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Research Article

PLASMA MEMBRANE INTEGRITY OF CAUDA EPIDIDYMAL BUCK SPERMATOZOA IN TRIS YOLK CITRATE EXTENDER SUPPLEMENTED WITH COCONUT WATER AT REFRIGERATION TEMPERATURE

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ABSTRACT

The study was carried out on total ten pairs of testis from matured non-descript buck irrespective of breed presented for slaughter at government approved slaughter house, over a period of four months from August to November, 2018 with the objective to evaluate the effect of coconut water supplementation in Tris egg yolk citrate extender on plasma membrane functional integrity of cauda epididymal buck spermatozoa preserved at refrigeration temperature. The paired cauda epididymal spermatozoa were diluted with Tris egg yolk citrate (TEYC) extender and made five equal aliquots i.e. T1 to T5. T1 was kept as a control while T2, T3, T4 and T5 were supplemented with 5, 10, 15 and 20 % coconut water, respectively and preserved at refrigeration temperature (4-5 ºC). The plasma membrane integrity was measured by hypo-osmotic swelling (HOS) test at 12 h intervals up to 48 h. The highest HOS reacted sperm count, irrespective of preservation time, was found in T4 followed by T3, T5, T2 and T1 groups. In conclusion, supplementation of coconut water in TEYC extender improves the functional integrity of plasma membrane in cauda epididymal buck spermatozoa preserved at refrigeration temperature with best result at 15 % coconut water supplementation.

Keywords: Buck, Cauda epididymal, Coconut water, HOS, Spermatozoa

Goat semen can be preserved either at room temperature temporarily, at refrigerated temperature for 24-48 hours (Ferdinand *et al.,* 2012) or cryopreserved (Beltran *et al*., 2013) for long term storage. Liquid goat semen destined for use within 12 hours should be stored at 4 ºC (La Falci *et al.,* 2002), because refrigerator temperature helps to stop metabolic process of stored liquid semen which resulted in the utilization of nutrients such as fructose by the sperm cells (Aboagla and Terada, 2003).

The presence of sugars, amino acids, minerals and vitamins found in coconut water, are not only nutrients but also cryoprotectants in addition to presence of antioxidants (Yong *et al*., 2009). Coconut water is poor in phospholipids and rich in complex organic molecules, such as proline, glycine, glutamic acid and indole-acetic acid (IAA) which protects and extends the life span of spermatozoa, based on cell membrane protection that reinforces its molecular structure (Nunes and Combarnous, 1995).

The amino acid component of coconut water protects the spermatozoa plasmalemma from temperature related injury (Yong *et al*., 2009) through coating of the plasma membrane and combining with phospholipids on the plasma membrane (Atessahin *et al*., 2008). Several studies have demonstrated that coconut water after correcting osmolality and pH is effective in the maintenance of *in vitro* and *in vivo* spermatic cell characteristics (Nunes, 1998). Positive effect of coconut water supplementation in Tris-citric acid-fructose-egg yolks for functional integrity of plasma membrane in freeze-thawed cattle semen was reported by El-Sheshtawy *et al.* (2017).

There is diminutive information available regarding epididymal sperm retrieval and preservation at refrigerated temperature. Similarly, except few reports on addition of certain fruit filtrate as non-enzymatic antioxidant in buck epididymal semen preservation, no any literature was found on use of coconut water as buck epididymal spermatozoa preservative, particularly in India. Therefore, in present study the coconut water-based extenders has been used in search for alternative semen extenders that are non-toxic, buffering, low cost, practical, and effective for maintenance of functional integrity of sperm's plasma membrane.

MATERIALS AND METHODS

Sample collection and processing: Ten pairs of testicles were collected immediately after slaughter under strict hygienic conditions from apparently healthy matured non-descript buck irrespective of breed at government approved slaughter house and transferred to the laboratory in ice packs as early as possible. The laboratory processing of testes was carried out within 30 minutes of reaching the laboratory. Testes were washed and cleaned with R.O. water. Fascia, blood vessels and sheath of testes were removed with the help of BP blade and thumb forceps. Care was taken to prevent the damage to the epididymis. Spermatozoa were retrieved separately from the right and left cauda epididymis at room temperature by the incision method. Several small incisions were made on the cauda of epididymides with a BP blade to enable spermatozoa swim out in to five ml pre warmed (37º C) tris egg yolk citrate (TEYC) diluter in a petri dish. Cauda epididymal sperm samples having ≥ 70% individual motility were selected for further analysis and extended with TEYC diluter to make a final volume of 20 ml. Retrieved spermatozoa of right and left cauda epididymis were extended with diluter separately.

Preparation of coconut water: Coconut water (CW) was prepared on the day of experiment from tender, green,

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healthy and undamaged coconut fruits. The coconut water collected from coconut fruits was first filtered through Whatman filter paper No. 4 followed by syringe filter (0.2µ). Finally, it was centrifuged at least for 5 minutes at 3000 rpm in centrifuge machine and the supernatant from each tube was obtained carefully in a sterile glass bottle.

Experimental groups: To study the effect of coconut water supplementation on plasma membrane functional integrity of refrigerated cauda epididymal buck spermatozoa, 20 ml of diluted samples from individual epididymides were divided equally into five aliquots (T1 to T5) and supplemented with different concentrations of coconut water. T1 is kept as a control while T2, T3, T4 and T5 were supplemented with 5, 10, 15 and 20 % coconut water, respectively and preserved at refrigeration temperature (4-5 ºC) up to 48 hrs and evaluated at every 12 hrs interval at 37 ºC temperature in water bath.

Evaluation of plasma membrane functional integrity: To evaluate the plasma membrane functional integrity of spermatozoa, Hypo Osmotic Swelling (HOS) test was determined by mixing 0.1 ml of diluted semen with 1.0 ml of hypo osmotic solution (0.735 g Sodium citrate + 1.351 g Fructose + 100 ml Triple glass distilled water). The tube containing the mixture was incubated at 37º C for 30 minutes. A drop of semen from the mixture placed on a clean dry glass slide and covered with cover slip. The sperms characterized by varying degrees of coiling or swelling of tail were considered to have an intact plasma membrane (HOS reacted sperm) and the sperms without tail curling were considered to have damaged membrane (HOS non-reacted sperm). Total 200 spermatozoa were counted in different fields and percentage of spermatozoa exhibiting tail curling (reacted) was calculated.

Statistical analysis: The data pertaining to various aspects were suitably tabulated and analysed using R-3.3.2 software. The differences among the parameter means were carried out using appropriate statistical methods, *viz.* ANOVA, DNMRT (Duncan's New Multiple Range Test). The mean differences were considered significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS AND DISCUSSION

The HOS reacted sperm percentage of paired (right and left) cauda epididymal spermatozoa supplemented with different concentrations of coconut water in tris egg yolk citrate extender was monitored in all the groups at different time intervals and presented in Table 1.

The HOS reacted sperm count observed at 0 hour differed significantly ($p<0.001$) between the various groups however, found lower in T1 as compared to other groups. Similarly, at 12, 24, 36 and 48 hours post- chilling, it also differed significantly ($p<0.05$, $p<0.01$ & $p<0.001$) among the various groups but found lower in T1 as compared to other groups. The overall mean value of HOS reacted sperm count irrespective of various groups were reduced with increased preservation time as 81.69±2.51, 77.68±2.58, 72.62±2.75, 69.39±2.79 and 63.45±3.09 per cent at 0, 12, 24, 36 and 48 hours, respectively.

Moreover, in T1 group, the HOS reacted sperm count was found non-significantly higher at 0 as compared to 12 hour but significantly ($p<0.001$) higher as compared to 24, 36 and 48 hours post- chilling. However, in T2 group, it was found significantly ($p<0.001$) higher at 0 as compared to 12, 24, 36 and 48 hours post-freezing. Again, in T3 group, it was observed non-significantly higher at 0 as compared to 12 and significantly ($p<0.001$) higher as compared to 24, 36 and 48 hours post- chilling. Further, in

Table 1

Effect of different concentrations of coconut water in tris egg yolk citrate extender and storage duration on HOST reacted sperm count (%) of paired cauda epididymal buck spermatozoa preserved at refrigeration temperature (Mean ±SE)

Groups	HOST reacted sperm count (%) (n=20)					Overall (n=50)	F value	P value
	0 hr	12 hr	24 hr	36 hr	48 hr			
T1	$76.25\pm2.73^{b}_{x}$	$72.85\pm2.94^{b}_{x}$	$64.25\pm2.68^{b}_{y}$	$62.20\pm2.84^{b}_{y}$	$54.05\pm2.70^{b}_{z}$	65.92±3.31	25.87***	0.000
T2	$83.45\pm2.40^{a}_{w}$	$79.45\pm2.36^{a}_{x}$	$74.45\pm2.50^{a}_{y}$	$70.85\pm2.62^{a}_{y}$	$63.95\pm2.67^{a}_{z}$	74.43±3.03	28.33***	0.000
T3	$82.80\pm2.46^{a}_{w}$	$78.45\pm2.60^{a}_{wx}$	$74.95\pm2.40^{a}_{xy}$	$70.65\pm2.60^{a}_{yz}$	$66.55\pm3.02^{a}_{z}$	74.68±2.99	16.57***	0.000
T4	$83.05\pm2.13^{a}_{w}$	$78.70\pm2.12^{a}_{x}$	$74.40\pm2.58^{a}_{y}$	$71.60\pm2.45^{a}_{yz}$	$68.05\pm3.05^{a}_{z}$	75.16±2.87	16.51***	0.000
T5	$82.90\pm2.17^{a}_{w}$	$78.95\pm2.28^{a}_{wx}$	$75.05\pm2.48^{a}_{xy}$	$71.65\pm2.67^{a}_{y}$	$64.65\pm2.93^{a}_{z}$	74.64±2.99	22.96***	0.000
Overall (n=50)	81.69±2.51	77.68±2.58	72.62±2.75	69.39±2.79	63.45±3.09	—	—	—
F value	5.48***	3.72**	10.59***	6.63***	8.66***	—	—	—
P value	0.000	0.007	0.000	0.000	0.000	—	—	—

$^{a-b}$ Means with different superscript within a column (between the groups) differs significantly at $p<0.01$ and $p<0.001$.

$_{w-z}$ Means with different subscript between a column (between time intervals) differs significantly at $p<0.001$.

***Significant at $p<0.001$; **Significant at $p < 0.01$.

T1-Control; T2- Tris CW 5%; T3-Tris CW 10%; T4-Tris CW 15%; T5-Tris CW 20%

T4 group, it was found significantly ($p<0.001$) higher at 0 as compared to 12, 24, 36 and 48 hours post-freezing. However, in T5 group, it was observed non-significantly higher at 0 as compared to 12 hours but significantly ($p<0.001$) higher as compared to 24, 36 and 48 hours post freezing.

The HOS reacted sperm count in various treatment groups as compared to control were higher at all the time intervals with significant differences and it was also noted that the HOS reacted sperm count in all the groups revealed a waning trend with increasing preservation time.

The overall mean values of HOS reacted sperm count irrespective of preservation time was found lower in T1 (65.92 ± 3.31 %) as compared to T2 (74.43 ± 3.03 %), T3 (74.68 ± 2.99 %), T4 (75.16 ± 2.87 %) and T5 (74.64 ± 2.99 %) groups with highest value in T4 group.

Like our findings, Daramola *et al.* (2016) also reported significantly higher HOS reacted sperm against control (84.0 ± 1041 vs 64.0 ± 2.31 %; $p<0.05$) in West African Dawrf (WAD) buck spermatozoa. However, they observed the best improvements in HOS reacted sperm count at 10% coconut water. Similarly, Silva *et al*. (2011) reported, higher HOS reacted sperm count in powdered coconut water ACP-109c (40.1 ± 4.5 %) as compared to TRIS extender (38.1 ± 4.5 %) in cryopreserved epididymal sperm of agouti. Likewise, Luzardo *et al.* (2010) reported significantly higher percentage of HOS reacted sperm count in deionized coconut water based extender (55.26 ± 1.78 %) as compared to control (51.39 ± 1.46%) and natural coconut water based extender (41.57 ± 1.50 %) in frozen boar semen.

However, El-Sheshtawy *et al.* (2017) reported the highly significant percentage of HOS reacted sperms in 4 % coconut water based extender (76.25 ± 0.59%) as compared to control, 6% and 8% coconut water based extender (65.00 ± 1.10, 61.63 ± 0.63 and 70.88 ± 1.14 %) in freeze-thawed cattle semen which was somewhat diverse from the findings of the present study where, we found higher HOST reacted sperm count at 15% concentration of coconut water in tris egg yolk citrate extender.

In conclusion, the percentage of HOS reacted sperm count found in control was lower as compared to all supplemented groups. Further, the highest percentage of HOS reacted sperm count was found in 15% coconut water supplemented group proves its beneficial effects on maintaining the functional integrity of plasma membrane in cauda epididymal buck spermatozoa preserved at refrigeration temperature.

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