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The Effect of *Phoenix dactylifera* Pollen Grains Tris-Infusion on Semen Preservability of Local Bull Breeds

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Abstract: The benefits of dates palm and their pollen grains in the human nourishment system have their consideration and importance in medical and nutritional point of view. The present study aimed to investigate a new perspective for the use of date palm pollen grains (DPPG) aqueous extract or infusion in preservation of chilled and freezed bovine semen. Pooled bovine semen were diluted, at the Artificial Insemination Center, Abbasiah, Egypt, by Tri-Citrate-Fructose egg yolk (TCFY) diluent (control) and diverse concentrations of clarified soaked pollen grains in Tri-Citrate-Fructose (TCF) diluent in concentration of 50, 100, 150, 200 and 250 mg DPPG / 5 ml TCF. Five concentrations were kept without egg yolk (TPG) while other 5 concentrations contained egg yolk (TPGY). Each of the two dilutions was enumerated according to the concentration of DPPG. Extended semen is then chilled (for 7 days) and cryopreserved. Motility, intact sperm membrane (HOST), alive and abnormal sperm % were evaluated as characteristic sperm parameters. After 7 days of chilling, significant ($P<0.0001$) high percent of motile sperms was recorded for TPGY 150 (42.50 ± 4.79%). While, after freeze-thawing, TPG 50 (43.75 ± 2.39) and TPGY 250 (42.50 ± 5.20) showed the significant ($P<0.0001$) high percent of motile sperms. TPGY 250 revealed moderate results concerning HOST and alive sperm %. Conception rate (CR) field test had confirmed the results of lab where, TPG 50 gave 80% CR and TPGY 250 gave 75% CR. In conclusion, the aqueous infusion or extract of the DPPG, added to the tris-citrate-fructose extender with or without the addition of egg yolk, proved its good preserving and maintaining capacity of chilled (TPGY 150) and thawed (TPGY 250) bull sperms which was expressed mainly by the sperm motility.

Key words: Palm Dates Pollen Grains · Tris-Citrate-Fructose Extender · Semen · Bull

INTRODUCTION

The use of natural extract and infusions from fruits, vegetables and their seeds in extenders for preserving animal semen was introduced for their protective properties in preserving cattle and caprine sperms [1]. This innovative technique has resolved some problems in semen cryopreservation especially the bacterial contamination of extended semen, the presence of phytohormones in these natural products that protect the spermatozoa against the phospholipase Aenzyme in the ejaculate [2]. Coconut water [3, 4] and tomatojuice [5, 6] had given good results in semen preservation.

Palm dates pollen grains extract is also one of the potent fruits that has been investigated for its reproductive impact in rabbit bucks [7]. Gu *et al*. [8], Al-Farasi *et al*. [9] and Mansouri *et al*. [10] had examined the potent antioxidant activity of the aqueous extract of dates. This activity was attributed to the wide range of phenolic compounds in dates including *p*-coumaric, ferulic and sinapic acids, flavonoids and procyanidins.

Admission of date palm pollen grains (DPPG) suspension into the nourishment system of male increased sperm counts and their motility, consequently it improves fertility through normalization of serum testosterone [11]. Date palm pollen is used as a traditional medicine for male fertility by improving sperm count, motility, morphology and DNA quality with increase in weight of testis and epididymis. These effects are due to the increase in plasma testosterone levels as DPPG is rich in flavonoids [12].

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Hence, the present study aimed to investigate the potency of different concentrations of aqueous infusion or extract of the DPPG, added to the tris-citrate-fructose extender with addition of egg yolk (TCFY) or without the addition of egg yolk (TCF), in preserving and maintaining good chilled and after thawing fertile sperms.

MATERIALS AND METHODS

Preparation of Different Semen Extenders:

TRIS Base Extender: Tris-citric acid-fructose with egg yolk (TCFY) or without egg yolk (TCF) diluents, were prepared according to Foote *et al*. [13]. TCFY was used as control extender.

TRIS-Pollen Grain Extender (TPG): Pollen grain extract was prepared after well grinding of DPPG in a morter to acquire a fine powder grade. 50, 100, 150, 200 and 250 mg of this powder was soaked in 2x5 test tubes each containing 5 ml of TCF diluent (5 tubes were kept without egg yolk addition (TPG 50, TPG 100, TPG 150, TPG 200 and TPG 250) while the other 5 tubes received 20% egg yolk (TPGY 50, TPGY 100, TPGY 150, TPGY 200 and TPGY 250) after soaking DPPG and centrifugation). All tubes were put in cooling incubator (adjusted at 5°C) for five days with daily vortex and finally centrifugated to get the supernatant TPGor TPGY extenders.

Semen Collection and Initial Evaluation: Three mature cattle bulls maintained at Artificial Insemination Center, General Organization for Veterinary Services Ministry of Agriculture, Abbasia, Egypt, were used as semen donors. Ejaculates were collected using a bovine adapted artificial vagina at weekly intervals for 3 weeks. Semen samples were initially evaluated for subjective sperm motility and sperm concentration. Ejaculates fulfilling minimum standard of sperm motility (70%) and sperm morphology pooled in order to have sufficient semen for a replicate and to eliminate the bull effect. The semen was given a holding time for 10 minute at 37°C in the water bath before dilution.

Semen Processing: Semen samples were diluted with TCFY extender (control) and other aliquots of pooled semen samples were diluted with TPG and TPGY extenders (5 concentrations for each extender) containing the different concentrations of DPPG clarified aqueous infusion in order to provide a concentration of 60 million sperm/ml. Extended semen was slowly cooled to 5°C and equilibrated for 2 hrs. Then, they were packed into 0.25 ml polyvinyl French straws. After equilibrium periods, the straws were horizontally placed on a rack and pre-frozen in a liquid nitrogen vapour 4 cm above liquid nitrogen surface for 10 minutes and were finally dipped in liquid nitrogen. A fraction of extended semen from control and each concentration of the additives was kept at 5 °C for 7 days (chilling) and sperm motility was evaluated daily.

Assessment of Semen Quality Parameters: The assessment was undertaken on cooled and after thawing of bull spermatozoa. Also, sperm motility was evaluated for raw semen, 2 hours after cooling and chilled semen daily up to 7 days. Frozen straws were thawed at 37°C/ 1 minute. The parameters studied were subjective semen characteristics (motility, alive, abnormality and hypoosmotic swelling test (HOST) %) [14].

Conception Rate Field Test (CR): The results of a conception rate in a field test practice on 102 cows were recorded in villages at Fayoum governorate. Cows were inseminated with the different concentrations of TCFY (control semen), TPG and TPGY diluted semen after thawing. Pregnancy was confirmed 2 months later after insemination via rectal palpation. Results were obtained as a percentage via the application of the following equation:

$$CR = \frac{\text{No. Of conceived cows}}{\text{Total no. Of inseminated cows}} \times 100$$

Statistical Analysis: Statistical analysis were analyzed using the SAS [15] computerized program v. 9.2 to calculate the analysis of variance (ANOVA) for the different parameters between control and additives replications. Significant difference between means was calculated using Duncan multiple range test at $P<0.05$.

RESULTS

Effect of DPPG Addition with Different Concentrations to TCF on Sperm Motility of Cooled and Chilled Semen: Sperm motility didn't show any significant changes after 2 hours of cooling. Although, after 7 days of chilling, the TPGY 150 (42.50 ± 4.79%) revealed a significantly ($P<0.0001$) higher percent of motile sperms compared to the control and the other TPG and TPGY concentrations (Table 1).

Table 1: Sperm motility% of chilled TRIS-Pollen extender in cattle bulls

Parameter ---------- Treatment	2Hours after cooling at 5°C	After 7 days of chilling at 5°C
Control	87.50 ± 1.44^{A}	31.25 ± 3.15^{B}
TPG 50	67.50 ± 12.67^{A}	7.50 ± 1.44^{EF}
TPG 100	68.75 ± 10.08^{A}	8.75 ± 3.75^{DEF}
TPG 150	65.00 ± 18.37^{A}	17.50 ± 2.50^{CDE}
TPG 200	71.25 ± 7.74^{A}	5.00 ± 2.04^{F}
TPG 250	62.50 ± 7.77^{A}	5.00 ± 2.04^{F}
TPGY 50	85.00 ± 2.04^{A}	20.00 ± 4.08^{CD}
TPGY 100	88.75 ± 1.25^{A}	23.75 ± 7.47^{BC}
TPGY 150	83.75 ± 2.39^{A}	42.50 ± 4.79^{A}
TPGY 200	86.25 ± 2.39^{A}	17.50 ± 2.50^{CDE}
TPGY 250	86.25 ± 3.15^{A}	11.25 ± 3.15^{DEF}
F-value	1.57	10.03
P<	0.16	0.0001

Duncan P<0.05
mg DPPG / 5 ml TPG
mg DPPG / 5 ml TPGY

Table 2: Effect of Pollen grain addition to Tris diluent on post thawing semen characteristics of cattle bulls

Parameter ---------- Treatment	Motility % after thawing	HOST %	Alive %	Abnormality %
Control	41.25 ± 1.25^{A}	60.33 ± 0.88^{D}	52.64 ± 1.22^{E}	20.61 ± 0.61^{CD}
TPG 50	43.75 ± 2.39^{A}	61.72 ± 0.92^{D}	51.72 ± 1.98^{E}	22.49 ± 1.25^{BC}
TPG 100	21.25 ± 1.25^{B}	72.33 ± 1.45^{AB}	91.44 ± 0.72^{AB}	18.15 ± 0.60^{DEF}
TPG 150	27.50 ± 4.79^{B}	70.00 ± 1.15^{B}	49.94 ± 1.20^{E}	17.13 ± 0.59^{EF}
TPG 200	17.50 ± 4.79^{B}	66.00 ± 1.00^{C}	42.62 ± 2.62^{F}	19.17 ± 0.60^{DE}
TPG 250	18.75 ± 5.15^{B}	71.74 ± 0.94^{B}	87.49 ± 1.32^{B}	28.19 ± 1.90^{A}
TPGY 50	20.00 ± 2.04^{B}	66.77 ± 0.91^{C}	92.22 ± 1.17^{A}	18.54 ± 0.32^{DE}
TPGY 100	26.25 ± 5.54^{B}	75.00 ± 0.58^{A}	88.49 ± 0.29^{AB}	19.84 ± 0.44^{CDE}
TPGY 150	22.50 ± 1.44^{B}	58.87 ± 1.16^{D}	59.85 ± 1.02^{D}	22.69 ± 0.89^{BC}
TPGY 200	21.25 ± 1.25^{B}	70.48 ± 0.48^{D}	58.67 ± 1.86^{D}	15.00 ± 0.58^{F}
TPGY 250	42.50 ± 5.20^{A}	73.00 ± 0.58^{AB}	81.74 ± 0.90^{C}	25.30 ± 1.99^{AB}
F-value	7.61	33.34	180.85	13.16
P<	0.0001	0.0001	0.0001	0.0001

Duncan P<0.05
mg DPPG / 5 ml TPG
mg DPPG / 5 ml TPGY

Table 3: Effect of addition of pollen grain (to the TCF and TCFY on a field conception rate test

Treatment	Conception rate
Control (TCFY)	70.00
TPG 50	80.00
TPG 100	66.67
TPG 150	50.00
TPG 200	60.00
TPG 250	33.33
TPGY 50	71.43
TPGY 100	46.15
TPGY 150	66.67
TPGY 200	50.00
TPGY 250	75.00

Effect of DPPG Addition with Different Concentrations to TCF on Post Thawing Semen Characteristics: TPG 50 showed that its post-thawing motility (43.75 ± 2.39) and TPGY 250 (42.50 ± 5.20), was apparently higher than the control TCFY (41.25 ± 1.25) while all of the three showed higher significant (P<0.0001) increase in motility than the other concentrations of TPG and TPGY. Concerning the intact sperm membrane test (HOST%), TPG 100 (72.33 ± 1.45) and TPGY 100 (75.00 ± 0.58) and TPGY 250 (73.00 ± 0.58) showed higher significant (P<0.0001) difference than the control and other TPG and TPGY extended semen. Regarding the alive sperm %, TPG 100 (91.44 ± 0.72), TPGY 50 (92.22 ± 1.17) and TPGY 100 (88.49 ± 0.29)

revealed higher significant (P<0.0001) difference than the control TCFY (52.64 ± 1.22) and other TPG and TPGY extended semen. About, abnormal sperm %, the lowest significant (P<0.0001) percentage pertained to TPGY 200 (15.00 ± 0.58) while worst percentage pertained to TPG 250 (28.19 ± 1.90) (Table 2).

The conception rate field test showed apparent good results (Table 3). This was obvious concerning the TPG 50 (80.00%), TPGY 250 (75.00%) and TCFY control group (70.00%) which gave the most superior results.

DISCUSSION

Cryopreservation of bovine semen often induce an additional source for reactive oxygen (ROS) attack on sperm due to decreased activities of antioxidant enzymes and the sperm membrane become more susceptible to lipid peroxidation [16] which affect the membrane permeability [17]. Natural antioxidants exert a protective effect preserving the metabolic activity and cellular viability of cryopreserved bovine spermatozoa [18]. The results of the present study revealed good sperm motility after 7 days of chilling especially TPGY 150; so, it can be used in artificial insemination (AI) up to 7 days of chilling and better post thawing characteristics with TPGY 250. These results could be attributed to the antioxidant effect of DPPG through reduction of ROS level exerted by its high content of flavonoids [11, 12, 19]. Flavonoids are excellent scavengers of free radicals and the number of hydroxyl group on the phenyl ring seems to enhance the antioxidant capacity of polyphenolic molecule [20, 21]. The antioxidant effect of DPPG is due to its high concentration of vitamin C, B_1 (Thiamine), B_2 (Riboflavin), nicotinic acid (Niacin) and vitamin A [22, 23]. DPPG improved semen preservability through reduction of bacterial growth in the semen extender that used egg yolk as anti-cold shock [24].Concerning the conception rate, it was influenced by many parameters of the semen characteristics, among which sperm motility is the main item. The results of the present study revealed superior conception rate (80.00%) in cows inseminated by TPG 50 and (75.00%) in cows inseminated with TPGY 250, these results are in accordance with their high post freezing sperm motility (43.75 and 42.50% respectively). In conclusion, the aqueous infusion or extract of the DPPG, added to the tris-citrate-fructose extender with without the addition of egg yolk, proved its good preserving and maintaining capacity of chilled (TPGY 150) and after thawing (TPGY 250) fertile bovine bull sperms which was expressed mainly by the sperm motility.

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