Influence of date palm pollen grain extract on rabbit buck semen characteristics throughout chilled storage period of 72 h

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Background
The demand for using stored semen in artificial insemination programs of livestock animals is increasing. Therefore, developing and improving methods for semen preservation would provide adequate fertility rates that maintain the high production rates for rabbit industry. Several studies on preservation protocols and extender composition have been carried out.
Objective
The current study was designed to examine the effect of various concentrations of date palm pollen grain extract (DPPE) on postchilling quality of rabbit semen.
Materials and methods
Total phenolic and flavonoid substances and antioxidant activity were assessed in DPPE. High-performance liquid chromatography was used for identification and separation of goal metabolites. Semen was gathered from 10 male rabbits, grouped, and then split into five fractions (500 µl each). The first fraction represented as control, whereas DPPE was supplemented at concentrations of 1.6, 2.0, 2.4, and 2.8 mg/5 ml tris-citric extender. Extended semen specimens were cooled at 4°C for 72 h. Motile, life, abnormal, membrane, and acrosome integrity percentages of sperms were appraised in chilled semen all over the refrigeration period.
Results and conclusion
Total phenolic and total flavonoids contents in the DPPE were 4.15 mg GAE/g extract and 0.74 mg CE/g extract, respectively. The DPPE specimen showed various antiradical activity gauged toward 2,2-azino-bis/3-ethil-benothiazoline-6-sulfonic acid (12.37 mM TE/g) and 2,2-Diphenyl-1-picryl-hydrazyl (4.06 mM TE/g). However, the reducing capacity assessed by ferric reducing activity power method was 9.19 mM TE/g/g. The most effective compounds in the DPPE were pyrogallol (4150.92 µg/g extract), ferulic acid (2935.50 µg/g extract), and rutin (2163.99 µg/g extract). The enrichment of semen extenders with 2.4 mg DPPE/5 ml tris-citric extender had preserved the sperm forward motility, sperm livability, sperm acrosome integrities, and sperm membrane integrities in an upright state during cooling till 72 h related to control treating. No adverse effects were recorded on sperm abnormalities. It could be concluded that the enriching of rabbit bucks' semen tris-basic extender by 2.4 mg DPPE/5 ml tris-extender (the perfect and harmless concentration) sustained the sperm features in decent conditions all over a cooling period of 72 h.

Keywords:
antioxidants, date palm pollen grain extract, high-performance liquid chromatography, rabbit buck, semen extender

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Introduction

The application stored semen in artificial insemination (AI) plan of farm animals is increasing [1]. Therefore, there is a need to improve the methods for preserving semen for providing high fertility rates in the rabbit industry. Many studies on semen diluent composition and preservation procedures have been carried out [2–4]. Unfortunately, the capability of buck spermatozoa to stay alive *in vitro* after cooling [2] or frozen storage [5] is limited. Lipid peroxidation caused by overproduction of reactive oxygen species deteriorates sperm proteins, nucleic acids, lipids, and sugars [6,7]. Most of reactive oxygen species are steadily counteracted by antioxidants present in buck semen [8]. The endogenous antioxidants are inadequate to neutralize the peroxidation of lipid throughout the process of semen storage [9]. Numerous efforts have been applied with supplementation of nutritional supra-dietary

antioxidants to improve the quality of buck semen [10] or its livability throughout cooling [9]. However, studies estimating the effectiveness of adding of natural antioxidants in the rabbit seminal diluents are deficient.

Natural extracts and infusions from fruits and vegetables were used in semen diluent for conserving sperm quality [11,12]. Phytochemicals in plants are potential sources that confront free radicals due to their antioxidant activity [13].

Date palm pollen grains (DPPG) has been used since ancient times to enhance reproductive ability in humans as an alimentary addition. It has a powerful nutritional value as it contains high amounts of phytochemicals, flavonoids, phytohormones, and a crude gonadotrophic material [14]. Palm pollen grain extract is one of the powerful fruits that have been explored for its reproductive effect on male rabbit [15].

Gu *et al.* [16] and Mansouri *et al.* [17] assessed the antioxidant power of the date's water extract. This activity was related to a broad variety of phenolic composites in dates including ferulic, p-coumaric and sinapic acids, flavonoids, and procyanidins. Addition of DPPG suspension to the feeding system of male not only increased sperm counts and motility but also improved fertility through the normalization of serum testosterone [18]. In the traditional medicine, pollen grain of date palm had been used for male fertility by improving sperm characteristics with increase in epididymis and weight of testis. Flavonoids in DPPG were responsible for the increase in testosterone levels [19]. Polyphenolic compounds and vitamins of DPPG had a strong antioxidant activity through removing excessive radical formation [20–22]. DPPG has antibacterial, antiviral, and antifungal properties [23,24]. Therefore, the current study aimed to explore the effectiveness of some variant concentrations of the date palm pollen grain extract (DPPE) added to the Tris-based semen extender in maintaining good chilled semen characteristics.

Material and methods

Material

Date palm pollen grain samples

Fresh DPPG were collected in March 2020 from El-Behira Governorate, Egypt. The pollens were detached from the kernels with a fine gauze sieve and stored at 4°C until further use.

Standards of phenolic acids

Rutin, naringenin, catachine, scoplatine, hisperdin, myricetin, quercetin, apegnin, kaempferol, chlorogenic, syringic, p-coumaric, vanillic, caffeic, ferulic, sinapic, rosmarinic, gallic, protocatechuic, gentisic, and cinnamic acid standards were purchased from Sigma–Aldrich Inc. (Louis, Missouri, USA).

Folin and radical precursor

Folin-Ciocalteu reagent and DPPH (2,2-Diphenyl-1-picryl-hydrazyl), ABTS (2,2-azino-bis/3-ethil-benothiazoline-6-sulfonic acid), and TPTZ (2, 4, 6-tripyridyl-s-triazine) were obtained from Sigma–Aldrich Inc.

Solvents and other chemicals

Petroleum ether, diethyl ether, ethyl acetate, tetrahydrofuran, and methanol (analytical grade) were purchased from Tedia Company Inc. (Fairfield, Ohio, USA). Acetonitrile was obtained from Aldrich Chemical (GmbH & Co KG, Steinheim, Germany). Potassium persulfate, dinitrosalicylic acid, sodium hydroxide, aluminum chloride, sodium nitrite, sodium carbonate, hydrochloric acid, sulfuric acid, and acetic acid were of analytical grade.

Methods

Preparation of plant extract

A sample of powdered pollen grains (30 g) was extracted with water (300 ml) for 24 h at room temperature. The macerate was filtered through filter paper (Whatman: No 1) in a Buchner funnel. The filtered solution was evaporated in a rotary evaporator under vacuum at 40°C [25]. The dry final product was reconstituted in 10 ml DMSO and stored at −80°C until further analysis.

Determination of major phytochemicals in the prepared extract

Determination of total phenolic content

The total phenolics were determined according to the Folin-Ciocalteu procedure [26]. Total content of phenolics was expressed as mg/g gallic acid equivalent using a derived equation from the calibration curve: $Y=0.034x+0.111$, $R^2=0.999$, where x is the absorbance and Y is the gallic acid equivalent (mg/g).

Determination of total flavonoid content

The total flavonoids were determined using aluminum chloride colorimetric assay [26]. Total flavonoid contents were calculated as catechin equivalent (mg/g) using the following equation from the

calibration curve: $Y=0.012x+0.008$, $R^2=0.998$, where x is the absorbance and Y is the catechin equivalent (mg/g).

Determination of antioxidant activity of prepared extracts

Determination of 2,2-Diphenyl-1-picryl-hydrazyl radical scavenging activity

Free radical scavenging capacity of extract was determined using the stable DPPH [27]. The standard curve was prepared using Trolox ($Y=2.441x+2.113$, $R^2=0.999$, where x is the inhibition % and Y is the trolox equivalent mg/g). Results were expressed as mg of Trolox equivalent per g of extract.

Determination of 2,2-azino-bis/3-ethil-benothiazoline-6-sulfonic acid radical scavenging activity

ABTS radical scavenging capacity of extract was determined [27]. The standard curve was prepared using Trolox ($Y=2.965x+0.693$, $R^2=0.999$, where x is the inhibition % and Y is the trolox equivalent mg/g). Results were expressed as mg of Trolox equivalent per g of extract.

Ferric reducing activity power assay

The ferric reducing activity power assay was done [27]. The standard curve was prepared using Trolox ($Y=0.041x+0.006$, $R^2=0.999$, where x is the inhibition % and Y is the trolox equivalent mg/g). Results were expressed as mg of Trolox equivalent per g of extract.

High-performance liquid chromatography analysis

The phenolic compounds in the DPPE samples were analyzed by a high-performance liquid chromatography (HPLC) system using a Varian Prostar HPLC equipped with diode array detector (280 nm). Active compounds were separated on a C-18 reverse phase HPLC column (Varian, 150 mm×4.6 mm, particle size 5 µm) and elution gradient at 25°C. Eluent A was pure methanol and eluent B was a 0.05% acetic acid aqueous solution. Gradient conditions were initial=35% A and 65% B, 30 min=50% A and 50% B, and 40 min=90% A and 10% B. The flow rate was 1 ml/min, and the injection volume was 20 µl [25].

Animal management and semen collection

A total of 20 sexually mature and fertile New Zealand white male rabbits were purchased from the same herd of a commercial farm for the purpose of this study. Rabbits were aged 26–30 weeks and had 2.3–2.9 kg initial weight. Bucks were individually housed in metal wire mesh cages provided with separate facilities for feeding and water supply. Rabbit bucks were trained to mount teaser females and then ejaculated in artificial vagina (IMV, France) adapted at 40–42°C. Semen was collected twice weekly. Each ejaculate was assessed for initial semen quality. White ejaculated semen of more than 200 µl volume, more than or equal to 300×10^6 cells/ml concentration, and with more than or equal to 70% motile spermatozoa were included in the study.

Experimental design

Pilot experiment for selection of useful extract concentrations

Immediately after semen collection, selected ejaculates were pooled to avoid individual differences and to obtain sufficient volume for each treatment. Pooled sample was divided into 11 aliquots (each of 500 µl). The first aliquot was diluted 1 : 10 in tris-citrate-glucose (TCG) basic extender (250 mM tris-hydroxymethylaminomethane, 88 mM citric acid, 47 mM glucose) [28]. The other 10 aliquots were diluted 1 : 10 in the TCG basic extender enriched with 10 concentrations of DPPE (Table 1).

Experimental design to select the optimal extract enriched extender

Immediately after semen collection, selected ejaculates were pooled to allow sufficient volume for each treatment. The pooled sample was divided into five subsamples (each of 500 µl) to prepare one of the five treatments as follows:

(1) The first aliquot was diluted 1 : 10 in TCG basic extender and served as control.
(2) The other four aliquots were diluted 1 : 10 in the TCG extender supplemented with the selected four concentrations of the DPPE that were obtained from the pilot experiment (Table 1).

Semen evaluation

The diluted semen samples were refrigerated in an incubator at 4°C for 72 h. Forward motility, sperm viability, sperm membrane integrity (SMI), and acrosome integrity were assessed after 2, 24, 48, and 72 h of chilling.

Sperm motility

A drop of semen was placed in prewarmed slide (37°C) and covered with cover slip. Sperm motility subjectively was assessed by using phase contrast hot stage microscope set at magnification of ×400 and equipped with a heating plate (37°C).

Sperm morphology and viability

Stained smear was prepared as soon after ejaculation using an eosin nigrosine staining mixture at 1 : 4

Table 1 Sperm motility percentages (mean±SE) of rabbit semen after chilled storage in tris-citrate-glucose extenders enriched with different concentrations of date palm pollen grain extract

	Chilling duration (h)			
Concentration (mg/5 ml)	2 h	24 h	48 h	72 h
Control	90.00 ^{AB}±0.00	72.50 ^{CD}±1.44	32.50 ^{E}±1.44	22.50 ^{E}±1.44
0.4	87.50 ^{B}±1.44	72.00 ^{CB}±0.00	37.50 ^{D}±1.44	27.50 ^{D}±1.44
0.8	87.50 ^{B}±1.44	77.50 ^{B}±1.44	42.50 ^{C}±1.44	27.50 ^{D}±1.44
1.2	92.50 ^{A}±1.44	77.50 ^{B}±1.44	62.50 ^{B}±1.44	42.50 ^{BC}±1.44
1.6[a]	92.50 A±1.44	82.50 ^{A}±1.44	67.50 ^{A}±1.44	47.50 A±1.44
2.0[a]	92.50 ^{A}±1.44	77.50 ^{B}±1.44	62.50 ^{B}±1.44	45.00 ^{AB}±0.00
2.4[a]	90.00 ^{AB}±0.00	75.00 ^{BC}±0.00	62.50 ^{B}±1.44	47.50 ^{A}±1.44
2.8[a]	92.50 ^{A}±1.44	77.50 ^{B}±1.44	67.50 ^{A}±1.44	47.50 ^{A}±1.44
3.2	87.50 ^{B}±1.44	70.00 ^{D}±0.00	60.00 ^{B}±0.00	40.00 ^{C}±0.00
3.6	87.50 ^{B}±1.44	62.50 ^{E}±1.44	37.50 ^{D}±1.44	22.50 ^{E}±1.44
4.0	82.50 ^{C}±1.44	57.50 ^{F}±1.44	27.50 ^{F}±1.44	17.50 ^{F}±1.44
$P<$	0.0002	0.0001	0.0001	0.0001

[a]Concentration is used in the experimental design. Different superscripts (A, B, C, D, E, F) within the same column are significant at *P* value less than 0.05.

Table 2 Total phenolics, total flavonoids, and antioxidant activity of date palm pollen grain extract as determined by the 2,2-azino-bis/3-ethil-benothiazoline-6-sulfonic acid, 2,2-Diphenyl-1-picryl-hydrazyl and ferric reducing activity power assays

		Antioxidant activity (mM TE/g extract)		
Total phenols (mg GAE/g)	Total flavonoids (mg CE/g)	ABTS	DPPH	FRAP
4.15±0.22	0.74±0.04	12.37±0.44	4.06±0.33	9.19±0.35

ABTS, 2,2-azino-bis/3-ethil-benothiazoline-6-sulfonic acid; DPPH, 2,2-Diphenyl-1-picryl-hydrazyl; FRAP, ferric reducing activity power.

dilution rates [29]. The principle of these techniques is dye exclusion, as red eosin stains dead sperm head, whereas nigrosine provides a blue-black background.

Sperm membrane integrity: hypo-osmotic swelling test

Hypo-osmotic swelling test is a relatively simple test to evaluate the functional integrity of the spermatozoa membrane. Amorim *et al.* [30] developed this assay for rabbit spermatozoa. The swollen spermatozoa characterized by coiling of the tail are considered to have an intact plasma membrane.

Acrosome integrity

In the present study, Giemsa was used to stain the acrosome dark purple. Staining technique was as follows [31]: fresh ejaculate was diluted at 1 : 5 in warm normal saline. Diluted semen was smeared and air dried. Smear was fixed in 10% neutral formal saline for 15 min. Fixed smear was washed in running water for 20 min. Fixed smear was immersed in Giemsa working solution overnight. Stained smear was rinsed in two changes of distilled water and air dried. One hundred spermatozoa per sample were examined at ×1000 for acrosome integrity in each stained smear.

Statistical analysis

Statistical analysis was analyzed using SAS (Cary, NC, USA: SAS Inst. Inc; 2008) computerized program v. 9.2 [32] to calculate the analysis of variance for the different parameters between control and additive replications. Significant difference between means was calculated using Duncan multiple range test at *P* value less than 0.05.

Results

Total phenolic content, total flavonoids content, antioxidant activities, and high-performance liquid chromatography of date palm pollen grain extract

Total phenolics of DPPE were 4.15 mg GAE/g extract. The total flavonoids were 0.74 mg CE/g extract. The DPPE aliquot revealed antiradical activities calculated against ABTS (12.37 mM TE/g) and DPPH (4.06 mM TE/g). Ferric reducing activity power assay was 9.19 mM TE/g (Table 2). These results were explained by HPLC analysis (Table 3, Fig. 1), where the pyrogallol, the ferulic acid, the rutin, and the apeginin-7-glucoside were 4150.92, 2935.50, 2163.99, and 338.95 μg/g extract, respectively.

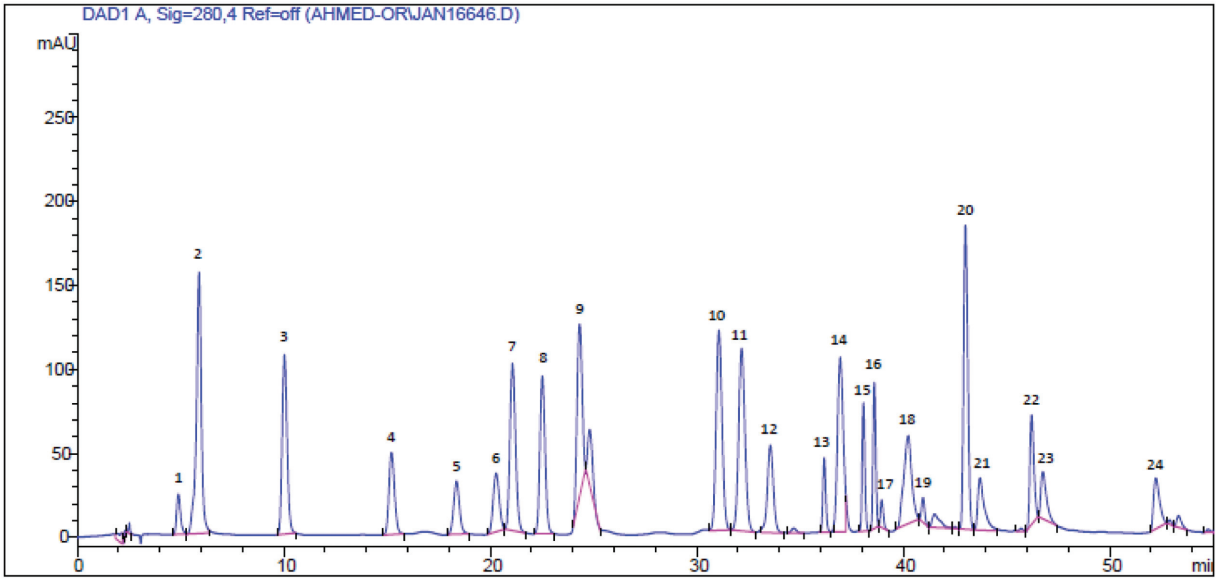
Sperm forward motility percentage

Cooling declined ($P<0.0001$) sperm forward motility (SFM) from 87.50% at 2 h to 30.00% at 72 h (Table 4). This was concurrently with the SFM percentage within every treatment [0, 1.6, 2.0, 2.4 and 2.8 mg DPPE/5 ml tris-citric extender (TCE)] from 2 to 72 h of refrigeration ($P<0.0001$). Regarding the

Table 3 High-performance liquid chromatography analysis of polyphenolic compounds in date palm pollen grain extract

Compounds	Retention time (min)	Concentration (μg/g extract)	Compound	Retention time (min)	Concentration (μg/g extract)
Pyrogallol	4.90	4150.92	Rutin	36.18	2163.99
Gallic acid	5.90	0.00	P-coumaric acid	36.95	0.00
Protocatechuic acid	10.03	0.00	Naringenin	38.07	0.00
P-hydroxybenzoic acid	15.22	0.00	Hesperidin	38.60	0.00
Catechin	18.37	0.00	Apeginin-7-glucoside	38.96	338.95
Chlorogenic acid	20.28	0.00	Myricetin	40.24	0.00
Caffeic acid	21.08	0.00	Rosmarinic acid	40.95	0.00
Syringic acid	22.52	0.00	Cinnamic acid	41.52	0.00
Vanillic acid	24.82	0.00	Quercetin	43.01	0.00
Scopoletin	31.07	0.00	Apigenin	43.72	0.00
Ferulic acid	32.17	2935.50	Kaempferol	46.22	0.00
Sinapic acid	33.56	153.49	Chrysin	52.24	0.00

Figure 1



HPLC of standard metabolites showing signal from diode array detector at wavelength 280 nm. Peak 1, pyrogallol; 2, gallic acid; 3, protocatechuic acid; 4, P-hydroxybenzoic acid; 5, catachine; 6, chlorogenic acid; 7, caffeic acid; 8, syringic acid; 9, vanillic acid; 10, scoplatine; 11, ferulic acid; 12, sinapic acid; 13, rutin; 14, p-coumaric acid; 15, naringeen; 16, hisperdin; 17, apeginin-7-glucoside; 18, myricetin; 19, rosmarinic acid; 20, cinnamic acid; 21, quercetin; 22, apegnin; 23, kaempferol ; 24, chrysin. HPLC, high-performance liquid chromatography.

supplementation of 5 ml TCE with various concentrations of DPPE inside rows (Table 4), the concentration 2.4 mg DPPE/5 ml TCE was the best ($P<0.0010$). DPPE supplements sustained high SFM percentage at 72 h in comparison with the control (0 mg DPPE), 1.6, 2.0, and 2.8 mg DPPE/5 ml TCE. This supported the equivalent overall mean ($P<0.0001$) with its respective concentration.

Sperm livability percentage

Cooling reduced ($P<0.0001$) the overall mean percentage of sperm livability (SL) from 92.08% at 2 h to 83.75% at 72 h (Table 5). This concurrently matched the SL percentage within treatment [control (0), 1.6, 2.0, 2.4, and 2.8 mg DPPE/5 ml TCE] from 2 to 72 h ($P<0.0002$). Regarding the supplementation of 5 ml TCE with variant DPPE concentrations (inside rows; Table 5), the only significant ($P<0.0418$) was at 24 h, whereas there was no significance ($P<0.0598$) difference between means at 48 and 72 h compared with the control. The comparison of the overall means for the Tris-DPPE showed that the use of concentrations 1.6, 2.0, and 2.4 DPPE had no significance in between although all of them were significantly ($P<0.0004$) different from the additive of 2.8 mg DPPE/5 ml TCE concerning the SL percentage.

Table 4 Mean±SEM of sperm motility percentages of diluted rabbit buck semen in tris-citrate-glucose extender enriched with different concentrations of date palm pollen grain extract after chilled storage

		Concentrations of DPPE (mg)/tris-extender (5 ml)					
Chilling duration (h)	Control (TCG)	1.6	2.0	2.4	2.8	*P*<	Overall mean
2	91.67 ^{Aa}±1.67	88.33 ^{ABa}±1.67	86.67 ^{ABa}±1.67	91.67 ^{Aa}±1.67	83.00 ^{Ba}±2.89	0.0245	87.50^{a}
24	73.33 ^{Ab}±1.67	61.67 ^{Bb}±4.41	65.00 ^{ABb}±2.89	71.67 ^{ABb}±1.67	68.33 ^{ABb}±3.33	0.1037	66.67^{b}
48	61.67 ^{ABb}±6.01	38.33 ^{Ac}±6.01	38.33 ^{Cc}±4.40	65.00 ^{Ab}±2.89	45.00 ^{BCc}±7.63	0.0159	46.67^{c}
72	30.00 ^{Bc}±5.77	23.33 ^{Bd}±3.33	23.33 ^{Bd}±1.67	51.67 ^{Ac}±4.41	21.67 ^{Bd}±1.67	0.0010	30.00^{d}
P<	0.0001	0.0001	0.0001	0.0001	0.0001		0.0001
Overall mean		52.92^{B}	53.33^{B}	70.00 ^{A}	54.58^{B}	0.0001	

DPPE, date palm pollen grain extract; TCG, tris-citrate-glucose. Different superscripts within the same row (A, B, C), within the same column (a, b, c, d) indicate significant at *P* value less than 0.05.

Table 5 Mean±SEM of sperm livability percentages of diluted rabbit buck semen in tris-citrate-glucose extender enriched with different concentrations of date palm pollen grain extract after chilled storage

		Concentrations of DPPE (mg)/tris-extender (5 ml)					
Chilling duration (h)	Control (TCG)	1.6	2.0	2.4	2.8	*P*<	Overall mean
2	93.33 ^{Aa}±1.67	92.33 ^{Aa}±0.88	93.00 ^{Aa}±1.00	93.00 ^{Aa}±1.00	90.00 ^{Aa}±1.15	0.2815	92.08 ^{a}
24	88.67 ^{ABb}±0.67	90.00 ^{Ab}±0.00	90.33 ^{Aab}±1.45	89.33 ^{Ab}±0.67	86.00 ^{Bb}±1.00	0.0418	88.92 ^{b}
48	87.67 ^{Ab}±0.67	88.33 ^{Abc}±0.88	87.33 ^{Ab}±1.45	85.67 ^{Ac}±0.67	85.33 ^{Abc}±1.20	0.2422	86.67 ^{c}
72	86.33 ^{Ab}±0.88	86.33 ^{Ac}±0.88	82.67 ^{ABc}±1.45	84.33 ^{ABc}±0.67	81.67 ^{Bc}±1.67	0.0598	83.75 ^{d}
P<	0.0076	0.0011	0.0036	0.0002	0.0120		0.0001
Overall mean		89.25 ^{A}	88.33 ^{A}	88.08 ^{A}	85.75 ^{B}	0.0004	

DPPE, date palm pollen grain extract; TCG, tris-citrate-glucose. Different superscripts within the same row (A, B, C), within the same column (a, b, c, d) indicate significant at *P* value less than 0.05.

Table 6 Mean±SEM of sperm abnormality percentages of diluted rabbit buck semen in tris-citrate-glucose extender enriched with different concentrations of date palm pollen grain extract after chilled storage

		Concentrations of DPPE (mg)/tris-extender (5 ml)					
Chilling duration (h)	Control (TCG)	1.6	2.0	2.4	2.8	*P*<	Overall mean
2	10.67 ^{Bb}±0.67	12.67 ^{ABb}±1.45	15.00 ^{ABc}±1.15	15.33 ^{ABb}±1.76	16.33 ^{Aa}±1.67	0.0925	14.83^{c}
24	15.00 ^{Aa}± 1.52	16.33 ^{Aa}±0.88	16.67 ^{Abc}±0.88	16.33 ^{Ab}±0.67	18.33 ^{Aa}±0.33	0.2488	16.91^{b}
48	14.67 ^{Bab}±1.20	17.67 ^{Aa}±0.33	19.00 ^{Ab}±0.58	18.33 ^{Aab}±0.33	17.00 ^{Aa}±0.58	0.0102	18.00^{b}
72	16.00 ^{Ca}±1.15	18.67 ^{BCa}±0.88	22.33 ^{Aa}±0.33	20.67 ^{ABa}±0.67	19.33 ^{ABa}±1.45	0.0109	20.25^{a}
P<	0.0529	0.0112	0.0010	0.0251	0.3242		0.0001
Overall mean		16.33 ^{B}	18.25 ^{A}	17.67 ^{AB}	17.75 ^{AB}	0.0606	

DPPE, date palm pollen grain extract; TCG, tris-citrate-glucose. Different superscripts within the same row (A, B, C), within the same column (a, b, c, d) indicate significant at *P* value less than 0.05.

Abnormal spermatozoa percentage

The overall mean percentage of anomalous sperm significantly ($P<0.0001$) augmented from 14.83% at 2 h to 20.25% at 72 h (Table 6). This was simultaneous with the atypical sperm morphology % inside treating (0, 1.6, 2.0, and 2.4 mg DPPE/5 TCE; $P<0.0010$), whereas the concentration of 2.8 mg showed no significant increase ($P<0.3242$) in abnormal sperm percentage at 2–72 h. Regarding the supplementation of 5 ml TCE with various DPPE concentrations (inside rows; Table 6), the abnormal sperm percentage significantly ($P<0.0102$) differed between the concentrations 1.6, 2.0, 2.4, and 2.8 of DPPE compared with the control at 48 and 72 h. There was no significant ($P<0.0606$) difference between the related overall means.

Sperm membrane integrity percentage

The SMI overall mean % was reduced significantly ($P<0.0226$) from 65.83% at 2 h to 58.75% at 72 h (Table 7). This was parallel with the SMI % inside treatment of control (0 mg DPPE/5 ml TCE) at 2–72 h ($P<0.0032$). The concentrations of 1.6, 2.0, 2.4, and 2.8 mg DPPE showed no significant difference ($P<0.2559$) in SMI % from 2 to 72 h, although the apparent slight decrease. Regarding the supplementation of 5 ml TCE with various DPPE concentrations inside rows (Table 7), the 2.4 mg DPPE/5 ml TCE was significantly ($P<0.0388$) the excellent DPPE supplement that preserved superior SMI % at 72 h compared with the control (0 mg DPPE), 1.6, 2.0, and 2.8 mg DPPE/5 ml TCE. These results supported the

equivalent overall mean ($P<0.0005$) with its respective concentration.

Sperm acrosome integrity percentage

The sperm acrosome integrities (SAI) overall mean percentages had decreased ($P<0.0001$) from 93.25% at 2 h to 80.25% at 72 h (Table 8). This matched the SAI % inside treatment (0, 1.6, 2.4, and 2.8 mg DPPE/5 TCE) at 2–72 h, whereas the concentration of 2.0 mg DPPE showed no significant ($P<0.0928$) difference for the SAI % from 2 to 72 h, although slow decreases. Regarding the supplementation of 5 ml TCE with various DPPE concentrations (inside rows) (Table 8), the 1.6, 2.0, and 2.4 mg DPPE/TCE significantly ($P<0.0008$) maintained higher SAI % at 72 h compared with the control (0 mg DPPE) and 2.8 mg DPPE/5 TCE. This supported the equivalent overall mean ($P<0.0001$) with their respective concentrations (Fig. 2).

Discussion

The perfection of the male buck reproductive performance is a double-faced coin: one is endogenous, represented in the nutritive high-quality stuffs supplementation for augmenting the power of the spermatogonia germ cells to increase mature sperm concentration and hence the fertilizing ability of the male [33–35], and the other is exogenous through the increase ability of sperm in diluted semen to overcome the environment hazardous action, especially the oxidative stress during preservation [2,3,12]. These nutritive additives could be synthetic [36] or extracted from natural stuffs [11]. Pollen grains are from the last category as we obtained them from the date palm tree. According to our results, the antioxidant activity is referred to their phenolic and flavonoid compounds. The effect of pyrogallol with the other phenolics and flavonoids had a synergistic effect in scavenging the free radicals that are produced from the sperm activities [37]. Laghouati *et al.* [38] used an aqueous extract of DPPG for *in vitro* preservation of rabbit semen. They found that the use of 40 and 80 mg DPPG/ml tris-based extender was better than the tris control in preserving rabbit semen. Di lorio *et al.* [3] reported that the damage in spermatozoa function does not go further beyond 48 h. Although our results had proved that the preserved rabbit semen with DPPG extract kept their viability and motility till 72 h, this may be due to the presence of phenolics and flavonoids and others that can elongate the time of preservation during chilling.

Our study showed that the addition of 2.4 mg DPPE/5 ml TCE had significantly preserved the sperm's motility, livability, membrane, and acrosome integrities in a high-quality state during cooling till 72 h compared with the control nontreated group. This

Table 7 Mean±SEM of sperm membrane integrity (hypo-osmotic swelling test) percentages of diluted rabbit buck semen in tris-citrate-glucose extender enriched with different concentrations of date palm pollen grain extract after chilled storage

		Concentrations of DPPE (mg)/tris-extender (5 ml)					
Chilling duration (h)	Control (TCG)	1.6	2.0	2.4	2.8	$P<$	Overall mean
2	70.00 ^{Aa}±0.00	65.00 ^{Aa}±2.88	63.33 ^{Aa}±1.67	70.00 ^{Aa}±2.88	65.00 ^{Aa}±2.88	0.2154	65.83 ^{a}
24	65.67 ^{ABab}±0.67	65.00 ^{ABa}±0.00	65.00 ^{ABa}±2.88	71.67 ^{Aa}±1.67	61.67 ^{Ba}±4.40	0.1516	65.83 ^{a}
48	62.33 ^{ABb}±1.45	61.67 ^{ABa}±1.67	61.00 ^{ABa}±3.05	71.67 ^{Aa}±1.67	55.00 ^{Ba}±5.77	0.0473	62.33 ^{ab}
72	51.67 ^{Bc}±4.40	61.67 ^{ABa}±1.67	53.33 ^{Ba}±3.33	66.67 ^{Aa}±1.67	53.33 ^{Ba}±4.41	0.0388	58.75 ^{b}
$P<$	0.0032	0.4158	0.2559	0.3300	0.3868		0.0226
Overall mean		63.33 ^{B}	60.67 ^{B}	70.00 ^{A}	58.75 ^{B}	0.0005	

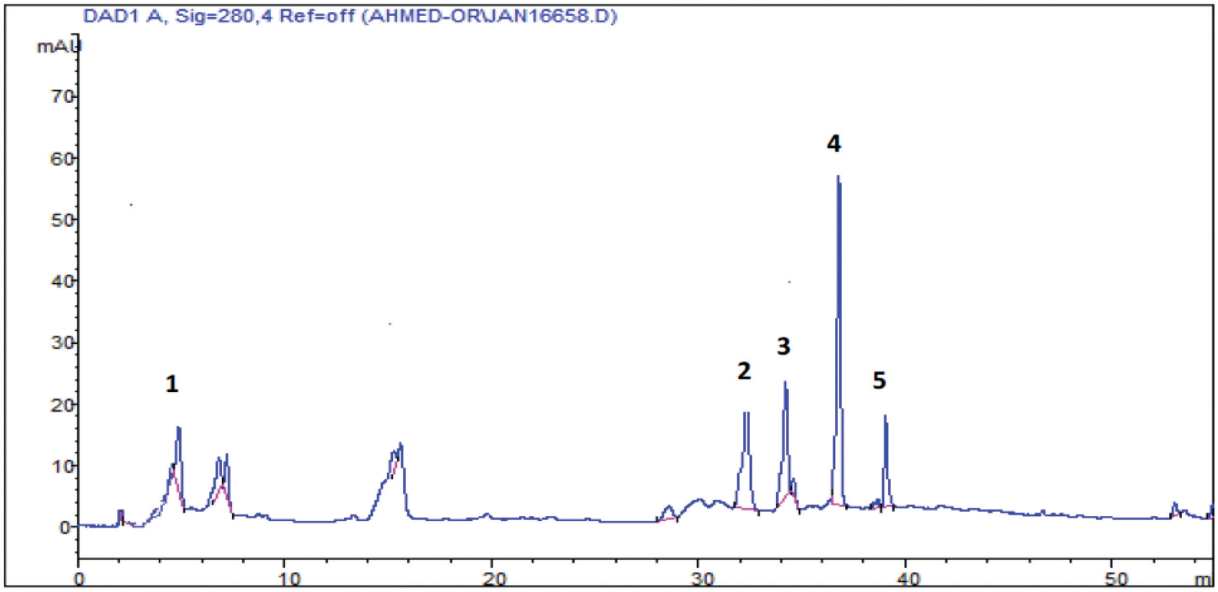
DPPE, date palm pollen grain extract; TCG, tris-citrate-glucose. Different superscripts within the same row (A, B), within the same column (a, b, c) indicate significant at *P* value less than 0.05.

Table 8 Mean±SEM of sperm acrosome integrity percentages of diluted rabbit buck semen in tris-citrate-glucose extender enriched with different concentrations of date palm pollen grain extract after chilled storage

		Concentrations of DPPE (mg)/tris-extender (5 ml)					
Chilling duration (h)	Control (TCG)	1.6	2.0	2.4	2.8	$P<$	Overall mean
2	91.00 ^{Ba}±1.00	95.67 ^{Aa}±0.67	91.67 ^{Ba}±1.67	95.00 ^{Aa}±0.57	90.67 ^{Ba}±0.67	0.0130	93.25 ^{a}
24	85.00 ^{Bab}±2.88	90.67 ^{Aab}±0.67	87.67 ^{ABab}± 1.45	91.00 ^{Aab}±1.00	83.33 ^{Bb}±1.67	0.0365	88.17 ^{b}
48	76.67 ^{Bbc}±4.40	86.67 ^{Ab}±1.67	83.33 ^{ABb}±1.67	86.67 ^{Ac}±1.67	76.67 ^{Bc}±1.67	0.0303	83.33 ^{c}
72	68.33 ^{Bc}±4.40	87.33 ^{Ab}±2.33	82.67 ^{Ab}±3.92	87.67 ^{Abc}±1.45	63.33 ^{Bd}±3.33	0.0008	80.25 ^{d}
$P<$	0.0083	0.0106	0.0928	0.0058	0.0001		0.0001
Overall mean		90.08 ^{A}	86.33 ^{B}	90.08 ^{A}	78.50 ^{C}	0.0001	

DPPE, date palm pollen grain extract; TCG, tris-citrate-glucose. Different superscripts within the same row (A, B), within the same column (a, b, c, d) indicate significant at *P* value less than 0.05.

Figure 2



HPLC of DPPE showing signal from diode array detector at wavelength 280 nm. Peak 1, pyrogallol; 2, ferulic acid; 3, sinapic acid; 4 rutin, 5, apeginin-7-glucoside. DPPE, date palm pollen grain extract; HPLC, high-performance liquid chromatography.

was attributed principally to the high content of rutin (quercetin-3-rhamnosyl glucoside) (2163.99 µg/g extract). Rutin possesses a strong dropping result toward the peroxidation of membrane phospholipids and exhibited strong DPPH, hydroxyl radical, and superoxide radical scavenging activities [39,40]. In agreement with our results, the presence of pyrogallol, ferulic acid, and apeginin-7-glucoside showed strong free radical removing power, interpreted as an advantage of supplementing buck semen TCE via beating the peroxidation of membrane phospholipids provoked through the refrigeration period [41–44]. Similarly, DPPH supplementation elongated the interval of cooling in rabbit buck diluted semen till 72 h [3,43]. In contrast to DPPH, addition of L-carnitine in rabbit semen TCE did not preserve the motility and viability of chilled semen beyond 48 h of cooling [34].

As such, further studies are needed to confirm this result and evaluate the conservation of chilled rabbit semen with the supplementation of DPPG extract to extender and determine the optimal concentration and the possible effect on *in vivo* fertility.

Conclusions

The enrichment of rabbit semen diluent with 2.4 mg DPPE/5 ml TCE (as the most excellent and harmless concentration) could retain the semen parameters in high-quality state during a cooling period of 72 h.

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Conflicts of interest

There are no conflicts of interest.

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