World Rabbit Sci. 2023, 31: 133-145
doi:10.4995/wrs.2023.18703
WRSA, UPV, 2003

# EFFECT OF AN EXTENDER ENRICHED WITH ALGERIAN DATE PALM POLLEN ON CHILLED SEMEN CHARACTERISTICS OF RABBIT BUCKS AT DIFFERENT AGES

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**Abstract:** In the present study, we evaluated the effect of various concentrations of date palm pollen (DPP) aqueous extract on rabbit sperm during storage at 4°C for 48 h. Semen was collected from old and young rabbit bucks using an artificial vagina and initially evaluated for sperm quality. The sperm were diluted in Tris buffer supplemented with (20, 40, 80 mg/mL) of DPP aqueous extract. The extended samples were stored at 4°C for 48 h. Sperm motility and motion kinetics were assessed after 2, 4, 24 and 48 h of storage. At each time, an aliquot was frozen for the analytical evaluation of thiobarbituric acid reactive substances (TBARS) (lipid peroxidation) and tocols (vitamin E). Proximate composition, antioxidant and sugar content were evaluated in DPP. The results showed that DPP was characterised by a high proportion of protein (27.10%) and ash (18.43%), whereas the lipid fraction was very low (0.51%) and total sugar was also high (16.25 g/100 g of fresh matter, f.m.). Regarding the antioxidants content, the sum of tocols showed a total value of 26.48 mg/g f.m. The total polyphenols content was 5.01 mg gallic acid equivalents/g f.m, and polyunsaturated fatty acids was around 30%. Date palm pollen extract had a dose-dependent effect on sperm parameters (curvilinear velocity [VCL] mainly) of old rabbit, although a null or negative effect was recorded at doses >DPP40. Conversely, in optimal conditions (i.e. young rabbit semen), the addition of pollen had no effect on sperm traits (motility and VCL). The progressive increase in DPP, despite providing sperm with an additional amount of tocols, also caused higher tocol consumption and an increase in lipid oxidation. In particular, DPP80 increased the TBARS level in sperm of both rabbit ages. The better trend was found in DPP20, where the tocol consumption figures were 10.46 % and 15.28 %, respectively, in old and young bucks, and the lipid oxidation was lower compared to the higher doses of DPP. In conclusion, the findings of this study demonstrated that supplementation of DPP extract to Tris buffer extender enhanced chilled rabbit semen traits only if administered in old rabbit semen at concentration lower than 40 mg/mL.

**Key Words:** rabbit, semen analyzer, *Phoenix dactylifera* L, chilling sperm, oxidative stress, tris buffer.

## INTRODUCTION

In rabbit, freshly diluted or cooled semen is preferred for insemination (Nagy *et al.,* 2002) because semen storage processes such as cryopreservation induce physical and chemical stress in the sperm membrane, resulting in reduced post-thaw sperm survival (Ahmad *et al.,* 2021).

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https://doi.org/10.4995/wrs.2023.18703

**Cite as**: Laghouati A., Belabbas R., Mattioli S., Dal Bosco A., Benberkane A., Bravi E., Sileoni V., Marconi O., Castellini C. 2023. Effect of an extender enriched with Algerian date palm pollen on chilled semen characteristics of rabbit bucks at different ages. *World Rabbit Sci., 31: 133-145. https://doi.org/10.4995/wrs.2023.18703*

Furthermore, spermatozoa are particularly sensitive towards oxidative damage due to their richness in polyunsaturated fatty acids; the double bonds facilitate the hydrogen abstraction process that initiates the peroxidation cascade (Aitken *et al.,* 1989), which is responsible for alterations in sperm function, such as mitochondrial dysfunction, reduced sperm motility and ability to undergo acrosome reaction, in addition to alterations to the sperm morphology (Aitken and Drevet, 2020).

Semen antioxidant defence systems, present in both sperm and seminal plasma, can counteract the excessive generation of reactive oxygen species (ROS). However, oxidative stress can be caused by an imbalance between ROS production and available antioxidant defences (Sikka, 1996). In this respect, it is essential to strengthen the semen diluent with appropriate antioxidant supplements to reduce ROS damage during sperm storage.

Recently, the search for antioxidant substances to preserve and protect spermatozoa under *in vitro* conditions has attracted the scientific community's attention.

In traditional medicine, date palm pollen (DPP) has been widely used as a folk remedy for curing male infertility (Bahmanpour *et al.,* 2006). It was used to improve reproductive performance in men and women as a dietary supplement (El-Sheshtawy *et al.,* 2014). In fact, in Algerian Sahara, DPP is also used as a food and sexual booster (Selmani *et al.,* 2017).

Additionally, phytochemical studies of DPP grains have revealed that this drug is an excellent candidate for antioxidant processes, due to its richness in high concentrations of total phenolic, flavonoids, and anthocyanins, as well as the presence of a significant quantity of selenoproteins (Baliga *et al.,* 2011). The DPP grains contain triterpenoids, saponins, a crude gonadotropic substance, a D-glucan, heteroxylon, galactomannans, estrone and cholesterol (El-Desoky *et al.,* 1995). In the same regard, Hassan *et al* (2008) and Hassan (2011) revealed the presence of estrone, estradiol and estriol, as well as flavonoid compounds (β-sitosterol, quercetin and rutin). Some studies also linked the antioxidant properties of DPP to phytosterols (Tahvilzadeh *et al.,* 2016). A previous study related the effects of DPP on spermatogenesis boost thanks to these antioxidants (Abed El-Azim *et al.,* 2015). The DPP also contains salts and minerals, including zinc, selenium, iron, copper, cobalt, manganese and nickel, as well as vitamins such as B1, B2, B12, A, E, and C (Tahvilzadeh *et al.,* 2016), many of which could exert antioxidant properties.

Vitamin E is one of the major membrane protectants against ROS and lipid peroxidation (LPO) and is believed to be the primary component of the antioxidant system of spermatozoa (Yousef *et al.,* 2003). It is a lipid-soluble, major chain-breaking antioxidant directly neutralising superoxide anion, hydrogen peroxide and hydroxyl radical (Sharma and Agarwal, 1996).

As a result, the addition of DPP extract to freezing extenders may help spermatozoa withstand oxidative stress. Previous studies revealed that supplementation of DPP with cryopreservation media prevented oxidative damage to spermatozoa and improved cryo-survival in bull (El-Sheshtawy *et al.,* 2014), buffalo bull (El-Sheshtawy *et al.,* 2016), Arabian stallion (El-Sisy *et al.,* 2018), Holstein bulls (Mohamed and Abdulkareem, 2020), bovine (Amsah *et al.,* 2020) and human (AL-Dujaily *et al.,* 2012).

On the other hand, a recent previous study showed that DPP (20, 40, 80 mg/mL) has been successfully used to preserve rabbit sperm motility parameters at 37°C until 2 h (Laghouati *et al.,* 2021); this positive impact was attributed to the synergic effect of bioactive compounds along with the antioxidant activity of DPP. This effect was more evident in poor dilution media (i.e. saline solution), with DPP having the capacity to furnish additional elements needed for sperm metabolism.

Moreover, DPP seems particularly effective in improving semen quality in low fertility individuals (El Neweshy *et al.,* 2013). Considering DPP as a potential powerful antioxidant for sperm that enhances and preserves semen quality, this study was designed to explore the functional characteristics and oxidative status of rabbit semen belonging to two groups of rabbit bucks (young *vs.* old) with different basic seminal traits and stored for 48 h at 4°C after dilution with a tris base extender supplemented with (20, 40, 80 mg/mL) of DPP aqueous extract.

## MATERIAL AND METHODS

### *Sampling of plant material*

Date palm (*Phoenix dactylifera L.*; Dokkars) pollen grains were collected from Touggourt, located about 660 km south of Algiers in March 2021. The pollen was air dried for 48 h under dark conditions, lyophilised and then stored at 4°C until use.

### *Determination of main pollen compounds*

All compounds of pollen were analysed in duplicate.

#### Proximate composition

Moisture, crude protein, ether extract, ash and crude fibre were determined for DPP according to the Official Methods of Analyses (AOAC, 2016)

#### Sugar content

The lyophilised pollen (1.00±0.08 g) was dissolved in 100 mL of high performance liquid chromatography (HPLC) grade water and left to shake for 30 min. The extract obtained was filtered with a PVDF syringe filter and injected into the HPLC (HPLC-ELSD) using a Shodex-NH2P-50, 250 mm polymeric amino column (Shodex Inc., Tokyo, Japan) following the method proposed by Floridi *et al* (2001). An evaporative light scattering detector (ELSD C-650, BÜCHI, Flawil, Switzerland) was used with a drift tube temperature of 110°C and 2.2 mL/min.

#### Antioxidant content

*Tocols*- The α and γ, δ-tocopherol, α and γ-tocotrienol content was quantified using the HPLC (Hitachi Primade, Milan, Italy), according to Hewavitharana *et al.* (2004). Five millilitres of distilled water and 4 mL of ethanol were added to 2 g of the sample and vortexed for 10 s. After mixing, 4 mL of hexane containing BHT (200 mg/L) was added and the mixture was carefully shaken and centrifuged at 8000× *g* for 10 min. An aliquot of the supernatant (3 mL) was dried under a stream of nitrogen and dissolved in 200 µL of acetonitrile; 50 µL was then injected into the HPLC system (Hitachi Primade consisting of a cooling autosampler 1210, pump 1110, fluorometric detector 1440 and a Synergi Hydro-RP column, Phenomenxsrl, Bologna, Italy). Tocols were expressed as mg/g.

*Polyphenols*- One g of DPP was dissolved in water and methanol (1/1, v/v). Compounds were extracted twice and diluted 1:10 with Folin-Ciocalteu solution (Sigma-Aldrich). After this, a 20% aqueous sodium carbonate solution was added to each sample. Cuvettes were prepared with this solution and absorbances were read at 765 nm using a Shimadzu 5020 spectrophotometer (Milan, Italy). The activity of the compounds is expressed in terms of mg gallic acid (GAE)/g of the sample.

#### Fatty acid profile

Fatty acid profile was evaluated on the lipid fraction extracted from DPP following the method reported by Folch *et al* (1957). To obtain the fatty acid methyl esters, the lipid extract was dried with a rotary evaporator and 1 mL of n-hexane was added. Finally, the trans-methylation procedure was performed with 0.5 mL of 2 M KOH methanol solution at 60°C for 15 min. One µL was added to the gas chromatography system (CP 3800 VARIAN, Milan, Italy) equipped with a FID detector and a capillary column of 100 m length×0.25 mm×0.2 µm film (Supelco, Bellefonte, PA). To calculate the amount of each fatty acid, heneicosanoic acid was used as the internal standard (C21:0, Sigma-Aldrich analytical standard). Fatty acids were expressed as g/100 g. The amount of each fatty acid was used to calculate the total saturated (SFA), total monounsaturated (MUFA), and total polyunsaturated (PUFA) fatty acids from the n-3 and n-6 series.

### *Preparation of sperm extender*

Tris pollen extender (TPE)

Pollen grain extract was prepared in tris base extender [TBE: Tris-hydroxymethylaminomethane (3.79 g), citric acid (2.16 g), glucose (0.59 g), penicillin G (0.1 g), dihydrostreptomycin (0.1 g) and distilled water (100 mL) at 300 mOsm/L and pH 7.1].

Twenty (20), 40, and 80 mg of DPP were soaked in 3 test tubes each containing 1 mL of TBE (Laghouati *et al.,* 2021). After the vortex, all tubes were stored in a refrigerator (adjusted at 4°C) for 24 h and finally centrifuged (to obtain the supernatant representing the used extender).

### *Animals*

Rabbits were housed in individual WRSA cages (Menegin, Italy; 40 cm width, 100 cm length and 40 cm height) and trained for semen collection with an artificial vagina in the Perugia University experimental farm. Rabbits were reared in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, and the National Research Council's Guide for the Care and Use of Laboratory Animals.

### *Sperm collection*

A pool of semen was collected from 60 rabbit bucks (New Zealand White strain) belonging to two groups (30 young: 6-9 mo of age *vs.* 30 old: >3.5 yr) with different basic seminal traits, using a rabbit doe as a dummy. Semen was collected twice a week from each rabbit for a total of 3 wk. The individual samples collected and suitable for experimentation were 20: 10 from old rabbits and 10 from young rabbits, from which 6 pools were formed and analysed (n. samples=6). The sperm was routed to the laboratory in a thermostatted incubator maintained at 35-37°C.

### *Sperm quality assessment*

After collection, the semen was immediately subjected to the following analyses:

*Volume* (mL), which was determined by graduate tubes.

*Sperm concentration* (number of sperm x $10^6$/mL) was measured by a Thoma–Zeiss cell counting chamber with a 40X objective and optical microscope (Olympus CH-2, Milan, Italy).

*Kinetic characteristics,* which were analysed by Computer Assisted Semen Analyzer (CASA) (model ISAS®4.0, Valencia, Spain) after appropriate dilution (1/20) of pooled semen with a Tris buffer. This system consisted of a negative phase-contrast optic system (Olympus CH-2) equipped with a CCD Sony camera. The setup parameters were established previously and the acquisition rate was set at 100 Hz (Castellini *et al.,* 2011).

### *Sperm processing*

Pooled sperms (n=6/age) were divided into 4 samples with a concentration of 600±50 spermatozoa×$10^6$/mL (Alvarino, 2000) and diluted with TBE extender (1:10) without DPP (control) and other aliquots with the different concentrations of TBE extenders. All samples were then stored for 2, 4, 24 and 48 h at 4°C. At each time, an aliquot was used for the analytical evaluation of thiobarbituric acid reactive substances (TBARS, lipid peroxidation) and tocols (vitamin E).

### *Assessment of sperm motility*

Sperm motility was evaluated at 37°C using the CASA method (PROiSER R+D Systems, Valencia Spain) after 0, 2, 4, 24 and 48 h of storage. For each sample, two drops and six microscopic fields were analysed for a total of 300 spermatozoa. Recorded sperm motion parameters were total motility rate (percentage of motile sperm/total sperm) and track speed (VCL - µm/s, the sum of the incremental distances moved by the sperm in each frame along the sampled path divided by time).

### *Oxidative status of rabbit spermatozoa*

The extent of the spermatozoa membrane lipid peroxidation was assessed by measuring the malondialdehyde (MDA), along with other substances that are reactive to 2-thiobarbituric acid (TBA), as reported by Mourvaki *et al* (2010). The molar extinction coefficient of the MDA was $1.56\times10^5$ 1/M cm. The results are expressed as nmol MDA/mL.

### *Assessment of antioxidant status (Tocols)*

The tocols (α and γ tocotrienols and α, γ, δ tocopherols) on semen were assessed according to (Mourvaki *et al.*, 2010) with the HPLC system previously reported. Tocols were identified using an FD detector (model Hitachi 1440) set at excitation and emission wavelengths of 295 nm and 328 nm, respectively and were quantified using external calibration curves prepared with increasing amounts of pure tocopherols in ethanol (Sigma-Aldrich, Steinheim, Germany; Extra syntheses, Genay, France). The results are expressed as nmol/mL.

The consumption of tocols was estimated as the difference (in %) between the amount of tocols supplied by DPP and what was originally detected in the seminal samples (sperm and seminal plasma plus DPP).

### *Statistical analysis*

The statistical analysis was done with a linear model comprising the effects of DPP, age of bucks, storage time with their interactions. Spearman regression coefficients were used to measure of rank correlation between variables (DPP addition, TBARS, total tocols and tocol consumption).

Data were analysed using the SPSS software (v18). The results are expressed as mean±standard error. The significance of differences was determined using Fisher's test. Values were considered significant when ($P\leq0.05$).

## RESULTS

### *Pollen analysis*

The DPP composition is presented in Table 1. The DPP was characterised by a high proportion of protein (27.10±1.26% f.m.) and ash (18.43±0.55% f.m.), whereas the lipid fraction was very low (ether extract: 0.51±0.01% f.m.). The total content in sugar was 16.25±2.47 g/100 g f.m. Sucrose was the major sugar, followed by glucose and fructose (14.23±2.23 g/100 g f.m., 1.08±0.15 g/100 g f.m. and 0.94±0.05 g/100 g f.m., respectively). Regarding the antioxidants content, the sum of tocols showed total value of 26.48±1.12 mg/g f.m. where the main isoform was the α-Tocopherol, followed by g-Tocotrienol. The total polyphenols content was 5.01±0.02 mg GAE/g f.m.

Fatty acids profile showed the following rank SFA>PUFA>MUFA. The main fatty acid was palmitic (C16:0; 24.21±3.10 g/100 g), followed by linoleic (LA, C16:2n-6; 20.25±3.81 g/100 g) and myristic (C14.0; 16.20±3.41 g/100 g). α-linoleic (ALA, C18:3n-3), palmitoleic (C16:1n-7) and oleic (OL, C18:1n-9) acids showed appreciated concentrations (8.77±2.19, 7.25±2.74, 7.15±1.25 g/100 g, respectively). The Long Chain Fatty Acids (LC-PUFA; more than 20 carbon atoms) were very scarce (i.e. arachidonic acid, ARA: 0.58±0.08 g/100 g f.m.).

### *Sperm motility and track speed of sperm*

An analysis of motility (%) and track speed (μm/s) in relation to pollen addition for both buck ages was done during storage time (0-48 h) (Figure 1a, b).

Apart from the effect of buck age, no significant differences were found for all sperm kinetic traits (Table 2). As expected, at T0, sperm of old bucks showed lower VCL (155.37 *vs.* 200.05 μm/s) and motility rate (40.12 *vs.* 64.32%) values than young bucks ($P<0.05$). During the storage time progress, the motility rate and VCL generally declined, with different trends in the experimental groups ($P>0.05$). The DPP treatment showed similar results to those observed in the control group over 48 h.

Table 1: Proximate composition, sugar, antioxidants (tocols and polyphenols) and fatty acids profile (mean±standard error) of DPP (in fresh matter).

| | Units | Values |
|---|---|---|
| Proximate composition | | |
| Moisture | g/100 g | 7.34±0.52 |
| Crude proteins | % | 27.10±1.26 |
| Ether extract | % | 0.51±0.01 |
| Ash | % | 18.43±0.55 |
| Crude fibre | % | 12.06±0.18 |
| Sugar | | |
| Fructose | % | 0.94±0.05 |
| Glucose | % | 1.08±0.15 |
| Sucrose | % | 14.22±2.27 |
| Total sugar | % | 16.25±2.47 |
| Antioxidants | | |
| γ-Tocotrienol | % | 7.29±0.27 |
| α-Tocotrienol | % | 0.42±0.01 |
| δ-Tocopherol | % | 0.03±0.01 |
| γ-Tocopherol | % | 0.05±0.04 |
| α-Tocopherol | % | 18.68±1.44 |
| Total tocols | % | 26.48±1.12 |
| Total polyphenols | mg GAE/g | 5.01±0.02 |
| Fatty acids | | |
| 10:0 | % | 0.45±0.12 |
| 12:0 | % | 5.11±1.05 |
| 14:0 | % | 16.20±3.41 |
| 16:0 | % | 24.21±3.10 |
| 18:0 | % | 3.45±0.05 |
| 20:0 | % | 6.58±0.45 |
| SFA | % | 56.00±5.63 |
| 16:1n-7 | % | 7.25±2.74 |
| 18:1n-9 | % | 7.15±1.25 |
| MUFA | % | 14.40±3.10 |
| 18:2n-6 | % | 20.25±3.81 |
| 18:3n-3 | % | 8.77±2.19 |
| 20:4n-6 | % | 0.58±0.08 |
| PUFA | % | 29.6±3.12 |
| n-6/n-3 | | 2.38±0.52 |

DPP: Date Palm Pollen; GAE: gallic acid equivalents; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

### *Oxidative status of rabbit spermatozoa: tocols, TBARS and their interaction*

Table 2 shows the oxidative status of rabbit spermatozoa, highlighting the significant effects. Apart from tocopherol, DPP addition did not show a significant effect on tocol content in seminal samples. At the same time, the lowest value of TBARS, as an indicator of LPO, was obtained in control samples (7.34 and 10.55 nmol MDA/mL, respectively in old and young rabbits); DPP80 drastically increased the lipid oxidation in both classes of bucks compared to the control (Figure 2).

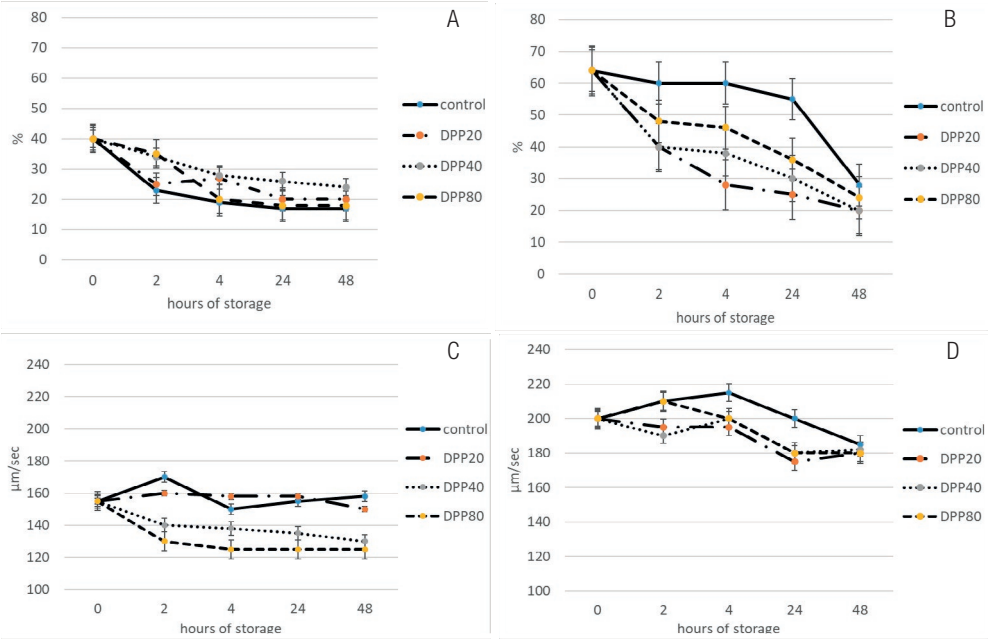


Figure 1: Motility rate (%, A and B) and track speed (µm/s; C and D) of rabbit sperm of old (A and C) and young (B and D) rabbit bucks in relation to date palm pollen (DPP) addition during 48 h of storage (mean±standard error).

Table 2 and Figure 2 show the tocol consumption (%) in relation to LPO. Tocol consumption significantly increased with the age of bucks ($P$=0.05), the DPP level ($P$=0.042) and obviously storage time ($P$=0.033).

The higher DPP doses progressively increased tocol can consumption, although the lipid oxidation remained higher in spermatozoa of both buck ages ($P$<0.001); the higher consumption was recorded in the semen of old rabbit (more than 60% compared to the 50% of DPP80). The better trend was found in DPP20, where the tocol consumption (10.46 and 15.28 % respectively, in old bucks and young bucks) and lipid oxidation were lower compared to the higher doses of DPP (12.90 *vs.* 14.37 nmol MDA/mL, respectively, in old bucks and young bucks).

From the Spearman correlation coefficients (Table 3) it should be underlined that the progressive increase of DPP in the semen was positively correlated with seminal tocols but, in the same time, with their consumption and also with TBARS value.

## DISCUSSION

To the best of our knowledge, there are no studies on the effect of DPP addition to rabbit semen extender for short time storage. Therefore, the aim of this study was to investigate whether the addition of different concentrations of DPP (20, 40, 80 mg/mL) protects rabbit spermatozoa, collected from old and young bucks, during 48 h storage at 4° C.

First, we analysed the main chemical characteristics of pollen. The chemical compounds observed confirmed and detailed that reported by other authors. However, a lower concentration of protein (–1.34 fold), polyphenols (–3.48 fold) and LC-PUFA was found compared to that found in Egyptian DPP (El-Kholy *et al.,* 2019), whereas total sugars were higher (+2.50 fold) than that. Accordingly, DPP can be considered a rich source of soluble sugars (16.25±2.47 g/100 g f.m.), with a concentration 30-fold higher than standard media or sugar-based cryoprotectant (~0.3M of sucrose or trehalose; Aljaser, 2022). Sucrose (14.23±2.23 g/100 g f.m.) was the major sugar, followed

Table 2: Sperm traits of old and young rabbit bucks.

| | Old bucks | | | | Young bucks | | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| n. samples | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | RMSE | TRT | Age | Time of storage | TRT×Age ×Time |
| | C | DPP20 | DPP40 | DPP80 | C | DPP20 | DPP40 | DPP80 | | | | | |
| Track speed (VCL µm/s) | 157.67$^{b}$ | 156.25$^{b}$ | 139.64$^{a}$ | 132.02$^{a}$ | 202.08$^{d}$ | 189.20$^{c}$ | 190.40$^{c}$ | 194.52$^{c}$ | 2.58 | 0.966 | <0.001* | 0.206 | 0.007* |
| Motility rate (%) | 23.23 | 26.43 | 30.4 | 26.25 | 53.4 | 35.43 | 38.48 | 43.67 | 6.14 | 0.160 | 0.029* | 0.002* | 0.125 |
| γ-T3 (nmol/mL) | 0.01 | 0.11 | 0.02 | 0.00 | 0.01 | 0.06 | 0.06 | 0.01 | 0.10 | 0.072 | 0.455 | 0.247 | 0.110 |
| α-T3 (nmol/mL) | 1.06 | 1.31 | 0.58 | 0.00 | 0.86 | 1.16 | 1.33 | 1.29 | 0.34 | 0.489 | 0.041* | 0.742 | 0.104 |
| δ-T (nmol/mL) | 0.84 | 1.29 | 1.28 | 0.36 | 1.46 | 1.46 | 1.50 | 1.13 | 0.29 | 0.082 | 0.020* | 0.999 | 0.185 |
| γ-T (nmol/mL) | 0.02 | 0.02 | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.03 | 0.012* | 0.067 | 0.177 | 0.915 |
| α-T (nmol/mL) | 1.14 | 1.25 | 1.17 | 2.63 | 0.84 | 0.97 | 1.29 | 1.28 | 0.35 | 0.104 | 0.006* | 0.086 | 0.177 |
| Tocols (nmol/mL) | 3.07 | 3.98 | 3.06 | 2.99 | 3.18 | 3.66 | 4.19 | 3.72 | 0.45 | 0.612 | 0.419 | 0.955 | 0.978 |
| Tocol consumption (%) | - | 10.46$^{a}$ | 66.57$^{e}$ | 63.78$^{e}$ | - | 15.28$^{b}$ | 34.61$^{c}$ | 53.13$^{d}$ | 0.82 | 0.042* | 0.050* | 0.033* | 0.028* |
| TBARS (nmol MDA/mL) | 7.34 | 12.90 | 17.01 | 22.92 | 10.55 | 14.37 | 16.42 | 21.24$^{z}$ | 0.91 | <0.001* | 0.199 | 0.290 | 0.619 |

Total n. samples=6 pools×5 storage times. DPP: date palm pollen. TBARS: thiobarbituric acid reactive substances. MDA: malondialdehyde.
$^{a,b,c,d,e}$ $P$<0.05 in TRT×Age×Time. TRT: pollen addition, time: incubation at 0-48 h.
*Significant at $P$<0.05.

by glucose (1.08±0.15 g/100 g f.m.) and finally fructose (0.94±0.05 g/100 g f.m.). Hence, the composition of DPP could be an indicator of its high nutritive value for supplying energy needed for sperm activity (Purdy, 2006).

It is also reported that DPP is a rich source of natural antioxidants and possesses a free radical scavenging capacity (El-Kashlan *et al.,* 2015; El-Sisy *et al.,* 2018). Hassan (2011) and Bishr and Desoukey (2012) attributed the antioxidant capacity of DPP to vitamins A, E and C and to its high content of phenolic, carotenoid and flavonoids. Furthermore, DPP is considered to have an antibacterial, antifungal and antiviral activity (Abdi *et al.,*2017; Mallhi *et al.,* 2014). Our results confirm that reported by Hassan (2011) on vitamin E level of DPP (~20.21 mg/g) and fatty acids profile with different concentrations.

Our data, once again, show that plant bioactive compounds have a great variability due to climate, season and genetics and that only an accurate determination of the main chemical compounds enables us to assess the effects and mechanisms of action (Ju *et al.,* 2021).

Vitamin E has the ability to penetrate the sperm membrane and protect the sperm cells against the detrimental influence of free radicals (Aitken and Clarkson, 1988). Vitamin E works synergistically with the co-antioxidant vitamin C (Castellini *et al.,* 2007); it is a major chain-breaking lipophilic antioxidant in sperm membranes that can break the covalent links formed by ROS between fatty acid side chains in membrane lipids (Kalthur *et al.,* 2011). It prevents the immobility of spermatozoa due to the peroxidation of phospholipids in the sperm cell mitochondria by scavenging all three types of radicals, namely superoxide, peroxyl and hydroxyl (Sinclair, 2000). A free radical chain reaction is broken by scavenging these radicals and forms a relatively stable complex (aldehyde). The efficacy of the vitamin E addition to media has been proven in rabbit semen after chilling and thawing (Amokrane *et al.*, 2020; Nabi *et al.,* 2017; Zhu *et al.,* 2015; Castellini *et al.,* 2002).

When evaluating the effect of DPP on qualitative traits of semen, some main findings (simple or/and interactive) could be underlined:

- DPP extract (20, 40, 80 mg/mL) has no effect on sperm motility traits, as no difference between the semen supplemented and control group up to 48 h of storage was found;

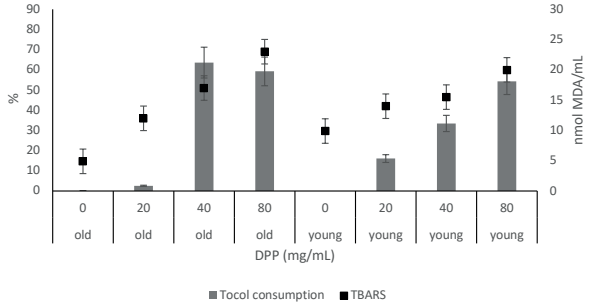


Figure 2: Lipid oxidation (TBARS, nmol MDA/mL) and tocol consumption (%) of sperm samples added with different date palm pollen (DPP) doses (mean±standard error). TBARS: thiobarbituric acid reactive substances. MDA: malondialdehyde.

- Old bucks generally presented semen samples with worse results (both kinetic traits and oxidative status); chilling and storage, even if in a non-significant way, progressively decreased sperm motility and track speed.

According to Agarwal (2005), DPP extract appeared to have a dose-dependent effect on sperm parameters (mainly VCL) of old rabbit, although no null or negative effect was recorded at doses >DPP40. Conversely, in optimal conditions (i.e. young rabbit semen), the addition of pollen had no effect on sperm traits (motility and VCL), probably because the sperm collected came from animals with excellent semen quality, thus, the sperm performance is not easily enhanceable (Castellini *et al.,* 2011).

Furthermore, although the levels of spermatozoa with good motility were lower in old rabbit semen compared to that of young animals, these spermatozoa are more oxidised, due to the poor sperm cell quality condition (higher ROS; Silva *et al.,* 2022).

On the contrary, in previous studies, DPP extract provided positive effects on rabbit semen and in sperm from various other species (Arabian stallion, bull, buffalo and human). An aforementioned study on rabbit (using the same DPP doses: 20, 40, 80 mg/mL but diluted into a different extender - saline) showed a beneficial effect of DPP on sperm motility and kinetic traits until 2 h of storage at 37°C (Laghouati *et al.,* 2021).

On bull sperm, motility was improved at 30 mg/mL in chilled sperm and at 10, 50 mg/mL in frozen-thawed sperm (El-Sheshtawy *et al.,* 2014). Similar results were reported in buffalo sperm in improving motility of chilled (10-50 mg DPP/mL) and thawed (30-50 mg DPP/mL) sperm (El-Sheshtawy *et al.,* 2016). Furthermore, 20 mg/mL DPP had a beneficial effect on the chilling and freezing of Arabian stallion sperm (El-Sisy *et al.,* 2018).

These discrepancies could be due to different concentrations of DPP in the extender or/and to their interactions with other compounds of the semen extender. It could also be hypothesised that the season and the area of collection affects the DPP composition (Altamimi *et al.,* 2020)

The main relationship between kinetic traits and oxidative status of sperm membrane could be assessed by evaluating the lipid peroxidation and the tocols content of samples. We measured the MDA in seminal plasma as marker of LPO and the content of vitamin E (all tocols isoform) as the first molecule that acts to counteract the lipid oxidation in sperm (Castellini *et al.,* 2007; Mourvaki *et al.,* 2008), and we estimated this interaction by analysing the proportion of tocol consumed during storage.

Table 3: Spearman coefficients of sperm oxidation.

| | DPP level | Total tocols | Tocol consumption |
|---|---|---|---|
| Total tocols | 0.53 | | |
| Tocol consumption | 0.84[b] | –0.69[b] | |
| TBARS | 0.98[a] | 0.58 | 0.89[b] |

DPP: date palm pollen. TBARS: thiobarbituric acid reactive substances. [a]$P<0.01$; [b]$P<0.05$.

Contrary to what expected, the addition of DPP to semen extender did not improve the antioxidant capacity or TBARS value. The progressive increase of DPP, despite providing sperm with additional amount of tocols, also caused higher tocol consumption and an increase in TBARS level. In particular, administration of the highest dose (DPP80) increased the lipid oxidation in sperm of both rabbit ages. This trend could be explained assuming that, mainly with the higher doses of DPP (40 and 80 mg/mL), the higher vitamin E consumption was probably due to the amount required to balance the oxidative thrust induced by lipid oxidation (Bartolini *et al.,* 2021). The pollen, on one hand, supplies an additional amount of tocopherols (antioxidants) to the spermatozoa, and on the other, increases the PUFA proportion (oxidative substrates) in rabbit sperm. It is widely demonstrated that many components of seminal plasma may modify the lipid bilayer of sperm membrane, and in turn affect the LPO and sperm quality (Mourvaki *et al*., 2010; Rodríguez *et al*., 2019). Sperm with higher PUFA concentrations are more susceptible to oxidation and, therefore, consume a greater amount of antioxidant molecules to counteract the LPO.

Moreover, mainly at higher doses, DPP seems pro-oxidant, probably interacting with other molecules: i.e. iron, copper and other transition metals, which are reported to be abundant in DPP (>200 mg/100 g; Hassan, 2011) and in seminal plasma (Chandel and Jain, 2014). Transition metals cause lipid peroxidation by stimulation of $H_2O_2$, promoting free radical mediated processes (Repetto *et al.,* 2010), and by another mechanism, they bind to negatively charged phospholipids that alter the physical properties of the bilayer, favouring the initiation and propagation reactions of LPO (Oteiza *et al.,* 2004).

This trend in lipid oxidation also justifies the previously discussed lack of DPP effect on motility parameters. Indeed, motility of spermatozoa is very susceptible to lipid peroxidation, as confirmed by *in vitro* studies that showed how ROS causes sperm immobility within 5-30 min (De Lamirande and Gagnon, 1992; Jones *et al.,* 1978). The protection of sperm cells against oxidative stress is mainly assured by seminal plasma (Castellini *et al.,* 2000). Indeed, sperm cells have a very limited antioxidant capability mainly during storage. Oxidative stress induces defective sperm function related to the capacity of oxygen metabolite to stimulate lipid peroxidation in spermatozoa (Álvarez *et al.,* 1987; Aitken *et al.,*1989; Jones *et al.,* 1978). The numerous double bonds of membrane fatty acids (Bollwein *et al.,* 2008) facilitate the hydrogen abstraction process that initiates the peroxidation cascade, particularly in rabbit sperm (Castellini *et al.,* 2019). Therefore, antioxidants should be *in vitro* supplemented to protect sperm against the damaging effects of ROS (Baker *et al.,* 1996; Bansal and Bilaspuri, 2009). On the other hand, the dilution of sperm causes a reduction of protective substances in seminal plasma (Cabrita *et al.,* 2011); membrane alterations induced by phase transitions that occur during cooling (Medeiros *et al.,* 2002) may also induce further damages.

In conclusion, the findings of this study demonstrated that supplementation of DPP extract to tris buffer extender did not enhance chilled rabbit semen traits and did not improve the antioxidant potential at doses higher than 40 mg/mL.

Therefore, further research is required to: i) assess the possible change in DDP due to area/season of production; ii) select the best concentration of DPP on chilled rabbit semen against oxidative damage to spermatozoa and to develop standard and reliable protocol for sperm; iii) evaluate in detail the complex relation between pro/antioxidants among substances contained in DPP (transition metals, polyunsaturated fatty acids, polyphenols, etc.).

Acknowledgements: The authors express their thanks to the laboratory team of Perugia University for their availability, their precious help and the support provided. We also wish to thank Mr Azzeddine Benghanima for his valuable assistance in pollen collection at the oasis of Touggourt.

Authors contribution: Laghouati A.: conceptualization, formal analysis of semen quality and writing – original draft. Belabbas R.: Validation and writing – review & editing. Mattioli S.: Formal chemical analysis of DPP or semen, data curation and writing – original draft. Dal Bosco A.: funding acquisition and writing – review & editing. Benberkane A.: conceptualization. Bravi E.: formal chemical analysis of DPP. Sileoni V.: formal chemical analysis of DPP. Marconi O.: formal chemical analysis of DPP and funding acquisition. Castellini C.: data curation, writing – original draft, visualization and supervision.

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