enzymes of sperm of the antioxidant defense system due to its antioxidant compounds (El-Seadawy et al., 2017; Khafaji et al., 2019). In this context, PEE activate TAC, GSH, GPx and SOD and reduced level of MD, in the serum and seminal plasma of bucks in our study. The inclusion of natural antioxidants appeared to improve oxidative stress of bucks under HS conditions (El-Rate et al., 2021). Under HS conditions, dietary supplementation of propolis could be used effectively to mitigate negative effects of elevated temperature on oxidative status of rabbit bucks (Hashem et al., 2013). Generally, antioxidants act as ROS reagents within tissues of the testes by reducing oxidative damage in these tissues by impact of enzymes of antioxidant, which play necessary function in modulating endogenous oxidative damage (El-Speiy et al., 2017). Propolis has been successfully used to decrease levels of MDA and activation of SOD and catalase against ROS (Jasprica et al., 2007). The biological and antioxidant effects exhibited by propolis could be related to the effect of the some minerals, certain vitamins and phenolic compounds of propolis (El-Seadawy et al., 2017; Khafaji et al., 2019). These biologically active contents are antioxidant agents having the ability to scavenge ROS and decreasing lipid peroxidation (Yousef and Salama, 2009). Also, propolis has various beneficial biological activities such as antimicrobial, antifungal, antiviral, antiinflammatory and immunostimulating properties (El-Harairy et al., 2018).

In addition, the obtained results observed an enhancement in the immune system functions of males by improving immunoglobulin's (IgA, IgG and IgM), and antibody titer, while lysozyme concentration was reduced. Propolis stimulated nonspecific and specific immunity factors (Cuesta et al., 2005). PEE are highly rich in flavonoids, saponins, sapogenins, and phenolic and vitamin compounds, which act as antioxidants, and may, therefore, improve immune function (El-Seadawy et al., 2017; Khafaji et al., 2019). The positive impact of PEE showed herein with bucks could be associated with its anti-bacterial, anti-viral, anti-parasitic, anti-inflammatory, imm-unomodulatory, and anti-oxidant activities (Attia et al., 2019). Also, PEE improved production of antibody and has potent impact on various cells of innate immune response (Nassar et al., 2012). In broiler chicks, propolis supplementation is suitable for promoting health and immunity status (Attia et al., 2017). Some propolis components increase T- lymphocyte proliferation and secretion of IL-1 and IL-2 by splenocytes (Park et al., 2004). Propolis could activate antigen presenting cells (macrophages) to produce cytokines which activate T and B lymphocytes (Chu, 2006).

As such, we handled the beneficial effects on fresh semen quality of bucks treated with PEE, but the main question 'Is the treatments of bucks with propolis, particularly, during hot climate, have benefits on freezing and fertilizing abilities on rabbit sperm after cryopreservation? The obtained results in this context revealed that oral administration of rabbit bucks with different levels of PEE significantly enhanced motility, livability, viable and plasma membrane integrity and reduced abnormality, apoptosis and of necrosis percentages, with the greatest marks of sperm function and apoptosis for propolis at a level of 300 mg/buck. These improvements were mainly attributed to enhancing sperm characteristic and antioxidant capacity in seminal plasma of fresh semen and post-thawed sperm medium (TAC, MDA, and POC). Accordingly, to reduce the oxidative stress, PEE buck administration can increase the capabili-

ty of fresh seminal plasma (El-Seadawy et al., 2017). Also, PEE can increase the activity of many defense enzymes due to its high content of polyphenolics and vitamins and its strong cell-protective effect against toxic stimuli (Bueno-Silva et al., 2013). During cryopreservation, PEE, as an antioxidant, decrease lipid and protein oxidation in the cell membranes of spermatozoa. Also, all levels of PEE appear to be a positive dose for improvement of activity of TAC and MDA. MDA and POC indicating higher membrane and DNA integrities and reduction of sperm apoptosis post-thawing (Mostek et al., 2017). Enhancing the antioxidant capacity and preventing or balancing oxidative stress synthesis is the main proposal for increasing semen freezability. POC is used as an oxidative stress indicator due to the relative early formation and relative carbonaceous proteins stability (Abdelnour et al., 2020). The levels of POC could be decreased by PEE. These properties of PEE may explain the enhancement in the heat-stressed buck semen quality in our study.

In rabbits, good semen-quality in fresh diluted or in cryopreserved form is important for satisfied fertilizability of spermatozoa (El-Desoky et al., 2017). In our study, treatment of bucks with PEE showed positive impact on quality of fresh and cryopreserved semen. Many studies confirmed that good quality semen pre-freezing reflects positive impacts on post-thawed sperm variables. Improving motility, normality and concentration of spermatozoa in bucks treated with PEE have been associated with increasing fertility (El-Ratel et al., 2021). Improving all semen characteristics of fresh and cryopreserved inseminated semen produced from bucks treated with 300 mg/buck reflected the highest pregnancy rate of does.

CONCLUSION

Based on the foregoing results obtained in this study, administration of propolis ethanolic extract (300 mg/buck) can enhance quality of semen and sperm fertilizing capacity of fresh and post-thawed semen of rabbit bucks under heat stress conditions. These enhancements might be associated with their capability to increase immune response and antioxidants status via decreasing the lipid and protein oxidations and sperm apoptosis.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

NOVELTY STATMENT

Propolis ethanolic extract oral administration (300 mg/buck) is recommended as a promising safe strategy for enhancing the reproductive efficiency of breeding rabbit bucks used in natural mating or artificial insemination under heat stress conditions.

AUTHORS CONTRIBUTION

Substantial contributions to conception and design (Ibrahim El-Ratel and Ibrahim Abu El-Naser); acquisition of data (Ibrahim Talat El-Ratel and Amira El-Gaml); analysis and interpretation of data (Ibrahim El-Ratel, Mostafa El-Moghazy and Amira El-Gaml); statistical analyses (Ibrahim Abu El-Naser), drafting the manuscript (Ibrahim Talat El-Ratel, Ibrahim Abu El-Naser and Amira El-Gaml); critically revising the manuscript for important intellectual content (Ibrahim Talat El-Ratel , Ibrahim Abu El-Naser and Mostafa El-Moghazy); and final approval of the manuscript for publication (all authors).

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