

## Effect of extender supplemented with date palm pollen grain on bovine semen qualities

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## Abstract

Natural extract from plant-based has grown in popularity as protective properties in extender for preserving animal semen. Date palm pollen grain (DPPG) is commercially used for male fertility by enhancing sperm count, motility and DNA quality because DPPG contains flavonoids. Thus, the present study aimed to evaluate the effect of a) extender supplementation with different concentration of DPPG on sperm motility, viability and membrane integrity b) different preservation storage of chilled and frozen bovine semen after seven days. The semen was collected through electrical stimulation and assigned to four treatment groups. The semen were diluted in Tris citric fructose egg yolk (TCFY) diluent (control group; CG) or supplemented with DPPG with different concentration (G1 = 2%, G2 = 4% and G3 = 6% in 20 mg DPPG/40 mL of Tris citric fructose (TCF). Semen samples were chilled (experiment 1) in the refrigerator (4°C) for seven days and cryopreserved in liquid nitrogen (experiment 2) prior to dilution to four treatments. The samples were thawed in a water bath (37 °C) and analysed for motility, membrane integrity and viability by conventional laboratory methods. No significant difference was observed among treatment groups in experiment 1. However, in the second experiment, the addition of 6% DPPG resulted significantly higher ( $p < 0.05$ ) in sperm viability compared to control groups ( $71.25 \pm 1.04$ ) vs ( $56.47 \pm 4.69$ ). The supplementation of 6% DPPG showed the ability to protect the viability of bovine sperm, respectively.

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## 1. INTRODUCTION

Application of natural extract from plant-based sources to improve reproductive performance in livestock is vital. Optimised extender elucidate problems in semen storage such as protect sperm against cold shock and bacterial contamination. Natural extract from plant-based such as coconut oil (Tarig *et al.*, 2017), tomato (Al-Daraji, 2014) and pomegranate (El-sheshtawy *et al.*, 2016) proved to improve semen preservation. Plus, the natural extract contains antioxidant that helps to minimise the reactive oxygen species (ROS) and oxidative stress (OS) in the cell. Date palm pollen grain (DPPG) is a fine powder produced from male flowering which contains herbal antioxidant to against the action of ROS and OS of sperm membrane in the cryopreservation process. In fact, DPPG is the second-highest plant extraction that contains antioxidant activity among 28 fruit commonly consumed in China (Ghnimi *et al.*, 2017). In Malaysia, dates fruits have grown in popularity due to the health benefit. It showed an increase in export due to the increasing demand by local Muslim consumers (Haris *et al.*, 2019). Malaysia has been successful in planting dates fruits to fulfill the consumers' demand (Haris *et al.*, 2019). However, recent evidence

showed that DPPG was used as a dietary supplement to improve the reproductive performance of male and women (El-sisy *et al.*, 2016).

Studies on semen preservation are well versed, but there was scarcity on the best commercial supplementation in the extender based on natural resources. Previous studies have been proved that the addition of DPPG in the semen extender can improve the quality of the sperm in buffaloes through the expression of sperm motility (El-sheshtawy *et al.*, 2014; El-sisy *et al.*, 2016). Few reports mentioned that the cryopreservation of semen could cause functional damage and reduce the quality of spermatozoa. Cryoprotectant helps to protect and provide sperm in an appropriate medium. In addition of DPPG to the freezing extender has a beneficial effect on sperm quality instead of has antibacterial properties, nutritive and antioxidant. Therefore, this study was conducted to investigate the potency of reducing the addition of DPPG (20 mg) at different concentration in extender on sperm motility, viability and membrane integrity and to explore the effect of semen qualities in different preservation of chilled and frozen after seven days of storage.

## 2. MATERIALS AND METHODS

### 2.1. Experimental design

This study was to compare the different concentrations of DPPG in extenders on the semen quality of bovine. The experimental design was used randomised completely block design (RCBD), block with different temperature on the sperm quality and operators for four treatment groups as Tris citric fructose yolk (TCFY; control), (TCFY + 2% DPPG; G1), (TCFY + 4% DPPG; G2), (TCFY + 6% DPPG; G3). The study was replicated three times for each of the experiment. Experiment 1 and 2 were investigating the effect of semen extender of four treatment groups on sperm quality (motility, viability and membrane integrity) in chilled and frozen semen after seven days of storage, respectively.

### 2.2. Preparation extender

Tris buffer: Tris buffer (control treatment) used in this study was composed of 3.028 g Tris, 1.250 g fructose, 1.675 g citric acid, 0.72% glycerol (v/v), 0.1 g penomycin and 20% (v/v) egg yolk (TCFY) was prepared as control extender in the experiment. Date palm pollen grain (DPPG) was purchased and imported from the Saudi Arabia. The stock solution was prepared according to El-sheshtawy *et al.*, (2014). The extenders of TCFY were supplemented with different concentration of DPPG (G1 = 2%, G2 = 4% and G3 = 6%) in 20 mg/40 ml. A total of semen extender solution was prepared in 12 ml of each treatment groups.

### 2.3. Semen collection and initial evaluation

The study conducted at the Princess of Naradhiwas University, Thailand. (6.4557° N, 101.7898° E). Semen collection was carried out from Brahman bull cattle (*Bos indicus*) aged three years old using ElectroJac IV ejaculator (Neogen, Lexington, KY, USA). The bull ejaculated one time per day early in the morning and three replications were performed. After collection, semen was placed in a water bath at 37°C and evaluated for volume (ml), colour, mass movement, sperm mortality and sperm concentration.

### 2.4. Semen processing

Semen samples were diluted and kept at 25 °C for 30 minutes. Then, all the diluted semen of all the groups was slowly cooled to 5 °C, which equilibrated for 5 hours. After equilibration, all the treatment groups were packed into paillette 0.25 ml orange pastel (Imv technologies, L'Aigle, France) and sealed with the polyvinyl powder at the end of the straws. The straws were horizontally placed on a rack and pre-frozen in liquid nitrogen vapour at 4 cm above the liquid nitrogen (8 cm) level in a Styrofoam box (25 x 35 x 30 cm) for 15 minutes and then the straws were plunged directly into the liquid nitrogen for 15 minutes. The sample was divided into two experiments which in the first experiment, all the treatment groups were store in the refrigerator and the second experiment, all the straws were

stored in liquid nitrogen. Thawing was performed at water bath and frozen straws were thawed at 38°C for 30 seconds after storage (Vidal *et al.*, 2013).

### 2.5. Assessment of semen quality parameters

#### 2.5.1. Assessment of sperm concentration

A total of 10 µl of the sperm was placed on a hemocytometer and covered with a cover slide and evaluated under a phase-contrast microscope at a magnification of 40x. The semen was diluted with sodium chloride (NaCl) with added 1 ml of semen to 99 ml of diluent (1:100). Only ejaculate above 1.0 ml volume with a concentration of  $1.19 \times 10^8$  sperm/ml and having more than 70% progressively motile sperm is used in this experiment.

#### 2.5.2. Assessment of motility

The percentage of mortality of spermatozoa in each sample was subjectively evaluated under a phase contrast microscope at 400x magnification by placing a 10 µl of pre warmed semen on slide and covered with cover glass. The mean from the semen sample was recorded as final mortality score.

#### 2.5.3. Sperm viability

The sperm viability was prepared according to Malik *et al.*, (2016). A total of 200 µl of diluted spermatozoa and 200 µl eosin-nigrosin stains were mixed and smeared on a warm slide by using hot plate. Two hundred spermatozoa from different microscopic fields were examined using a microscope (400x). Unstained sperm cells indicate viable sperm was counted and stained sperm indicate dead sperm with a damaged cell membrane.

#### 2.5.4. Sperm membrane integrity

Membrane integrity was observed by using hypo-osmotic swelling test (HOST). The ingredient of HOST solution is 1.8 g fructose and 0.98 g sodium citrate were dissolved in 200 ml distilled water. A total of 100 µl of semen were mixed with 200 µl of HOST solution and incubated for 1 hour at 37°C. A total of 10 µl of the sperm mixture was placed on a slide and covered with a cover glass and two hundred spermatozoa were evaluated under a phase-contrast microscope at a magnification of 400x. Spermatozoa with intact plasma membrane will swell the tail while the damage sperm membrane will not show swelled tail (Zubair *et al.*, 2013).

### 2.6. Calculation of percentage of spermatozoa

Percentage of motile, HOST reactive and viable was calculated by the following formula (Chae *et al.*, 2017):

$$\frac{\text{Number of motile or Host reactive or viable}}{\text{Total counted spermatozoa}} \times 1 \quad (1)$$

**2.7. Statistical analysis**

All the data in motility, membrane integrity and viability were analysed followed by ANOVA using the SAS software system to determine the differences between all the parameters (version 9.1, SAS Institute Inc, 1996, Cary, NC, USA). Tukey’s post hoc test was used to compare the differences between P values ( $p < 0.05$ ) consider as statically significantly. All data are express as mean  $\pm$  standard error of mean (SEM).

**3. RESULT AND DISCUSSION**

In the present study, DPPG was utilised in bovine semen extender as a beneficial antioxidant effect on sperm viability. Date palm pollen grains (DPPG) in these recent years categorised as a herbal remedy containing high natural antioxidant which provides as a solution to problems in semen cryopreservation (Bishr *et al.*, 2012). Based on the previous study, the addition of DPPG carried out in this study was reduce (20 mg) from the earlier studies. DPPG has been used with cryoprotectant extender at different concentration (2%, 4% and 6%) in different preservation (chilled and frozen) to investigate the potency of sperm qualities. As expected, the results of this study showed that the sperm qualities decreased after the freezing for all groups compared to the chilling sperm. These could result from ice crystal formation disruption during cryopreservation that may alter membrane protein and carbohydrates composition. Subsequently, leading to the damage of membrane structural and consequently resulting in viability sperm decrease (Hezavehei *et al.*, 2018).

In experiment 1, the effect of different concentrations of DPPG on semen qualities is shown in Table 1. There was the highest percentage of motility, membrane integrity and viability parameter on chilled semen in the extender with 6% of DPPG compared to the control group in all group. However, experiment 1 showed no significant differences were noticed for all sperm quality parameters (motility, viability and membrane integrity) during the chilling process ( $p > 0.05$ ) after seven days of storage.

**Table 1:** Percentage (mean  $\pm$  SEM) of the quality sperm parameters on chilled semen in a Tris buffer supplemented with different concentration of DPPG

Sperm parameters	DPPG Enrichment % (w/v)			
	Control	DPPG 2%	DPPG 4%	DPPG 6%
Total motility	70.90 $\pm$ 8.64	74.03 $\pm$ 2.31	80.70 $\pm$ 7.67	87.37 $\pm$ 0.42
Membrane integrity	86.30 $\pm$ 2.48	85.98 $\pm$ 0.56	88.75 $\pm$ 3.59	90.82 $\pm$ 2.39
Viability	84.31 $\pm$ 5.73	85.89 $\pm$ 0.13	89.03 $\pm$ 0.15	90.22 $\pm$ 1.22

(n)=3

In experiment 2 showed the enrichment 6% of DPPG is significantly higher compared to the control group (71.25  $\pm$  1.04b) vs (56.47  $\pm$  4.69a) (Table 2). The novel

finding in this study indicated that the viability of frozen semen in 6% concentration of DPPG was significantly higher ( $p < 0.05$ ) compared to control group. This proved that the concentration at 6% of DPPG helped to improve and protect the maintenance of sperm viability from damage after seven days of storage in frozen. DPPG as extender provides positive effects to various sperm species on viability and motility including rabbits (Faleh & Sawad, 2006), rats (Bahmanpour *et al.*, 2006) and humans (Rakesh *et al.*, 2015). DPPG proved that it could protect the viability of sperm. This may be due to the composition of DPPG, which contain vitamin C permits sperm protection viability by reducing cold shock effect. Vitamin C is essential cofactor enzymes that are free radicals scavengers (Michael *et al.*, 2008) which possibly improved sperm function by reducing cell damage (Gangwar *et al.*, 2015). Higher DPPG concentration as extender gives positive results and improves sperm survivability when compared to the control group which current study in agreement with the previous study (El-sheshtawy *et al.*, 2014; El-sisy *et al.*, 2016). In contrast, sperm viability was not affected by DPPG addition as reported by El-Sisy *et al.* (2016). This may be due to various factor including different breeds, locations and seasonal changes during semen collection, which sperm quality could be affected (Leboeuf *et al.*, 2000). Though, the result of this study showed that the addition 6% of 20 mg of DPPG are capable of improving sperm’s survivability in chilled and frozen semen compared to the control group after seven days of chilling and frozen.

**Table 2:** Percentage (mean  $\pm$  SEM) of the quality sperm parameters on frozen semen in a Tris buffer supplemented with different concentration of DPPG

Sperm parameters	DPPG Enrichment % (w/v)			
	Control	DPPG 2%	DPPG 4%	DPPG 6%
Total motility	45.32 $\pm$ 9.84	47.58 $\pm$ 5.58	52.08 $\pm$ 8.83	56.46 $\pm$ 9.55
Membrane integrity	85.05 $\pm$ 2.56	85.54 $\pm$ 4.28	85.12 $\pm$ 3.45	88.14 $\pm$ 3.03
Viability	56.47 $\pm$ 4.69 <sup>a</sup>	59.26 $\pm$ 6.15 <sup>ab</sup>	60.80 $\pm$ 4.38 <sup>ab</sup>	71.25 $\pm$ 1.04 <sup>b</sup>

<sup>a,b</sup> Mean  $\pm$  SEM within each rows, mean with different alphabetic superscript were significantly different (ANOVA-post hoc test at  $p < 0.05$ ). (n=3)

Next, the concentration of DPPG in the motility of chilled and frozen semen is non-significantly different ( $p > 0.05$ ) with other treatment groups. The sperm motility with the supplementation of DPPG in extender after seven days of storage has shown no effect. This may occur due to the effect of the concentration of DPPG, which is not significant enough to protect the sperm motility. As reported by El-Sisy *et al.* (2016), the addition of 100 mg and 150 mg of DPPG give the significant improvement in sperm motility in chilling and freezing of Arabian stallion sperm. Next, a previous study conducted by El-sheshtawy

*et al.* (2016) also mentioned that the addition of 100 mg DPPG in chilled and 250 mg DPPG in frozen semen showed positive impacts on buffalo sperm motility. Other than that, El-sheshtawy *et al.* (2014) also proved that the addition 250 mg of DPPG showed a significant improvement in bull sperm motility in chilled and frozen semen. In contrast, this study showed that the addition of 20 mg of DPPG does not affect sperm motility. However, the addition of DPPG provides a positive impact on sperm viability only.

DPPG can protect the sperm from cryoinjury. Malik *et al.* (2016) reported that natural sugar and bioactive compounds (Ghnimi *et al.*, 2017) in DPPG gives additional protection to the sperm during cooling, freezing and thawing (Holtz *et al.*, 2008). Purdy (2006) mentioned that sugar supply energies in the extender to maintain semen quality. During cryopreservation, sperms are subjected to tremendous chemical and physical damage caused by the phase transition of temperature. ROS can increase dramatically during the stress environment (Devasagayam *et al.*, 2014) especially after froze thawed (Kim *et al.*, 2010; Baumber *et al.*, 2013) which may lead to the severe deterioration of sperm (Hezavehei *et al.*, 2018). Excessive ROS production is unable to be effectively controlled by the antioxidant in the seminal plasma (Fingerova *et al.*, 2009). Thus, the limitation of ROS production of sperm during cryopreservation are possible with the addition of antioxidant contain in DPPG which can balance the ROS level and further improved the post-thawed (Agarwal *et al.*, 2014). Mansouri *et al.* (2005) reported that DPPG contains rich of phenolic compound and phytochemicals as a natural antioxidant such as p-coumaric, ferulic and sinapic acids, flavonoids, procyanidins and carotenoids which can help to protect the sperm against the ROS.

#### 4. CONCLUSION

Based on the results, it can be concluded that the addition of DPPG can improve sperm performance in the cryopreservation process compared without adding DPPG in the extender. The addition of 20 mg in 6% concentration of DPPG in the extender gives a positive impact on sperm viability ( $p < 0.05$ ). In contrast with this study, the addition of 20 mg was not significant enough to protect the sperm motility. Next, the quality of sperm decreased after freezing in all groups. It can be assumed that, despite using the same protocol of freezing, different DPPG concentration as extender used in this study and variability performance at experimental animals might also generate different results compared to previous literature. Other than that, further study on the use of DPPG extender, the experiment should use more sensitive techniques to access the fertilisation potential in semen samples such as artificial insemination or in vitro fertilisation to prove the efficiency of the cryoprotectant.

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