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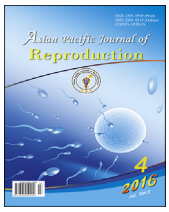

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Effects of pomegranate juice in Tris-based extender on cattle semen quality after chilling and cryopreservation

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ABSTRACT

Objective: To study the effect of adding different concentrations of the pomegranate juice (PJ) to the cattle bull semen extender on post-thawing semen quality.
Methods: Semen was collected from five cattle-bulls at weekly intervals for 5 weeks at the Semen Freezing Center, General Organization for Vet. Services, Ministry of Agriculture. Semen samples were diluted in Tris-citric acid-egg yolk-fructose extender and divided into six aliquots, the 1st served as control while PJ was supplemented at 10%, 20%, 30%, 40% and 50% in the aliquot 2, 3, 4, 5 and 6 respectively. Diluted semen samples were subjected to cooling and cryopreservation and stored in liquid nitrogen (LN2). Sperm motility in chilled semen (over 10 d) and post-thawing sperm parameters, including individual motility, alive sperm, membrane integrity, and total sperm abnormality were assessed.
Results: Obtained results clearly demonstrated that the addition of 10% PJ in the chilled extended cattle semen proved to be beneficial for maintaining sperm motility percentage. On the other hand, the addition of 40% and 50% PJ failed to preserve motility all over the 10 d. Also, supplementation of extender with 10–20% PJ significantly increases the post-thaw motility and viability as compared with control group.
Conclusions: Supplementation of bull semen extender with 10% and 20% PJ provides good chilling and improved frozen-thawed semen quality.

1. Introduction

Vegetables and fruits are of the natural sources that maintain life through their contents of multiple active remedies compounds. Pomegranate is one of the beneficial fruits in medicinal treatment [1,2]. Some authors have investigated mainly the antioxidant activities of its polyphenols [3,4].

Populations in the Middle East used pomegranate (*Punica granatum*) as a herbal medicine [5]. Tezcan *et al*. showed that fructose and glucose were the major sugars and that citric and malic are the major acids [4]. The nutritive value of the pomegranate fruit has been demonstrated by Virgili and Marino [6] who explained that daily consumption of 250 mL of PJ covers about 50% of the daily requirements of vitamins A, C and E. Moreover, the fruit contained antioxidant polyphenols that present half of the fruit's antioxidant ability for counteracting the free radicals [7]. The strong antioxidant capacity of PJ was useful in fighting certain cancers [8–10]. Besides many experimental studies described PJ in improving semen quality [11,12] and erectile dysfunction in male patients [13]. El Ghazzawy *et al*. mentioned that PJ had the ability to counteract structural changes in the rat epididymis caused by plasticizer Bisphenol, which interfere with its function and contribute to infertility, *via* increasing the number of caudal epididymal sperm, decreasing sperm abnormalities and improving male fertility [14]. Administration of pomegranate extract could improve sperm characteristics and antioxidant activity in adult male Wistar rats [12] and men [15]. Al-Daraji revealed that supplementation of semen diluent with PJ significantly improved storage ability of roosters' semen and increased the protective effects against lipid peroxidation during liquid storage of roosters' semen for up to 36 h [16].

Hence, the present study was designed to investigate the effect of pomegranate juice when incorporated as a semen extender for maintaining the quality of chilled and frozen-thawed cattle bull semen.

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2. Materials and methods

2.1. Semen collection and initial evaluation

Five mature cattle-bulls reared at the Semen Freezing Center, General Organization for Vet. Services, Ministry of Agriculture, Abbasia, Egypt, were included in this study. Semen was collected from these five cattle-bulls using an artificial vagina at weekly intervals for 5 weeks. The semen samples were transferred to the adjacent lab within few seconds and initially evaluated for volume (in a graduated tube), sperm motility and live sperm percent. The neat semen samples with more than 70% motility and 80% morphologically normal spermatozoa were admitted to freezing procedure. The ejaculates were 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 min at 37 °C in a water bath before dilution.

2.2. Pomegranate processing

Pomegranates with intact peel were sold from the Egyptian market. They were washed, peeled and the red grains were collected in a clean dish. The grains were squeezed with gauze to obtain a clear watery juice. The juice was filtered and stored at −18 °C till used [1].

2.3. Semen processing

A basic control extender (Tris-citric acid-egg yolk-fructose [TCYF]) was prepared according to Foote [17]. TCYF/PJ (pomegranate juice enriched extender, PJEE) [v:v] (0.5/4.5 (10%), 1.0/4.0 (20%), 1.5/3.5 (30%), 2.0/3.0 (40%) and 2.5/2.5 (50%)) were prepared and centrifuged to discard any precipitate. Semen samples were diluted in TCYF (control, 0% PJEE) and the former concentrations of PJEE to ensure 60 million motile spermatozoa/mL, cooled slowly up to 5 °C and equilibrated for 4 h. Semen was packed into 0.25 mL polyvinyl French straws (IMV, France). After equilibration periods, the straws were placed horizontally on a rack and frozen in a vapor 4 cm above liquid nitrogen (LN_2) for 10 min and were then dipped in liquid LN_2.

2.4. Semen quality assessment

The assessment was undertaken on after freeze-thawing of bull spermatozoa. Also, sperm motility was evaluated for raw semen, 2 h after cooling and chilled semen daily up to 10 d. Frozen straws were thawed at 37 °C/30 s. The parameters studied were subjective semen characteristics (motility %, alive %, abnormality % and membrane integrity (hypo-osmotic swelling test HOST) %).

2.4.1. Subjective motility

Subjective motility was assessed using a phase-contrast microscope (100× magnification), with a warm stage maintained at 37 °C. A wet mount was made using a drop of semen placed directly on a pre-warmed slide and covered by a pre-warmed cover slip under the same temperature conditions. Sperm motility estimations were performed in three different microscopic fields for each semen sample. Visual motility was assessed microscopically with closed circuit television system [18].

2.4.2. Live and abnormal spermatozoa (%)

This was evaluated using eosin-Nigrosin stained smear as described by Sidhu and Guraya [19]. Two hundred spermatozoa were assessed.

2.4.3. Sperm membrane integrity

Sperm membrane integrity was assessed using the hypo-osmotic swelling test (HOST) [20]. Two hundred spermatozoa were assessed and the percentage of spermatozoa with curled tails (swollen/intact plasma membrane) was calculated.

2.5. Statistical analysis

Statistical analysis data were analyzed using the SPSS (2005) computerized program v. 14.0 to calculate the analysis of variance (ANOVA) [21] for the different parameters between control and additives replications. A significant difference between means was calculated using Duncan's multiple range test at $P < 0.05$.

3. Results

Data analysis revealed a gradual decline concerning the motility percentage of spermatozoa chilled at 5 °C for the control (0% PJEE) and the first treatment (10% PJEE) from the 1st day to the 10th day. On the contrary, the four other treatments (20–50% PJEE) abruptly declined at the 7th and 10th days. The use of 10% PJEE for extended chilled cattle semen showed the highest significant (at least $P < 0.058\,1$) motility percentage all over the 10 d. The worst results were obtained on using the 40% and 50% PJEE (Table 1).

Table 1
Sperm motility percentage of chilled semen in PJEE in cattle-bulls.

Treatment	Days					*F*-value for Treat × Days	*P* <
	1	2	3	7	10		
Control (0% PJEE)	86.67 ± 1.67^{A}	81.67 ± 3.33^{A}	78.33 ± 1.67^{A}	45.00 ± 10.41^{AB}	23.33 ± 14.53^{AB}	3.55	0.000 1
10% PJEE	88.33 ± 1.67^{A}	85.00 ± 5.00^{A}	85.00 ± 2.89^{A}	53.33 ± 8.82^{A}	33.33 ± 13.33^{A}		
20% PJEE	85.00 ± 5.00^{AB}	83.33 ± 4.41^{A}	75.00 ± 2.89^{A}	30.00 ± 7.64^{BC}	3.33 ± 3.33^{B}		
30% PJEE	78.33 ± 4.41^{AB}	78.33 ± 4.41^{A}	60.00 ± 2.89^{B}	13.33 ± 6.01^{CD}	3.33 ± 3.33^{B}		
40% PJEE	61.67 ± 13.02^{B}	50.00 ± 10.00^{B}	26.67 ± 6.67^{C}	0.00 ± 0.00^{D}	0.00 ± 0.00^{B}		
50% PJEE	18.33 ± 10.93^{C}	8.33 ± 6.01^{C}	1.67 ± 1.67^{D}	0.00 ± 0.00^{D}	0.00 ± 0.00^{B}		
P <	0.000 2	0.000 1	0.000 1	0.000 4	0.058 1		

Means with different superscripts are significantly different using Duncan's multiple range test at $P < 0.05$.

Table 2
Effect of PJEE on post-thawing characteristics of cattle-bulls extended semen (%).

Treatment	Motility	HOST	Alive sperm	Total abnormality
Control (0% PJEE)	45.56 ± 1.55^{B}	63.00 ± 1.15^{A}	58.33 ± 0.33^{D}	21.00 ± 3.21^{A}
10% PJEE	53.89 ± 2.61^{A}	69.67 ± 1.45^{A}	69.00 ± 0.58^{A}	24.67 ± 3.84^{A}
20% PJEE	51.67 ± 1.86^{AB}	67.00 ± 3.61^{A}	60.00 ± 2.31^{CD}	23.00 ± 0.00^{A}
30% PJEE	45.56 ± 2.82^{B}	67.33 ± 2.85^{A}	64.33 ± 0.67^{ABC}	21.67 ± 1.86^{A}
40% PJEE	37.78 ± 2.78^{C}	62.67 ± 3.18^{A}	62.00 ± 3.06^{BCD}	20.33 ± 1.20^{A}
50% PJEE	33.33 ± 2.20^{C}	62.33 ± 4.33^{A}	66.67 ± 0.89^{AB}	21.67 ± 1.76^{A}
$P <$	0.000 1	0.429 5	0.005 1	0.813 6

Means with different superscripts are significantly different using Duncan's multiple range test at $P < 0.05$.

Concerning to frozen-thawed semen, the present study revealed that supplementation of extender with 10% and 20% PJ increased the post-thaw motility [(53.89 ± 2.61)% and (51.67 ± 1.67)%, respectively], and these values are significantly ($P < 0.000\,1$) higher than the control group [(45.56 ± 1.55)%]. The addition of 10% PJ increased the live percentage count [(69.00 ± 0.58)%], this value was significantly ($P < 0.000\,1$) higher than the control group [(58.33 ± 0.33)%] (Table 2). No significant ($P < 0.429\,5$) differences could be detected between groups in sperm membrane integrity and total sperm abnormality %.

4. Discussion

Pomegranate is known for its antioxidant activity, both *in vivo* and *in vitro* [3,4]. Fresh juice contains high amount of vitamin C, and polyphenolic compounds (anthocyanins, punicalagin, ellagic and gallic acid) [4]. The main objective of the current study was to add PJ to cattle semen extender for chilling and cryopreservation and to assess its effect on sperm motility, functional sperm membrane integrity, viable sperm and total sperm abnormality percentage. The PJ concentrations were chosen after pre-experiments we conducted, and according to the limited available data [16]. In the current study, PJ has been added to TCYF extender at different concentrations (10%, 20%, 30%, 40% and 50%).

Obtained results clearly demonstrated that PJ resulted in a gradual decline concerning the motility percentage of spermatozoa chilled at 5 °C for the control (0% PJEE) and the first treatment (10% PJEE) from the 1st day to the 10th day. The addition of 10% PJEE for chilled cattle semen proved to be beneficial for maintaining sperm motility percentage (at least, $P < 0.058\,1$) all over the 10 d. On contrast, the concentrations of 40% and 50% PJEE did not preserve motility. Similarly, Al-Daraji revealed that the inclusion of PJ (2 mL, 4 mL/100 mL) into rooster semen extender resulted in significant ($P < 0.05$) decreases the dead, the abnormal and the acrosomal integrity percentage of spermatozoa when semen samples were examined before storage or after certain storage periods (12, 24 or 36 h) [16]. The previous author attributed these positive results to the potent antioxidant activity of PJ.

With regard to frozen-thawed semen, the present study revealed that supplementation of extender with 10% and 20% PJ significantly increased the post-thaw motility ($P < 0.000\,1$) as compared with the control group. The addition of 10% PJ significantly increased the live percentage ($P < 0.000\,1$) than the control group. During freezing, there is progressed production of reactive oxygen species (ROS) [22] that cause changes in function and structure of sperm membrane in concomitant with an alteration in antioxidant defense systems [23], including a reduction in intracellular GSH content [24]. To counteract the destructive effects of ROS, seminal plasma possesses an antioxidant system that seems to be very relevant to the protection of sperm [25]. Unfortunately, this antioxidant capacity of spermatozoa is very limited to protect itself against ROS, compared with somatic cells. The addition of antioxidant to the freezing and thawing medium with antioxidants could be useful to improve the viability and subsequent fertilizing capacity of frozen-thawed farm animal's spermatozoa [26]. A lot of studies were carried out on the addition of different antioxidants in extenders to protect spermatozoa against detrimental effects of ROS [27,28]. Halvorsen *et al*. reported that pomegranate fruit contained very high concentrations of antioxidants (11.33 mmol/100 g) [29]. Flavonoids and other phenolic compounds appear to have antioxidant activities that are several times higher than those of vitamins E and C [30]. Longtin concluded that the antioxidant capacity of PJ is dependent not only on vitamin C content but also other antioxidant-rich like tannins and flavonoids compounds [31]. However, he suggested that the antioxidant capacity of PJ is a function of the combined action of a number of constituents. In addition, fresh PJ contains 10% total sugars, and 1.5% pectin, ascorbic acid, polyphenolic, flavonoids and the principal amino acids (glutamic and aspartic acid) [32,33]. These phytochemicals may act as antioxidants, and modulate bacterial populations in the body or media [34]. Moreover, Roger reported that PJ is also packed with vitamins A, C, and E, all of which boost sexual libido in men and women [35]. Gangwar *et al*. indicated that vitamin C at the level of 56.78 mmol/L can be used as an antioxidant in semen diluent in a routine freezing process for better post-thaw recovery of buck semen [36]. Vitamin C is naturally present in seminal plasma to scavenge and decrease numerous disruptive free radical processes, including lipid peroxidation [37]. The addition of vitamin C in an extender could possibly improve sperm function by reducing cell damage through its continuous radical-scavenging action. Alpha-tocopherol, one of the main sperm antioxidants, was found to be abundant in spermatozoa membrane [38,39] and protect sperm motility from oxidative damage [40]. Addition of 20 mmol/L of L-glutamine to ram semen extender prevented injuries to sperm and improved the post-thaw semen characteristics [41]. In conclusion, PJ supplementation improved sperm motility of chilled semen and post-thaw sperm motility, membrane integrity and viability and decreased total sperm abnormalities of cattle cryopreserved semen.

Conflict of interest statement

We declare that we have no conflict of interest.

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