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Effect of addition of pomegranate (*Punica granatum*) juice on the liquid storage (5°c) of mithun (*Bos frontalis*) semen

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ABSTRACT

A total 50 ejaculates from eight mithun bulls were collected twice a week over 8 months and semen pooled to eliminate individual differences and was split into four equal aliquots, diluted with the TEYC extender. Group 1: diluted 1:2 with TEYC extender (control), group 2 to 4: semen diluted with 1:2 TEYC extender supplemented with 6, 8 and 10 ml of PJ/ 100 ml of diluent, respectively. The seminal parameters and biochemical profiles were assessed at 5°C for 0, 6, 12, 24 and 30 h of incubation. Inclusion of PJ into diluent resulted in significant ($p < 0.05$) decrease in percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities at different hours of storage periods. Additionally, PJ at 6 and 10 ml were inferior to PJ 8 ml treatments as regards to these characteristics. It was concluded that the possible protective effects of PJ on sperm parameters are enhancing the function of antioxidant enzymes and prevent efflux of cholesterol and phospholipids from cell membrane during preservation.

Key words: Antioxidants, Mithun, Pomegranate (*Punica granatum*) juice, Seminal parameters.

INTRODUCTION

Mithun (*Bos frontalis*), a unique free – range bovine species is available in the North Eastern hilly region of India. The recent initiatives to popularize this species as an economic beef animal demand its rearing under semi-intensive system and adoption of systematic breeding programme. In this context, it is necessary to standardize an effective semen preservation protocol for this species.

Cold storage of semen is used to reduce metabolism and to maintain sperm viability over an extended period of time. But the quality of semen is deteriorated during this extended storage period. One cause of this decline may be due to the action of the reactive oxygen species generated by the cellular components of sperm, namely a superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and lipid hydroperoxides formed via lipid peroxidation of the membrane lipids of spermatozoa (Perumal *et al*., 2011a, Perumal *et al*., 2011b). Mithun semen normally contains antioxidants, including catalase (CAT) and superoxide dismutase (SOD) that can offset lipid peroxidation. Concentration of these antioxidants is reduced during dilution and storage that affects semen quality.

Evidence suggests that the nutritional antioxidants such as pomegranate (*Punica granatum*) juice (PJ) can reduce oxidative stress (Aviram *et al*., 2000), prevent cholesterol efflux from cell membrane (Kalpan *et al*., 2001), enhances action of low density lipoprotein (Aviram *et al*., 2000) and inhibits one or both of the enzymes of cyclooxygenase and lipooxygenase (Lansky *et al*., 2002). PJ, therefore, may prevent formation of the free radicals and neutralise the formed ROS in semen to maintain its quality for enhancing fertility. Therefore present study was designed to examine the probable role of PJ as a potent antioxidant in counteracting detrimental effects of lipid peroxidation that naturally occurred during *in vitro* storage of mithun semen at 5°C.

MATERIALS AND METHODS

Eight apparently healthy mithun bulls of approximately 4 to 6 years were selected from the herd derived from various hilly tracts of the north eastern region of India. The average body weight of the bulls was 501 kg (493 to 507 kg) at 4 - 6 yr of age with good body condition (score 5-6) maintained under uniform feeding, housing and lighting conditions. Each experimental animal was fed as per the farm schedule. Semen was collected from the animals through rectal massage method. During collection, the initial transparent secretions were discarded and neat semen drops were collected in a graduated test tube with the help of a funnel. During the study, all the experimental protocols met the Institute Animal Care and Use Committee regulations.

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Total numbers of 50 ejaculates were collected from the mithun twice a week over 8 months and semen pooled to eliminate individual differences. Immediately after collection, the samples were kept in a water bath at 37°C and evaluated for volume, colour, consistency, mass activity and pH. After the preliminary evaluations, samples were subjected to the initial dilution with pre-warmed (37°C) Tris egg yolk citrate extender (TEYC). The partially diluted samples were then brought to the laboratory in an insulated flask containing warm water (37°C) for further processing. The ejaculates were evaluated and accepted for evaluation if the following criteria were met: concentration: >500 million D ml; mass activity >3+, individual motility: >70% and total abnormality: <10%.

The pomegranate fruit was procured from the commercial market at ripened stage and it was opened with sterilized hand inside the laminar floor. The pomegranate seed was separated from the fruit and squeezed the seed for the pomegranate juice with hand. The squeezed juice was centrifuged at 4000 rpm for 15 minutes and the pure juice was separated. The juice was preserved in deep freezer for upto utilization.

Each pooled ejaculate was split into four equal aliquots and diluted with the TEYC extender with pomegranate juice. Group 1: semen diluted 1:2 with TEYC extender (control), group 2 to group 4: semen diluted with 1:2 TEYC extender supplemented with 6 ml, 8 ml and 10 ml of PJ / 100 ml of diluent, respectively. However, pH of diluents was adjusted to be 6.8 – 7.0 by using phosphate buffer solution. Diluted semen samples were kept in glass tubes and cooled from 37 to 5°C, at a rate of 0.2–0.3°C/min in a cold cabinet and maintained at 5°C during liquid storage for up to a 30 h period of the experiment. The percentage sperm motility, viability, total sperm abnormality, acrosomal integrity and the plasma membrane integrity by hypo-osmotic swelling test (HOST) were determined as per standard procedure in samples during storage at 5°C for 0, 6, 12, 24 and 30 h, respectively. The SOD activity of the seminal plasma was estimated using the method as described by Madesh and Balasubramanian (1997) with some modifications and catalase activity was estimated by the method of Cohen *et al*. (1970). Total cholesterol was estimated by commercial available kit. Results were analysed using the SPSS/PC computer program (version 15.0; SPSS, Chicago, IL).

RESULTS AND DISCUSSION

The effects of various doses of PJ on sperm motility, viability, total sperm abnormality, acrosomal and plasma membrane integrity at different hours of incubation in liquid state (5°C) were presented in Table 1, 2,3,4 and 5, respectively. Results also revealed that the inclusion of PJ into diluent resulted in significant ($p < 0.05$) decrease in percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities when semen samples were examined at different hours of storage periods compared with control group. Additionally, PJ at 6 and 10 ml were inferior to PJ 8 ml treatments as regards to these characteristics, while there were no significant differences between PJ at 6 and 10 ml in relation to these features. The antioxidant enzymatic profiles such as SOD and catalase of control and treatment group were presented in Table 6 and 7. Highest mean SOD and catalase activities were recorded PJ treated semen than control group. Both SOD and catalase were significantly ($p<0.01$) differed between control and supplemented group. Similarly, cholesterol concentration was significantly differed between the PJ treated and control (Table 8). It was obvious from the data of this experiment that the addition

TABLE 1: Effect of diluents supplementation with pomegranate juice on motility of spermatozoa of mithun semen

Additives	Storage Time				
	0 h	6h	12 h	24 h	30h
Control	75.23 ± 2.30 [a]	72.86 ± 2.85 [a]	63.56 ± 2.51 [a]	42.32 ± 2.54 [a]	32.32 ± 2.87 [a]
PJ 6ML	80.24 ± 2.10 [b]	77.63 ± 2.61 [b]	71.93 ± 2.15 [b]	50.63 ± 2.06 [b]	42.57 ± 2.39 [b]
PJ 8ML	86.36 ± 2.78 [c]	82.44 ± 2.13 [c]	78.54 ± 2.67 [c]	56.55 ± 2.85 [c]	50.33 ± 2.18 [c]
PJ 10ML	79.84 ± 2.62 [b]	76.12 ± 1.99 [b]	70.82 ± 2.39 [b]	48.47 ± 2.15 [b]	39.69 ± 1.43 [b]

Within columns means with different letters (a, b, c, d) differ significantly ($p < 0.05$)

TABLE 2: Effect of diluents supplementation with pomegranate juice on viability of spermatozoa of mithun semen

Additives	Storage Time				
	0 h	6h	12 h	24 h	30h
Control	75.36 ± 2.28 [a]	73.45 ± 2.23 [a]	62.33 ± 3.58 [a]	43.52 ± 1.74 [a]	34.81 ± 2.45 [a]
PJ 6ML	82.43 ± 2.00 [b]	76.23 ± 2.23 [b]	73.66 ± 1.94 [b]	48.43 ± 2.07 [b]	41.85 ± 2.27 [b]
PJ 8ML	84.62 ± 2.03 [c]	83.26 ± 2.16 [c]	76.87 ± 2.21 [c]	52.75 ± 1.86 [c]	48.56 ± 1.78 [c]
PJ 10ML	80.47 ± 2.04 [b]	75.34 ± 2.27 [b]	67.23 ± 2.52 [d]	44.23 ± 1.67 [a]	40.38 ±2.37 [b]

Within columns means with different letters (a, b, c, d) differ significantly ($p < 0.05$)

TABLE 3: Effect of diluents supplementation with pomegranate juice on total sperm abnormality of spermatozoa of mithun semen

Additives	Storage Time				
	0 h	6h	12 h	24 h	30h
Control	6.8 ± 1.55	10.4 ± 1.53 [a]	11.4 ± 1.67 [a]	13.3 ± 1.61 [a]	16.3 ± 1.60 [a]
PJ 6ML	6.1 ± 1.30	7.7 ± 1.34 [b]	9.3 ± 1.69 [b]	12.3 ± 1.46 [b]	13.5 ± 1.34 [b]
PJ 8ML	5.4 ± 1.06	6.5 ± 1.08 [c]	8.4 ± 1.39 [c]	10.8 ± 1.51 [c]	12.3 ± 1.37 [c]
PJ 10ML	6.5 ± 1.49	7.87 ± 1.73 [b]	9.7 ± 1.79 [b]	12.6 ± 1.80 [b]	14.7± 1.79 [d]

Within columns means with different letters (a, b, c, d) differ significantly ($p < 0.05$)

TABLE 4: Effect of diluents supplementation with pomegranate juice on acrosomal integrity of spermatozoa of mithun semen

Additives	Storage Time				
	0 h	6h	12 h	24 h	30h
Control	78.33 ± 2.58 [a]	74.38 ± 2.40 [a]	71.24 ± 2.99 [a]	47.34 ± 2.42 [a]	37.32 ± 2.53 [a]
PJ 6ML	81.25 ± 2.08 [b]	78.64 ± 1.52 [b]	75.26 ± 2.18 [b]	51.68 ± 2.05 [b]	43.25 ± 2.62 [b]
PJ 8ML	86.77 ± 1.42 [c]	85.26 ± 1.94 [c]	78.67 ± 2.87 [c]	60.88 ± 1.93 [c]	58.38 ± 1.73 [c]
PJ 10ML	82.32 ± 2.21 [b]	77.68 ± 1.96 [b]	72.35 ± 4.10 [a]	50.43 ± 2.26 [b]	44.64 ± 4.82 [b]

Within columns means with different letters (a, b, c, d) differ significantly ($p < 0.05$)

TABLE 5: Effect of diluents supplementation with pomegranate juice on plasma membrane integrity (HOST) of spermatozoa of mithun semen

Additives	Storage Time				
	0 h	6h	12 h	24 h	30h
Control	73.57 ± 2.43 [a]	70.76 ± 2.20 [a]	64.43 ± 3.15 [a]	43.38 ± 2.07 [a]	39.52 ± 1.76 [a]
PJ 6ML	80.76 ± 1.92 [b]	76.97 ± 2.18 [b]	70.26 ± 2.48 [b]	48.57 ± 1.55 [b]	46.77 ± 2.00 [b]
PJ 8ML	85.48 ± 1.54 [c]	81.34 ± 2.58 [c]	75.43 ± 1.92 [c]	60.42 ± 2.76 [c]	55.68 ± 1.69 [c]
PJ 10ML	78.92 ± 2.76 [b]	75.43 ± 1.94 [b]	68.56 ± 1.72 [b]	50.44 ± 2.51 [b]	47.53 ± 1.96 [b]

Within columns means with different letters (a, b, c, d) differ significantly ($p < 0.05$)

TABLE 6: Effect of diluents supplementation with pomegranate juice on SOD of mithun semen

Additives	Storage Time				
	0 h	6h	12 h	24 h	30h
Control	0.56 ± 0.12 [a]	0.52 ± 0.05 [a]	0.49 ± 0.14 [a]	0.47 ± 0.11 [a]	0.43 ± 0.06 [a]
PJ 8ML	0.68 ± 0.22 [b]	0.66 ± 0.16 [b]	0.64 ± 0.18 [b]	0.62 ± 0.05 [b]	0.61 ±0.10 [b]

Within columns means with different letters (a, b) differ significantly ($p < 0.05$)

TABLE 7: Effect of diluents supplementation with pomegranate juice on catalase of mithun semen

Additives	Storage Time				
	0 h	6h	12 h	24 h	30h
Control	5.34 ± 0.65 [a]	5.11 ± 0.82 [a]	4.95 ± 0.23 [a]	4.63 ± 1.05 [a]	4.37 ± 0.83 [a]
PJ 8ML	8.46 ± 1.12 [b]	7.21± 0.75 [b]	6.96 ± 0.89 [b]	6.73 ± 1.12 [b]	6.72 ± 0.57 [b]

Within columns means with different letters (a, b) differ significantly ($p < 0.05$)

TABLE 8: Effect of diluents supplementation with pomegranate juice on cholesterol efflux of mithun semen

Additives	Storage Time				
	0 h	6h	12 h	24 h	30h
Control	144.48 ± 5.89 [a]	145.88 ± 6.72 [a]	149.76 ± 6.25 [a]	156.74 ± 6.45 [a]	159.2 ± 6.82 [a]
PJ 8ML	143.08 ± 7.49 [b]	144.54 ± 8.26 [b]	145.12 ± 7.36 [b]	147.98 ±5.37 [b]	148.6 ± 6.38 [b]

Within columns means with different letters (a, b) differ significantly ($p < 0.05$)

of PJ especially at the concentrations of 8 ml / 100 ml of diluent to the semen diluent resulted in significant improvement in quality and antioxidant enzyme activity of mithun semen in *in vitro* stored for up to 30 h.

There was no report on effect of addition of PJ on seminal parameters in mithun. Analysis of various seminal parameters such as forward progressive motility, livability, acrosomal and plasma membrane integrity are important for extensive utilization of semen in artificial insemination. In the present study, PJ supplementation on these parameters revealed significant difference between the treatment groups.

In this study, improvements observed in sperm quality may be attributed to prevention of excessive generation of free radicals, produced by spermatozoa themselves, by means of their antioxidant property of PJ. It was concluded that the possible protective effects of PJ supplementation due to enhancing antioxidant enzymes content and preventing efflux of cholesterol and phospholipids from cell membrane. Thus it may protect the spermatozoa during preservation and enhancing the fertility in farm animals. Future studies by measuring the level of lipid peroxidation and also sperm preservation/cryoprotective studies are warranted to confirm the present findings.

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