Bulgarian Journal of Veterinary Medicine, 2018, **21**, No 1, 40–49
ISSN 1311-1477; DOI: 10.15547/bjvm.1044

Original article

EFFECTS OF *PHOENIX DACTYLIFERA* POLLEN GRAINS EXTRACT SUPPLEMENTATION ON POST-THAW QUALITY OF ARABIAN STALLION SEMEN

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Summary

El-Sisy, G. A., D. A. El-Badry, R. I. El-Sheshtawy & W. S. El-Nattat, 2018. Effects of *Phoenix dactylifera* pollen grains extract supplementation on post-thaw quality of Arabian stallion semen. *Bulg. J. Vet. Med.,* **21**, No 1, 40–49.

This study explored the effect of extender supplementation with different concentrations of date palm pollen grain (DPPG) on post-thawed sperm motility, viability index, membrane and acrosome integrities in Arabian stallions. Five ejaculates from each of four Arabian stallions were subjected to cryopreservation with a modified INRA-82, without any supplementation (control) or supplemented with 50, 100, 150, 200 and 250 mg DPPG. After thawing, all samples were maintained at 37 ^{o}C, while analyses were performed at 0, 1, and 2 and 3 hours. Sperm motility percentage, viability index, membrane integrity percentage and acrosome integrity of each sample were determined by conventional laboratory methods. The addition of 100 mg DPPG resulted in improved maintenance of sperm motility after 0 and 60 min post-thawing, as compared to the control and other treatment groups. Non-significant effects on viability index were observed after enrichment of extender with 100 and 150 mg DPPG. The addition of 100 and 150 mg DPPG resulted in significant ($P<0.0010$) improvement in post-thawing membrane integrity (41.33±0.83%; 41.33±2.33%) compared to the controls (34.33±1.55%). These concentrations exerted also a beneficial effect in preserving sperm acrosome integrity (38.33±1.01%; 38.67±1.64%) as compared with the control one (33.33±1.12%). Supplementation of modified INRA-82 with 200 mg DPPG failed to maintain sperm motiliy while 250 mg PG has a negative impact on all studied post-thawing semen parameters. In conclusion, adding 100 and 150 mg date palm pollen grain extract to modified INRA-82 seemed useful in the chilling and freezing process of Arabian stallion sperm.

Key words: Arabian stallion, cryopreservation, pollen grain, semen

INTRODUCTION

There is a continuous interest in the utilisation of herbal remedies in the developing countries. At present, the use of herbal antioxidants to counteract the deleterious

action of reactive oxygen species (ROS) and oxidative stress on body cells and tissues has attracted research attention. From ancient times, date palm pollen grain (DPPG, male germ powder of palm flowers) was used to improve reproductive performances in men and woman as dietary supplement. DPPG has a potent nutritive importance as it is rich in phytochemicals such as estrone, α-amirin, triterpenoidal saponins, flavonoids estrone, estradiol, estriol and a crude gonadotrophic substance (Abedi *et al*., 2014).

Various studies have concluded that dietary supplementation could improve spermatogenesis, increase sperm counts, motility, and increase the concentration of testosterone, FSH and LH (Salman *et al*., 2014; Baharara *et al*., 2015; El-Kashlan *et al*., 2015; Rasekh *et al*., 2015). Also, DPPG is a rich source of natural antioxidants. El-Kashlan *et al*. (2015) observed that DPPG extract possessed a powerful free radical scavenging capacity. Hassan (2011) and Bishr & Desoukey (2012) attributed the powerful antioxidant capacity of pollen grain to its high content of phenolic, carotenoid, flavonoid compounds, and considerable amount of vitamins A, E and C. Moreover, pollens have antibacterial, antifungal and antiviral activity (Abedi *et al*., 2012; Mallhi *et al*., 2014).

Cryopreservation causes a wide range of chemical, physical and mechanical injures to sperm membranes of all mammalian species (Watson, 2000), which are attributed to temperature changes, alterations in the transition from the lipid phase, production of reactive oxygen species (ROS) and osmotic stress (Ortega Ferrusola *et al*., 2009; Câmara *et al*., 2011). Moreover, the overproduction of ROS causes oxidative stress that involves structural damage of sperm membranes, fall of intracellular ATP levels causing decrease in the viability and motility of cryopreserved sperm (Baumber *et al*., 2000; Agarwall & Said, 2005). To alleviate the harmful effects of ROS, seminal plasma possesses powerful source of ROS scavengers which offer protection for equine sperm, including enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and small molecular antioxidants such as ascorbic acid and α-tocopherol (Aitken & Baker, 2004; Sikka, 2004). Unfortunately, removal of seminal plasma during equine semen processing and consequent loss of these antioxidants may increase the vulnerability of sperm oxidative stress (Ball, 2008). Owing to the evidenced damaging effect of ROS to genomic material and sperm membranes, it is necessary to establish whether semen extender enriched with various antioxidants could improve the survival and fertility of cryopreserved equine sperm (Sinclair, 2000). The addition of natural extracts and infusions from plant sources to semen extenders was indicated in cattle and caprine sperm preservation for their protective, nutritive, antioxidant and antibacterial properties (Sansone, 2000). Al-Dujaily *et al*. (2012) concluded that the addition of 20% *P. dactylifera* pollen extract to the culture medium of the *in vitro* sperm activation improved sperm motility. El-Sheshtawy *et al*. (2014) found out that the addition of aqueous extract of the DPPG to TRIS-citrate-fructose extender improved survival rates and semen quality after chilling and cryopreservation in bulls. To the best of our knowledge, only few studies have been conducted to evaluate the potency DPPG extract added to semen extender in preserving and maintaining sperm quality.

Despite the substantial flow of data on the promising quality and characteristics

of DPPG, few studies have so far been conducted to investigate the effect of enrichment of semen freezing extender with DPPG extracts on sperm viability after freeze thawing. The tendency for using natural antioxidants of plant origin is continuously increasing; therefore, the aim of this study was to investigate the efficacy of addition of aqueous extract of the DPPG to semen extender (modified INRA-82) on the stallion sperm's survival rates after freezing/thawing.

MATERIALS AND METHODS

Preparation of extender

Modified INRA-82 (mINRA-82) described by El-Badry *et al*. (2014) was used as base and control extender. This extender consists of 25 g/L glucose monohydrate, 1.5 g/L lactose monohydrate, 1.5 g/L raffinose pentahydrate, 0.4 g/L potassium citrate monohydrate, 0.3 g/L sodium citrate dihydrate, 4.76 g HEPES, pH 7.0, 500 mg/L gentamycin, 0.035% SDS and 0.15% skimmed milk. All ingredients were dissolved in one liter of distilled water.

Preparation of extract

Pollen grain extract was prepared from the chafe of DPPG obtained from the palm tree pollen at El-Fayoum governorate. To 5 test tubes each containing 5 mL of mINRA-82 50, 100, 150, 200 and 250 mg of this powder (El-Shehstawy *et al.,* 2014) were added. All tubes were put in cooling incubator (adjusted at 5 °C) for three days with daily vortexing and finally centrifuged to get the supernatant extenders (IPG-50, mIPG-100, mIPG-150, mIPG-200 and mIPG-250, respectively).

Semen collection and processing

On a once weekly collection schedule, five ejaculates per stallion were obtained from 4 Arabian stallions, aged 8–14 years, and individually housed at Police Academy stud, Cairo, Egypt. At the time of collection, early in the morning, a teaser estrus mare was used for the stallion to mount. Semen was collected using a lubricated and pre-warmed (45 to 50 °C) Colorado model artificial vagina with an inline filter to separate the gel fraction.

Immediately following collection, the gel-free portion of the ejaculate was evaluated for volume and progressive motility. Sperm concentration was determined with a haemocytometer. Only ejaculates with at least 60% progressively motile sperm and 250×10^6 sperm cell/mL were used for freezing. The semen was extended 1:1 (semen:extender) in mINRA-82 warmed to 38 ^{o}C. The diluted samples were placed into 15-mL tubes and centrifuged for 10 min at 400×g. (Cochran *et al*., 1984). At least 95% of the supernatant was removed (Loomis, 2006) and each pellet was re-suspended with mINRA-82 (containing 5% glycerol and 15% egg yolk) enriched with DPPG (mIPG-50, mIPG-100, mIPG-150, mIPG-200, mIPG-250) and mINRA-82 (control without DPPG) to a final sperm concentration of 100×10^6 motile sperm/mL. Each aliquot was cooled slowly to 5 ^{o}C over one hour under aerobic conditions, and then incubated at 5 ^{o}C for 30 min (Crockett *et al*., 2001). The extended semen was drawn into 0.5 mL straws (Minitube, Germany) and sealed thermally and placed 4 cm above liquid nitrogen in the vapour phase in foam box for 10 min before being plunged into the liquid phase (Cristanelli *et al*., 1985). The straws were then stored in goblets on canes and kept immersed in liquid nitrogen. For thawing, two straws

per treatment were warmed in a water bath at 38 °C for 30 s.

Evaluation of frozen-thawed semen

Sperm motility was examined and recorded using a pre-warmed stage of phase contrast microscope (200×) just after thawing, 1, 2 and 3 h post thawing. The post-thawing viability indices were calculated according to Milovanov (1962) to be equal to half of the post-thaw motility in addition to the summation of recorded motility at 1st, 2nd and 3rd hours post-thawing.

Hypo-osmotic swelling (HOS) positive cells percentage was determined using the procedure described by Nie & Wenzel (2001). In brief, 100 µL aliquot of each semen sample was mixed in 1.0 mL of a pre-warmed 100 mOsm sucrose solution (1.712 g sucrose dissolved in 50 mL of sterile, deionized water). The mixture was incubated at 37 °C for 60 min in a 1.5 mL microcentrifuge tube. Following incubation, a small drop of sample was placed on a microscope slide and cover-slipped for examination by using phase contrast microscopy (400×) to evaluate 100 spermatozoa for evidence of swelling and curling changes.

Sperm acrosomal integrity was estimated using fast green stain (Wells & Awa, 1970).

Statistical analysis

One way analysis of variance and Duncan's multiple range tests (using SPSS program version 16.0) were done for the obtained data of frozen-thawed semen qualities after transformation of percentages to their corresponding arc-sin values (Snedecor & Cochran, 1989). $P<0.05$ was considered as statistically significant.

RESULTS

The results in Table 1 indicate a highly significant ($P<0.0001$) effect of adding 100 mg DPPG to the mINA-82 extender on post-thawing stallion sperm motility. It resulted in improved maintenance of sperm motility after 0 and 60 min post-thawing, compared to the control and other treatment groups, while the addition of 250 mg DPPG failed to maintain sperm motility 60, 120, and 180 minute after thawing.

As shown in Table 2, non-significant effects were noticed for the addition of 100 and 150 mg DPPG to mINRA-82

Table 1. Effect of date palm pollen grain (DPPG) addition to modified INRA-82 on post-thaw stallion sperm motility. Data are presented as mean ± SEM, n=15

Pollen enrichment	% motility after thawing (arc-sin values)			
	0 hour	1 hour	2 hours	3 hours
mINRA-82 (control)	$41.67^{bc} \pm 0.63$	$36.67^{b} \pm 1.67$	$31.67^{ab} \pm 1.67$	$25.00^{a} \pm 1.09$
mINRA-82+50 mg DPPG	$38.33^{cd} \pm 0.63$	$31.67^{c} \pm 0.63$	$26.67^{c} \pm 0.63$	$21.67^{b} \pm 0.63$
mINRA-82+100 mg DPPG	$48.33^{a} \pm 1.67$	$41.67^{a} \pm 2.27$	$33.33^{a} \pm 1.67$	$26.67^{a} \pm 1.26$
mINRA-82+150 mg DPPG	$43.33^{b} \pm 2.27$	$36.67^{b} \pm 1.67$	$31.67^{ab} \pm 0.63$	$25.00^{a} \pm 1.09$
mINRA-82+200 mg DPPG	$38.33^{cd} \pm 1.67$	$33.33^{bc} \pm 1.67$	$28.33^{bc} \pm 1.67$	$21.67^{b} \pm 1.26$
mINRA-82+250 mg DPPG	$35.00^{d} \pm 1.09$	$25.00^{d} \pm 1.09$	$18.33^{d} \pm 0.63$	$11.67^{c} \pm 0.63$

Different superscript letters within columns (a, b, c) are significantly different ($P<0.05$) according to the Duncan multiple range test.

Table 2. Effect of date palm pollen grain (DPPG) addition to modified INRA-82 on post-thaw stallion sperm parameters. Data are presented as mean ± SEM, n=15

Pollen enrichment	Sperm parameters (arc-sin values)		
	Viability index	HOS (%)	Acrosome integrity (%)
mINRA-82 (control)	$114.00^{a} \pm 1.09^{*}$	$34.33^{b} \pm 1.55$	$33.33^{b} \pm 1.12$
mINRA-82+50 mg DPPG	$99.17^{b} \pm 0.63$	$32.33^{bc} \pm 0.45$	$31.00^{bc} \pm 0.22$
mINRA-82+100 mg DPPG	$125.83^{a} \pm 1.26$	$41.33^{a} \pm 0.83$	$38.33^{a} \pm 1.01$
mINRA-82+150 mg DPPG	$115.00^{a} \pm 1.09$	$41.33^{a} \pm 2.33$	$38.67^{a} \pm 1.64$
mINRA-82+200 mg DPPG	$102.50^{b} \pm 1.26$	$34.00^{b} \pm 1.57$	$32.00^{bc} \pm 1.43$
mINRA-82+250 mg DPPG	$72.50^{c} \pm 0.63$	$29.67^{c} \pm 0.98$	$29.00^{c} \pm 1.15$

Different superscript letters within columns (a, b, c) are significantly different (P<0.05) according to the Duncan multiple range test.

extender in terms of post-thawing sperm viability index while highly significant effects (P<0.0001) were recorded for membrane and acrosome integrities when compared to the control (mINRA-82). Post-thawing viability index was insignificantly higher in mIPG-100 and mIPG-150 treatments (125.83±1.26; 115.00±1.09) as compared with the control (114.00±1.09). The addition of 100 and 150 mg DPPG resulted in significant (P<0.0001) improvement in post-thawing membrane integrity (41.33±0.83%; 41.33±2.33%) compared to the controls (34.33±1.55%). Additionally, enrichment with 100 and 150 mg DPPG was beneficial for the sperm acrosome integrity (38.33±1.01%; 38.67±1.64%) as compared with the control one (33.33±1.12%). The supplementation of mINRA-82 with 200 mg DPPG had no effect on all post-thawing semen parameters studied and it was nearly similar to control, meanwhile 250 mg DPPG had negative impact on all studied post-thawing semen parameters.

DISCUSSION

In recent years, extensive research has been conducted to investigate the effect of natural (of herbal, natural and plant origins) antioxidants such as ginseng (Park *et al.,* 2016), curcumin (Shah *et al.*, 2016), date palm pollen grains (El-Sheshtawy *et al*., 2014), honey bee (El-Sheshtawy *et al*., 2016a) and pomegranate juice (El-Sheshtawy *et al*., 2016b) on the viability of animal sperm during cooling and cryopresevation. DPPG is a fine powder material produced by male flowering date palm plants, necessary for plants pollination. DPPG has been considered as an herbal remedy ever since ancient times without any scientific rationale.

Because of the continuous research interest in bioactive plant-derived medicinal ingredients, we attempted to deduce whether addition of *Phoenix dactylifera* pollen, as natural antioxidants, can improve sperm's survivability when added to frozen-thawed Arabian stallion extended semen.

The results of our study indicated that the addition of 100 mg DPPG to the stallion freezing extender proved to be beneficial for the sperm cells in terms of motility. Our results were in agreement with Al-Dujaily *et al.* (2012) who proposed that supplementing human sperm culture media with 20% *P. dactylifera* pollen ex-

tract resulted in improvement in the sperm motility. In a previous study, El-Sheshtawy *et al*. (2014) noticed significant improvement in bull sperm motility when 250 mg aqueous extract of the DPPG included to the Tris-citrate-fructose extender for chilling and cryopreservation. The current results disagreed with our previous study in bulls which may be related to species-specific differences. The capability of sperm from different species to withstand the freezing process is variable, bovine sperm spermatozoa are more tolerant to freezing-thawing than the equine spermatozoa (Rodgers *et al*., 2014).

Motility is the most essential indicator of the *in vivo* sperm fertilising ability (Vijayaraghavan, 2003). The beneficial effect of pollen grain, on motility and viability has been proved in rabbits, rats, mice, and humans (Marbeen *et al*., 2005; Al-Sanafi *et al*., 2006; Bahmanpour, 2006; Faleh & Sawad, 2006; Rasekh *et al*., 2015).

Our results showed that enriching stallion freezing extender with 100 and 150 mg pollen grain has advantageous effect on viability index, membrane and acrosome integrity compared to the control group. During cryopreservation, mammalian sperm is subjected to tremendous chemical and physical damage as a result of phase transition changes, osmotic stress and overproduction of reactive oxygen species (ROS) which results in increase lipid peroxidation of the membrane (Ortega Ferrusola *et al*., 2009; Câmara *et al*., 2011). In various animal species, including horses, lipid peroxidation has been documented as an important marker of oxidative stress and cryonic injury in spermatozoa (Aitken *et al*., 2007; Ortega Ferrusola *et al*., 2009). The main ROS scavengers in equine semen are glutathione peroxidase, superoxide dismutase, and catalase. Removal of seminal plasma during semen processing before freezing discard antioxidants present in semen and subjecting spermatozoa to extreme ROS injury (Aitken *et al*., 2007). Oxidative stress has deleterious effects on sperm membrane fluidity, integrity, and flexibility, characteristics linked with fertilising capacity (Said *et al*., 2005). Al-Farasi *et al*. (2005) and Mansouri *et al*. (2005) reported the potent antioxidant activity of the aqueous extract of dates which was attributed its inclusion to the wide range of phenolic compounds including *p*-coumaric, ferulic and sinapic acids, flavonoids and procyanidins. Palm pollen has also been recognised as a rich source of phytochemicals and nutrients mainly carotenoids and flavonoids (Daoud *et al*., 2015). Fresh pollen has a highly nutritive value as it is rich in mineral, multi-vitamins, sugars, lipids, growth factors and more than 100 enzymes and co-factors (Hassan, 2011). Moreover, it is a superior source of protein, amino acid, fatty acid, enzymes, hormones (estrone, estradiol and estriol) and mineral salts (Alferz & Campos, 2000).

Palm pollen contains an efficient material for improving semen quality and male fertility because it is rich in vitamins A, E and C and it is a good source of minerals such as zinc, selenium, iron, cooper and cobalt (Hassan, 2011) that stimulates sperm motility and the progressive forward movement. All of these above mentioned components could improve sperm motility. Vitamin C is a naturally occurring antioxidant in seminal plasma (Anane & Creppy, 2001) and is an essential cofactor of enzymes that are free radicals scavengers (Michael *et al*., 2008). It could possibly improve sperm function by reducing cell damage (Gangwar *et al*., 2015). On the other hand, α-tocopherol,

one of the major sperm antioxidants, was established to be abundant in spermatozoa membrane (Suleiman *et al*., 1996; Surai *et al*., 2000) and protect sperm motility from oxidative harm (Brzezińska-Ślebodzińska *et al*., 1995). Besides, date palm pollen suspension was reported to have potent antimicrobial, antifungal and anti-toxicant activities (Aba Al-Khail *et al*., 2003; Shakibaa *et al*., 2011; Daoud *et al*., 2015) and these effects could improve post-thawing semen quality through reduction of bacterial growth in the semen extender.

In conclusion, the inclusion of 100 and 150 mg date palm pollen grain extract in modified INRA-82 had a beneficial effect on the chilling and freezing process of Arabian stallion sperm.

ACKNOWLEDGEMENTS

The authors are greatly indebted to the National Research Center and the Police Academy stud for their helpful and valuable aids and all the facilities they presented to achieve the present article.

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Paper received 27.05.2016; accepted for publication 30.09.2016

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