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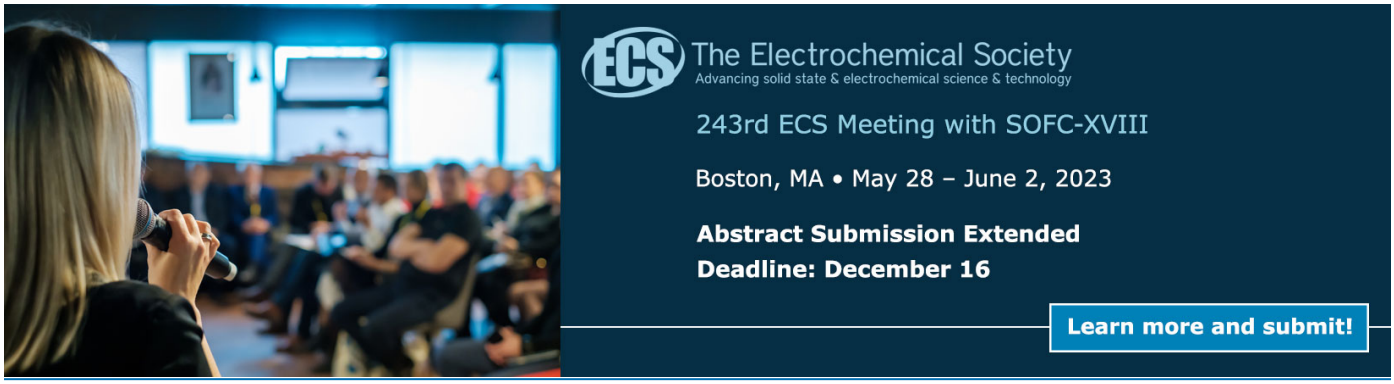

Effect of Extender Supplemented with Date Palm Pollen Grain on Caprine Semen Qualities

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Abstract. Date Palm Pollen Grain (DPPG) were proved to maintain semen characteristics and quality in bulls, equines, and buffaloes. However, DPPG as supplementation in the extender for goats are scarce. Hence, the aims of this research were to investigate the semen motility and viability and to examine the functional membrane integrity of semen in different concentrations of DPPG supplemented in goat semen extenders. In this study, DPPG was added with 4% and 8% in tris-citrate-fructose-yolk (TCFY) semen extender and chilled at 5^{o}C for 0, 24 and 48 hours. The diluted semen samples were assessed for sperm motility, viability, and membrane integrity. A total of four ejaculations from two Boer bucks were used in this study. The results indicated that the ability to maintain sperm viability was significantly higher in the extender supplemented with 8% DPPG in all storage times ($P<0.05$). Furthermore, 8% of DPPG in extender significantly improved sperm motility after 48 hours (56.67±5.57) compared to control (0% DPPG) (40.83±3.00) and 4% DPPG (43.33±5.24). Results also showed that sperm membrane integrity percentage in 8% DPPG (40.00±4.04) was significantly higher than control (30.33±2.19) at 0-hour storage time. The extender supplemented with 8% DPPG indicated the best result in maintaining sperm quality after 24 hours of chilled storage compared with other treatments. In conclusion, 8% of DPPG supplemented in semen extenders proved to maintain viability, motility and membrane integrity compared to control.

1. Introduction

Enhancement and elevating self-sufficiency level (SSL) of meat is vital in the agro-food industry to reduce dependency on importation of the commodity. The most widely use assisted reproductive technology which is the artificial insemination (AI) has proven to improve the production and genetic of the livestock. However, the successful rate of AI greatly depends on several factors including quality of chilled-thawed semen. Composition of preservation media or semen extenders also contributes to the success rate of pregnancy.

The most common semen extender is the tris-based egg yolk extender with other integral components such as buffer, cryoprotectant and antibiotics to provide protection for the spermatozoa collected. With the help of semen extender, issues about the protection of the sperm against bacterial contamination and temperature shock is solved. Even though research on semen preservation is quite common, research on the best commercial extender supplementation derived from natural resource is still lacking [5]. At present, natural extracts from fruits such as orange juice and apple juice [1] as well as date palm pollen grain [9] had proven to improve semen quality in preservation due to their antioxidant properties. Date palm pollen (DPP) is found to improve fertility of both men and women in

ancient times [16]. Benefits of natural resources included should be easily obtained, available all year round and cost effective to ease local livestock farmers.

The objective of the study were to investigate the effect of different concentrations of Date Palm Pollen Grain (DPPG) supplemented to goat semen extender on semen motility, viability and functional membrane integrity and to examine the effect of different concentrations of Date Palm Pollen Grain (DPPG) supplemented to goat semen extender on the length of chilled short-term storage time.

2. Materials and Methods

2.1 Animals and Treatments.

A total of two male Boer goats of age around 1 and a half years old, reared under intensive management. The study was conducted at Agro-Techno Park (ATP) located in University Malaysia Kelantan Jeli Campus. A total of three female Boer goats were selected for Controlled Internal Drug Release (CIDR) for semen collection. Prior to semen collection, CIDR was removed and used as dummy to arouse the sexual desire of the male goats for ejaculation.

2.2 Preparation of Tris-Citric Acid-Egg Yolk Fructose (TCYF) Extender.

The collected semen was diluted with Tris-Citric Acid-Egg Yolk Fructose (TCYF) extender and glycerol. Then, different amount of Tris Pollen Grain (TPG) stock solution was added with diluted semen to obtain extender supplemented with 4% (treatment 1) and 8% of DPPG (treatment 2) respectively, whereas the control group is diluted semen without DPPG supplementation (0% DPPG). After that, semen assessments were carried out at 0 hour, 24 hours and 48 hours after chilling at 5°C with 3 replications for control group and each treatment groups in each assessment respectively. A total of 3.028g of tris base, 1.675g of citric acid, 1.250g of fructose and 0.1g of penicillin were dissolved with 100mL of distilled water in a beaker. Then it was stirred for on a hot plate for 10 minutes. The membrane of the egg yolk was broken to get 20mL pure egg yolk. The egg yolk was mixed into the solution in the same beaker and stirred on hot plate for another 10 minutes. The TCFY solution was first filtered using filter paper and then again second filtered using filter syringe. After filtering, he TCFY extender was stored in a chiller at 4°C until further use.

2.3 Semen Collection.

The artificial vagina needs a temperature of approximately 45°C before being closed with a rubber stopper. The open side of the AV was connected with a 15mL graduated semen collecting tube. The lubricant was placed at the AV before collection took place.

Dummy on heat was restrained and introduced to male. When buck is ready to mount, a trained technician guided the penis for collection. Semen was analysed and covered with a layer of aluminium foil, followed by placing it in a thermos flask with water at 37°C [8].

2.4 Semen Dilution.

Semen samples were pooled together to avoid individual differences [10]. After that, 4mL of TCFY extender solution, 1mL of goat semen and 5mL of glycerol were taken using micropipette to make a total of 10mL of TCFY extender diluted semen. Then, the 10mL diluted semen was transferred to 3 different calibrated falcon tubes with 3mL of diluted semen each tube. Each of the falcon tubes were filled with different amount of TPG stock solution according to different concentration needed as showed in Table 1.

Table 1. Amount of TPG needed for different concentration of DPPG in semen extender

Concentration (%)	Amount of TPG stock solution needed in 3mL semen extender
0	0µL
4	120µL
8	240µL

2.5 Semen Processing.
All the diluted semen with TCFY extender were kept in water bath at 25°C for 30 minutes before being transferred into refrigerator at 4°C until 24 hours and 48 hours for semen analysis. The diluted semen samples were carefully transferred into water bath at approximately 37°C for 30 seconds to be thawed to carry out sperm analysis after chilling in the refrigerator at 4°C for 24 hours and 48 hours [10].

2.6 Semen Sample Assessment.
The semen samples collected were first be analyzed fresh, secondly, after diluted with extender on the same day of collection, then second analysis after 1st day (24 hours) and third analysis at 2nd day (48 hours) of chilling at 4°C and thawed. Macroscopic evaluation such as volume and color were carried out using naked eyes while microscopic evaluation such as viability, functional integrity of sperm plasma membrane and individual motility were carried out. Each semen samples supplemented with different concentration of TPG had three replications of microscopic semen evaluation.

2.7 Volume.
The volume of the freshly collected sperm in the calibrated semen collecting tube connecting the artificial vagina was observed using naked eyes and marked down before putting it in temperature 37°C water bath.

2.8 Viability.
A drop of diluted semen was used, followed by 2 drops of 1% eosin stain and 3 drops of 10% nigrosine stain. They were properly mixed using a needle and allowed them to stand for 10 to 20 seconds in order for the stain to be absorbed by dead spermatozoa. Then, another clean slide was taken, using one edge of the slide, some stain was taken, and a smear was made on the third clean slide. The smear was allowed to air dry. The smear was observed under the compound microscope with 40x objective. A total of 200 random sperm cells were observed and the number of dead and live sperm cells were recorded. Dead sperm cells were pink in color whereas live sperm cells were colorless.

2.9 Functional Membrane Integrity.
A total of 0.1mL of semen samples was taken using a micropipette and mixed with 1mL of hypo-osmotic solution evenly in a test tube. Then, the test tube was put in an incubator at 37°C for 45 minutes. Next, a drop of semen mixture was placed on a clean slide and covered with a cover slip. A total of 200 spermatozoa were randomly observed under 40x microscope, the number of sperm cells with curled tail as HOST reactive and non-curled tail were recorded and the percentage was calculated.

2.10 Individual Motility.
A drop of liquefied or diluted semen samples with dilution ratio of 1:20 (semen: prewarmed physiological saline), was placed on a clean pre-warmed glass slide and gently covered with a cover slip. The slide was immediately observed under 40x power microscope. A total of 200 random sperm cells were observed one by one using 2 ways counting method without overlaps. Thus, microscopic view was changed frequently to prevent repeated assessment of the same sperm cells. The percentage of motile and non-motile sperm cells were computed.

2.11 Statistical Analysis.
Data was presented as mean ± standard deviation (mean ± S.D.). The statistical analysis was done by using the IBM SPSS Statistics version 21.0 for the different parameters between control and additives replications [9]. One-way ANOVA was used to determine significant differences between parameters. Tukey's post hoc test at 5% significant level was used for data analysis to test the significant differences among averages. P-values less than 0.05 was considered significant and null hypothesis was rejected.

3. Results and Discussion
Plant-based natural extract has gained popularity in supplementing into semen extender, resulting from the protective properties of the plants to improve reproductive performances in livestock. In recent years, date palm pollen grain (DPPG) was categorized as an herbal remedy which contains high level of antioxidant [7]. In fact, DPPG places the second highest of plant extraction that possesses antioxidant

activity among 28 fruits commonly consumed in China [12]. Antioxidant is a compound that provides ability in minimizing the effect reactive oxygen species (ROS) from causing oxidative stress. Therefore, it is capable of protecting semen during preservation process from oxidative damage while maintaining the semen qualities. The statement was aligned with the studies shown by El-Sheshtawy *et al.* [9] and El-Sisy *et al.* [11] where supplementation of DPPG into semen extender can improve sperm motility in buffalo semen.

This study showed that the effect DPPG supplementation with different concentration (TCFY, TCFY + 4% DPPG and TCFY + 8% DPPG) on chilled sperm characteristics such as motility, viability and membrane integrity at 0 hour, 24 hours and 48 hours. The extender with 8% DPPG has the highest mean value compared to control and another treatment group in sperm motility and viability. Additionally, supplementation of 8% DPPG can help to preserve chilled semen quality for up to 48 hours. However, enrichment of 4% DPPG has no significant effect on sperm integrity and motility in all storage times.

3.1 Individual Motility, Viability and Membrane Integrity Assessment of DPPG Supplementation to TCFY extender on Chilled Goat Semen.

a) Individual Motility.

Table 2. Effect of diluents supplementation with different concentrations of DPPG on individual motility (%) of goat semen for different storage times (Mean ± SEM).

Storage time (hours)	Treatments		
	0% DPPG (control)	4% DPPG	8% DPPG
0	65.67±1.86	62.67±0.93	72.83±2.24
24	53.17±1.92	59.67±1.20	60.83±11.21
48	40.83±3.00[a]	43.33±5.24[a]	56.67±5.57[b]

[a,b] (n) = 9, Mean ± SEM (P<0.05).

The results of individual motility in percentage affected by different concentrations of DPPG (0%, 4%, 8%) supplemented in extender with different storage time (0h, 24h, 48h) were presented in Table 2.

At 48 hours of chilled storage time, motility rate was recorded significantly highest in diluted semen supplemented with 8% DPPG (56.67±5.57) compared to diluted semen supplemented with 0% DPPG and 4% DPPG (40.83±3.00 and 43.33±5.24, respectively).

Furthermore, El-Sheshtawy [9] stated that the motility was significantly highest in addition of 150mg and 250mg of DPPG into extender of buffalo semen after cooling for 2 hours and freeze thawing comparing to control group. To explain that the motility rate could be maintained due to the interaction between species and antioxidant effect of DPPG via alleviation of reactive oxygen species (ROS) level. The antioxidant compounds in DPPG such as flavonoid and vitamins A, B, C provide the fertility maintaining capacity of chilled and after-thawed sperms in terms of motility rate.

Other than that, our finding was in line with Adikwu and Deo [2], Ball *et al.* [6] and Reza *et al.* [15] that stated addition of vitamin C and vitamin E which are antioxidants in preserved semen can improve sperm motility. Adekunle *et al.* [1] also reported 10% of apple juice gave the highest motility rate in cooled goat semen at 216 hours of storage time. Moreover, motility rate in extender supplemented with 7.5% of apple juice was significantly higher than the control group (no supplementation added).

According to Ali et al. [4] there was positive effect (P<0.05) on semen of Ring-necked pheasant by using 3mM of ascorbic acid. The results of 3mM ascorbic acid in terms of sperm motility was better compared to control. However, the increased of ascorbic acid showed a negative effect on sperm motility of ring-necked pheasant. The researcher also stated that the research showed a different result from previous research. This may due to the different species and environment [4].

b) Viability.

Table 3. Effect of diluents supplementation with different concentrations of DPPG on viability (%) of goat semen for different storage times. (Mean ± SEM).

Storage time (hours)	Treatments		
	0% DPPG (control)	4% DPPG	8% DPPG
0	74.17±2.46[a]	64.83±1.59[ab]	84.67±1.74[ac]
24	41.33±8.29[a]	59.33±2.19[b]	59.33±7.31[b]
48	30.00±2.65[a]	56.00±1.53[b]	58.00±2.31[b]

[a,b,c](n) = 9, Mean ± SEM (P<0.05).

The results of viability rate in percentage affected by different concentrations of DPPG (0%, 4%, 8%) supplemented in extender with different storage time (0h, 24h, 48h) were presented in Table 3. Table 3 showed that viability rate was significantly higher in diluted semen supplemented with 8% DPPG (84.67±1.74) compared to 4% DPPG (64.83±1.59) at 0 hour of storage time. At 24 hours of storage time, the viability rate of diluted semen supplemented with 8% DPPG (59.33±7.31) is significantly higher than diluted semen supplemented with 4% DPPG (59.33±2.19) and control which is diluted semen supplemented with 0% DPPG (41.33±8.29). While at 48 hours of storage time, it gave similar results of viability rate with 24 hours of storage time where diluted semen supplemented with 8% DPPG (58.00±2.31) is significantly higher than diluted semen supplemented with 4% DPPG (56.00±1.53) and control which is diluted semen supplemented with 0% DPPG (30.00±2.65).

The results were similar with Al-Daraji [3] that stated addition of 8% tomato juice into chicken semen extender can significantly increase the percentage of live spermatozoa significantly at 0-hour storage time compared to 2%, 4% and 6% of tomato juice supplementation. In addition, 8% tomato juice significantly increased the viability rate at all liquid storage times up to 36 hours when compared to diluted semen with no addition of tomato juice as control group and other treatments (2%, 4%, 6%) as well. Improvement of the sperm viability may be due to the positive effect of tomato juice as an antioxidant rich source.

Besides, Malik *et al.* [13] also reported that inclusion of 0.4% and 0.3% of date palm juice can significantly increase the viability percentage of bull spermatozoa before freezing when compared to other concentrations. This may be contributed by date palm juice which is an outstanding material in producing refined sugar that provides protection for the spermatozoa against injury induced during freezing-thawing process.

On the other hand, the results of this study were aligned with El-Sisy *et al.* [11], stating that the addition of 100mg and 150mg of DPPG to modified INRA-82 (mINRA-82) extender can significantly increase post-thawed stallion sperm viability index when compared to higher amount of DPPG inclusion, but did not significantly differ from control group without DPPG inclusion. Furthermore, this is supported by the study from El-Sheshtawy [9] who stated that inclusion of 150mg DPPG on post-thawed buffalo semen extender showed significant differences on the highest alive sperm percentage compared to control (no DPPG), 50mg, 100mg, 200mg, 250mg treatment groups.

The beneficial effects of DPPG to post-thawed semen viability may be attributed to components in DPPG that may protect the outer layer of spermatozoa against cold so that they may survive under low temperature. Natural antioxidant compounds in DPPG exert a protective effect in preserving the metabolic activity and cellular viability of cryopreserved bovine spermatozoa. Besides, the antibacterial ability of DPPG improves the preservability of semen through minimization of bacterial growth in the extender [9].

c) Membrane Integrity.

Table 4. Effect of diluents supplementation with different concentrations of DPPG on functional membrane integrity (%) of goat semen for different storage times. (Mean ± SEM).

Storage time (hours)	Treatments		
	0% DPPG (control)	4% DPPG	8% DPPG
0	30.33±2.19[a]	35.33±3.28[ab]	40.00±4.04[b]
24	23.33±0.88	24.67±2.40	25.33±2.96

48	19.67±3.18	16.33±4.26	19.00±2.00

[a,b] (n) = 9, Mean ± SEM (P<0.05).

The results of functional membrane integrity in percentage affected by different concentrations of DPPG (0%, 4%, 8%) supplemented in extender with different storage time (0h, 24h, 48h) were presented in Table 4.

At 0 hour of storage, it was recorded that the functional membrane integrity rate of diluted semen supplemented with 8% DPPG (40.00±4.04) is significantly higher than control which is diluted semen supplemented with 0% DPPG (35.33±3.28).

The results were aligned with Adekunle *et al.* [1] that concluded inclusion of 10% apple juice gave the highest membrane integrity rate of chilled bucks spermatozoa at 240 hours of storage compared to control, 2.5%, 5% and 7.5%.

Moreover, El-Sheshtawy *et al.* [9] concluded that inclusion of 200mg of DPPG in extender showed the highest sperm membrane integrity percentage on post-thawed buffalo semen when compared to the control group. This may on account of the antioxidant capacity of DPPG in scavenging free radicals that could harm the sperm cell membrane. Therefore, the antioxidant effect could increase fertility rate of post-thawed semen by maintaining the functional membrane integrity of spermatozoa [9].

However, this is in contrast with Malik *et al.* [13] that reported inclusion of date palm juice did not significantly affect the membrane integrity rate of bull spermatozoa before freezing. This might be due to the supplementation of date palm in juice form in extender and the study was carried out in frozen thawed bull spermatozoa.

d) Comparison of The Chilled Goat Semen Quality at Different Storage Times.

Table 5. Effect of different storage times with different concentrations of DPPG on individual motility (%) of goat semen (Mean ± SEM).

Treatments	Storage time (hours)		
	0	4% DPPG	0
Diluted semen with 0% DPPG (control)	65.67±1.86[a]	53.17±1.92[b]	40.83±3.00[c]
Diluted semen with 4% DPPG	62.67±0.93[a]	59.67±1.20[a]	43.33±5.24[b]
Diluted semen with 8% DPPG	72.83±2.24[a]	60.83±11.21[ab]	56.67±5.57[b]

[a,b,c] (n) = 9, Mean ± SEM (P<0.05).

Table 6. Effect of different storage times with different concentrations of DPPG on viability (%) of goat semen (Mean ± SEM).

Treatments	Storage time (hours)		
	0	24	48
Diluted semen with 0% DPPG (control)	74.17±2.46[a]	41.33±8.29[b]	30.00±2.65[c]
Diluted semen with 4% DPPG	64.83±1.59	59.33±2.19	56.00±1.53
Diluted semen with 8% DPPG	84.67±1.74[a]	59.33±7.31[b]	58.00±2.31[b]

[a,b,c](n) = 9, Mean ± SEM (P<0.05).

Table 7. Effect of different storage times with different concentrations of DPPG on functional membrane integrity (%) of goat semen (Mean ± SEM).

Treatments	Storage time (hours)		
	0	24	48
Diluted semen with 0% DPPG (control)	30.33±2.19[a]	23.33±0.88[ab]	19.67±3.18[b]
Diluted semen with 4% DPPG	35.33±3.28[a]	24.67±2.40[b]	16.33±4.26[c]
Diluted semen with 8% DPPG	40.00±4.04[a]	25.33±2.96[b]	19.00±2.00[b]

[a,b,c] (n) = 9, Mean ± SEM (P<0.05).

Table 5 showed that semen diluted with 4% DPPG was managed to maintain sperm motility until 24 hours of chilling without a significant drop of motility rate whereas semen diluted with 8% DPPG managed to maintain the motility of sperm after 24 hours of storage but below 48 hours. Besides, results indicated that the percentage of motility for semen after 48 hours in chilled state was significantly lower than 0 hour.

For the viability aspect, semen can be stored until 48 hours when diluted with 4% DPPG as there were no significant decrease in viability rate across all storage time as shown in Table 4.5. Semen diluted with 8% DPPG was able to maintain viability rate after 24 hours of chilling. For membrane integrity aspect, semen diluted with 0% and 8% were able to maintain membrane integrity rate after 24 hours of chilled storage.

The results obtained from this study was aligned with Al-Daraji [3] who reported that inclusion of 8mL per 100mL of tomato juice in extenders can preserve chicken sperm viability and motility for up to 36 hours. However, motility rate cannot be maintained when less than 8mL of tomato juice was added. This could be due to lycopene, a type of organic pigment found in tomato that increased semen quality stored in vitro. It could also prolong the storage time while maintaining the semen characteristics.

On the contrary, El-Sheshtawy *et al.* [10] mentioned that the used 10% pomegranate juice enriched extender gave the highest motility percentage in chilled cattle semen all over 10 days compared to 40% and 50% pomegranate juice enriched extender. This proved that inclusion of 10% pomegranate juice is beneficial for maintaining sperm motility while higher levels of supplementation such as 40% and 50% did not make an impact on sperm motility. The positive results could be due to potent antioxidant activity of pomegranate juice.

4. Conclusion

In conclusion, DPPG was able to increase goat sperm performance during short term chilling process when compared to control (without supplementation of DPPG). Extenders supplemented with 8% of DPPG showed the highest values to maintain sperm viability and motility. Plus, it managed to maintain the semen qualities for approximately 24 hours of chilled storage. This proved that 8% of DPPG had a beneficial effect on chilled Boer goat semen. There was significance difference in 8% DPPG at all storage times in viability. In further enhance the work, it could investigate the storage time can be prolonged with an increase of supplementation of DPPG (10% DPPG or more).

Acknowledgement

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