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Technical note

Effect of vitamin C supplementation on freezability of Barbari buck semen

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ABSTRACT

Thirty six ejaculates from 6 adult Barbari bucks (2–4 years old) maintained at C.I.R.G under semi intensive management system were used to find out the freezability of buck semen at different levels of vitamin C (0.0 μM, 45.42 μM, 56.78 μM, 68.13 μM) by conventional method of freezing. The ejaculates were collected twice at weekly intervals by artificial vagina. The semen samples were diluted with tris–citric acid fructose diluents having 10% (v/v) egg yolk and 6% (v/v) glycerol as cry protectant agent. The semen samples were extended to maintain sperm concentration approximately 100–120 million per dose. Filling and sealing of straws were done at 5 °C in cold handling cabinet after 4 h of equilibration period, then straws were vapour frozen for 10 min above 2 cm of liquid nitrogen and finally stored into liquid nitrogen container. Post thaw motility, live sperm count, abnormalities, acrosomal integrity and hypo osmotic swelling test were conducted to check the freezability. Post thaw motility, live sperm count, acrosomal intergrity and hypo osmotic swelling positive spermatozoa differed significantly among groups and they were the highest in 1% group. The result indicated that vitamin C at the level of 56.78 μM can be used as an antioxidant in semen diluter in routine freezing process for better post thaw recovery of buck semen.

1. Introduction

An acceptable fertility of cryopreserved semen has always been a challenge and poor fertility of cryopreserved semen still remains a problem for the goat breeders and scientists engaged in cryopreservation of buck semen. Buck sperm cells contain a high proportion of polyunsaturated fatty acids, and therefore they are particularly susceptible to peroxidative damage (Asadpour et al., 2011). The production of Reactive Oxygen Species (ROS) is a normal physiological event in various organs and seminal plasma. However, the imbalance between ROS production and scavenging system can cause structural and functional damage to spermatozoa. To counteract the destructive effects of Reactive Oxygen Species (ROS), seminal plasma has an antioxidant system that seems to be very relevant to the protection of sperm (Alvarez and Storey, 1982). Their vulnerability increases especially following cryopreservation with a subsequent loss in membrane integrity, impaired cell function, decreased motility and the fertilizing capability of the spermatozoa. Reactive oxygen species i.e., super oxide, hydroxyl radical and peroxide are produced by spermatozoa, immune cells in semen and also environmental factors (especially UV) during cryopreservation stages (Alvarez and Storey, 1982; Aitken and Fisher, 1994; Aitken and Baker, 2004). The antioxidant capacity of spermatozoa is very limited to protect itself against ROS, compared with somatic cells. To control the level of ROS and promote motility and survival of sperm, numerous antioxidants have proven beneficial effect on improving buck fertility (Azawi and Hussein, 2013). Vitamin C (ascorbic acid, ascorbate) is naturally present in seminal plasma to scavenge free radicals and its presence also assists various other mechanisms in decreasing numerous disruptive free radical processes, including lipid peroxidation (Anane and Creppy, 2001).

Vitamin C represents the major water-soluble antioxidant in plasma. Ascorbic acid is required in vivo as a cofactor for at least eight enzymes and can also act as an antioxidant by reacting with free radicals (Michael et al., 2008). The concentration of vitamin C in seminal plasma is 10 times greater than in blood plasma (364 vs. 40 μmol/l). The addition of vitamin C in an extender could possibly improve sperm function by reducing cell damage through its continuous radical-scavenging action. However, a lot of studies were carried out on addition of different antioxidants in extenders to protect spermatozoa against detrimental effects of Reactive

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Oxygen Species (ROS) (Uysal and Bucak, 2007; Bucak et al., 2008) but there is limited information regarding the effects of the vitamin C in different extenders on buck semen quality. Therefore, the objective of this study was, to test the hypothesis that different levels of vitamin C might effectively protect buck semen from oxidative damage during cryopreservation in TRIS extender resulting in higher post thaw sperm viability, motility and fertility.

2. Materials and methods

2.1. Source of animal and its management

The six adult Barbari bucks, of 2–4 years old, were selected for the study. The bucks were kept under semi-intensive system of management at Indian Council of Agricultural Research-Central Institutes for Research on Goats (ICAR-CIRG) Makhdoom.

2.2. Semen collection and evaluation

Thirty six ejaculates were collected from six adult Barbari bucks at twice a week interval using artificial vagina. The semen was collected from February, 15th to March, 15th 2014. Immediately after collection, the volume, colour, consistency and mass motility of ejaculates were assessed. Semen samples having mass motility more than +4 were used for further study Ranjan et al. (2014).

2.3. Dilutor and dilution

Semen samples were extended with tris–citrate–fructose yolk diluents (tris—3.604 g; citric acid—1.902 g; fructose—1 g; streptomycin—100 mg; penicilline—100,000 I.U.; triple distilled water—100 ml; pH—6.8–6.9) having 10% (v/v) egg yolk and 6% (v/v) glycerol. The samples were diluted to maintain the sperm concentration 100–120 million per dose or 400 million sperm/ml.

2.4. Vitamin C concentration in dilutor

Semen was diluted with tris–citrate–fructose yolk dilutor having 0%, 0.8%, 1% and 1.2% vitamin C and diluted semen samples were equilibrated at 5 °C for 4 h before being frozen.

2.5. Semen cryopreservation

After equilibration period of 4 h semen was filled in 0.25 ml straws at 5 °C and straws were sealed with polyvinyl alcohol powder. After filling and sealing, straws were vapour frozen in vapours of liquid nitrogen by keeping them 2–4 cm above the liquid nitrogen for 10 min. Finally straws were plunged into liquid nitrogen and stored into liquid nitrogen container.

2.6. Live and dead sperm

This was estimated as per standard staining procedure as described by Hancock (1951). The staining solution contains Eosin (0.67 g/100 ml) and Nigrosin (5 g/100 ml) and water to make the volume 100 ml. A drop of diluted semen mixed with eight drops of stain was incubated at 30 °C for 2 min. Then smears made on pre-warmed slides were allowed to dry at 30 °C. The excess stain was washed off in running tap water. The slide was then immersed briefly in ethanol to remove water. Then mounted smear was observed under 400× objective lens of the phase contrast microscope. Sperm abnormalities were also counted with same staining technique. Approximately 400 sperm were counted.

2.7. Sperm staining and evaluation

Diluted semen (10 μl) was placed on a clean grease free pre warmed slide (37 °C) with cover slip and observed under 40× magnification of phase contrast microscope for assessing the progressive motility. The average values of two experts were considered for calculating the progressive motility. Giemsa stain was used to assess the acrosomal integrity of frozen thawed buck spermatozoa as per Watson (1975). Hypo osmotic swelling test was carried out as described by Gangwar et al. (2014).

2.8. Statistical analysis

Data were analyzed by General Linear Model of SPSS 16 data analysis software. The factorial model included the effect of vitamin C as independent variables and percent post thawed motility and live sperm count, abnormalities, acrosome intact sperm and hypo osmotic swelling positive sperm as dependent variables. Post Hoc Test Duncan LSD T3 was conducted to know the significant difference between different variables at $P < 0.05$. Homogeneity test was also conducted to assign different superscript to variables based on significant difference at $P < 0.05$ between different variables.

3. Result and discussion

Results of the present experiment are shown in detail in Table 1. In the present study the effects of vitamin C as an antioxidant in buck semen diluter on post thaw semen quality was evaluated. The percentages of motile spermatozoa live and dead spermatozoa, hypo osmotic swelled spermatozoa and acrosome integrity for each vitamin C concentration were averaged. Results indicated that the progressive motility, live sperm count, abnormality, acrosomal integrity and hypo osmotic swelling positive spermatozoa (mean ± SE) were 84.05 ± 1.23, 89.71 ± 0.94, 2.86 ± 0.64, 91.57 ± 0.91 and 75.43 ± 1.03, respectively, in fresh semen. Ranjan et al. (2009, 2014, 2015) studied the above parameters at different egg yolk levels and equilibration period in Marwari and Jamunapari goats. The post thaw quality of semen frozen in dilutor having different percent of vitamin C as an antioxidant was found suitable for storage and further use in artificial insemination programme. In the present study 45.42 μM and 56.78 μM of vitamin C level were found suitable and have positive effect on post thaw motility and viability. Addition of vitamin C at the level 45.42 μM and 56.78 μM increases the post thaw motility 47.29 ± 1.16% and 53.54 ± 1.29%, respectively, and these values are significantly ($P < 0.05$) higher than control group (41.04 ± 1.08%). Similarly addition of vitamin C at the level of 45.42 μM and 56.78 μM also increases the live percentage count 61.87 ± 1.43% and 68.21 ± 1.37%, respectively, and again these values are significantly ($P < 0.05$) higher than control group (56.92 ± 1.49%). But 56.78 μM of vitamin C has significantly ($P < 0.05$) improved the acrosomal integrity (83.62 ± 1.15%) and hypo osmotic swelling positive spermatozoa (53.92 ± 1.93%). In the present study 56.78 μM vitamin C level in diluter significantly ($P < 0.05$) improves the post thaw motility, live percentage, acrosomal intact spermatozoa and hypo osmotic swelling positive spermatozoa and the values were (mean ± SE) 53.54 ± 1.29, 68.21 ± 1.37, 83.62 ± 1.15 and 53.92 ± 1.93, respectively. Hence, the 56.78 μM level of vitamin C is optimum as an antioxidant for buck semen diluter. However, 45.42 μM vitamin C also significantly ($P < 0.05$) improves the post thaw motility (47.29 ± 1.16) and live sperm count (61.87 ± 1.43). However there was no effect on acrosomal integrity and hypo osmotic swelling positive spermatozoa. While 68.13 μM level of vitamin C had no significant effect on post thaw semen quality.

Caprine spermatozoa are highly sensitive to lipid peroxidation, which occurs as a result of the oxidation of membrane lipids by superoxide, hydrogen peroxide and hydroxyl radicals. Spontaneous lipid peroxidation of the spermatozoa membranes destroys the structure of the lipid matrix. The attacks of the reactive oxygen species ultimately lead to the impairment of sperm function, such as sperm motility, functional membrane integrity, leakage of intracellular enzymes and damage to the sperm DNA through the oxidative stress (Alvarez and Storey, 1989). Ascorbic acid is water soluble natural antioxidant present in epididymal fluid and seminal plasma of many species.

Earlier Sonmez and Demirci (2003) found that the intramuscular injection of ascorbic acid for 30 days increased the semen quality of normal rams and increased its concentration in blood and seminal plasma. Fazeli et al. (2010) also reported that the subcutaneous vitamin C injection for 90 days increased sperm motility and the effect was evident up to 30 days after the cessation of injections. However, the percentage of live sperm and mass motility showed similar trends with control animals. In the present study 45.42 μM and 56.78 μM of vitamin C level were found suitable and have positive effect on post thaw motility and viability. Azawi and Hussein (2013) reported that the addition of vitamin C to semen preservation media could improve longevity and quality of cooled sperm in Awassi ram semen. Similar to our findings Azawi and Hussein (2013) reported that motility was improved from 35.9 ± 1.9% to