EFFECT OF ANTIOXIDANT ADDITIVES ON FREEZABILITY OF BUFFALO SPERMATOZOA

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ABSTRACT

The cryopreservation induced sperm damage in buffalo is one of the hurdles responsible for its poor freezability and fertility. Therefore, the Present study was planned to understand the role of antioxidant additives on freezability of buffalo spermatozoa. Twenty four ejaculates were collected having mass motility ≥ +3 from 4 Murrah bulls (6 from each bull). Each ejaculate was divided into four groups *viz*., Group 1 as Control containing Tris-egg yolk-glycerol extender, Group 2 containing Pentoxiphylline 3.6 mM, Group 3 containing Theophylline 10 mM, Group 4 containing Theobromine 10 mM, Group 5 containing N-propyl gallate 15 µM and finally diluted upto 80×10^6 sperm/ml. Ejaculates were evaluated after freezing for seminal attribute *viz*., individual motility, live sperm, acrosomal integrity (AI) and hypo-osmotic swelling test (HOST). One way ANOVA was used to analyse the data. Significantly ($P<0.05$) higher sperm motility was observed in semen samples treated with pentoxiphylline in comparison to control, theophylline and theobromine and propyl gallate treated semen. At day 0, 7 and 30 days, semen treated with pentoxiphylline had significantly ($P<0.05$) higher percentage of viable spermatozoa as compared to theophylline and theobromine and propyl gallate treated semen. HOST and AI was significantly ($P<0.05$) higher in theophylline, theobromine and propyl gallate treated semen, however, among additive treated groups, HOST and AI were significantly ($P<0.05$) higher in pentoxiphylline treated semen. In conclusion among various antioxidant additives, pentoxyphylline addition 3.6 mM significantly improved semen quality of buffalo bulls.

Keywords: *Bubalus bubalis*, buffaloes, pentoxiphylline, theophylline, theobromine, N-propyl gallate, buffalo spermatozoa

INTRODUCTION

Artificial insemination (AI) technology using cryopreserved semen has played an instrumental role in the continuous progress of genetic improvement in livestock. In buffalo artificial insemination has been practiced last four to five decades but still conception rate is lower when compared with the cattle (Anzar *et al*., 2003; Barile, 2012). Many factors are responsible for lower conception rate in buffalo, but damage of sperm membrane during cryopreservation is one

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of the major challenges to achieve the success in buffalo AI (Watson, 1995). Higher proneness of buffalo spermatozoa in comparison to cattle sperm during cryopreservation is due to the variation of lipid content of sperm plasma membrane (Raizada *et al*., 1990; Tatham, 2000). Besides that during freeze-thaw process osmotic shock, intracellular ice crystals formation, and cold shock are also responsible for sperm damage (Watson, 1995) and these factors may also responsible for change in cell volume and osmotic tolerance of sperm (Gilmore *et al*., 1998). Abundance of polyunsaturated fatty acids (PUFA) in sperm plasma membrane is responsible for Reactive oxygen species production during cryopreservation process (Lone *et al*., 2016a; Balamurugan *et al*., 2017), lead to the lipid peroxidation of sperm plasma membranes, DNA damage of sperm (Lone *et al*., 2017), reduced antioxidant profile (Lone *et al*., 2016b; Lone *et al*., 2016c), which in turn result in reduced motility, viability and fertility of spermatozoa (Chatterjee and Gagnon, 2001). To overcome the problem researchers have tried various additives i.e. Butylated Hydroxy Toluene (BHT), Pentoxifylline (PTX) and α-tocopherol (Bhakat *et al*., 2011), glutathione (Shah *et al*., 2017), cholesterol loaded cyclodextrin (Lone *et al*., 2016a; Yadav *et al*., 2017) theobromine (Pankaj *et al*., 2009), trehalose (El-Sisy *et al*., 2016), and taurine (Reddy *et al*., 2010), in semen during cryopreservation process. Pentoxifyllin (PTX) is a derivative of methylxanthine and has been considered as an enhancer of sperm motility (Said *et al*., 2010; Nabi *et al*., 2017), hyperactivation (Tesarik *et al*., 1992), free radical scavenger (Zini *et al*., 2001) and acrosome reaction enhancer (Woolley and Richardson, 1978). PTX acts as inhibitor of cyclic adenosine monophosphate (cAMP) phosphodiesterase enzyme and increase the intracellular cAMP levels, which help in regulation of spermatozoa respiration, motility, and the acrosome reaction (Safarinejad, 2011). It has been reported that as compared to vitamin E and vitamin C, N-Propyl gallate has been found superior due to both antimicrobial and antioxidant activity (Rao *et al*., 2013). Therefore, the study was planned to compare the effect of various antioxidant addtives such as Pentoxifylline, Theophylline, Theobromine and N-propyl gallate on freezability of buffalo sperm after various days of cryopreservation.

MATERIALS AND METHODS

Experimental design

Semen of four Murrah buffalo bulls (4 to 6 years) was collected at Artificial Breeding Research Centre (ABRC), ICAR-National Dairy Research Institute, Karnal, Haryana, India. The bulls were maintained under standard and uniform management condition during the study period.

Semen collection

The semen was collected by Artificial Vagina twice in a week using standard procedure followed in the semen station during morning hours. Total 24 Ejaculates from 4 bulls (6 ejaculate from each bull) were selected. The semen samples were evaluated for Mass activity and individual progressive motility immediately after semen collection and the sample having +3 and above mass activity as well as 70% and above individual progressive motility were selected for further experiment.

Semen processing and preservation

Immediately after ejaculate collection,

it was split into five equal groups *viz*., Group 1 (Control), Group 2 (Pentoxiphylline 3.6 mM), Group 3 (Theophylline 10 mM), Group 4 (Theobromine 10 mM) and Group 5 (N-propyl gallate 15 µM). All aliquots were diluted with Tris-egg yolk-glycerol dilutor upto 80×10^6 sperm/ml. 20 million motile spermatozoa was packed in French mini (0.25 ml) straws and kept for 4 h equilibration at 5°C in racks. Then automatic freezing was carried out using biological cell freezer 5°C per minute for 4 to 10°C; 40°C per minute for -10 to -100°C and 20°C per minute for -100 to -140°C after transferring the rack along with the straws. Finally the straws were dipped in liquid nitrogen (-196°C) and stored till further assessment. Frozen semen was evaluated at 0, 7 and 30 days after cryopreservation for different seminal attributes such as individual motility, viability, acrosomal integrity and hypo-osmotic swelling test.

Semen analysis

After thawing the semen samples were evaluated for Individual motility using phase contrast microscope, live sperm percentage using Eosin-Nigrosin stain (Campbell *et al*., 1953), acrosomal intactness using Giemsa stain (Watson, 1975) and Hypo-osmotic swelling test (HOST) as depicted by Jeyendran *et al.* (1984) at 0, 7 and 30 days after cryopreservation.

Statistical analysis

One way ANOVA was used for data analysis using version 9.3 Statistical Analysis System (SAS, 2011) Software Programme. The results were presented in the table as Mean±SE.

RESULTS AND DISCUSSION

The results of seminal profile after addition of various additives and evaluated on various days after cryopreservation has been presented in Table 1. Significantly ($P<0.05$) higher sperm motility was observed in Pentoxiphylline, Theophylline and Theobromine treated semen in comparison to control (Group 1) and N-propyl gallate treated semen. However, at 0, 7 and 30 days of cryopreservation, highest sperm motility was observed in Theophylline treated group among additive containing groups. Semen fortified with additives such as Pentoxiphylline, Theophylline and Theobromine had significantly ($P<0.05$) higher viability compared to untreated semen (control). At day 0, 7 and 30 days, semen treated with Pentoxiphylline had significantly ($P<0.05$) higher percentage of viable spermatozoa compared to control, Theophylline, Theobromine and N-propyl gallate treated semen. Hypo-osmotic swelling test positive sperm was significantly ($P<0.05$) higher in Pentoxiphylline treated semen compared to control, Theophylline, Theobromine and N-propyl gallate treated semen at 3, 7 and 30 days of cryopreservation. Among the additive treated groups, highest HOS response was recorded in Pentoxiphylline and least in N-propyl gallate treated semen. In comparison of control; Pentoxiphylline, Theophylline and Theobromine treated semen showed significantly ($P<0.05$) higher intact acrosome, whereas among additive treated groups, Theophylline treated semen showed significantly ($P<0.05$) higher percentage of Acrosomal integrity.

In similar line Pankaj *et al.* (2009) reported enhanced sperm motility, viability, acrosomal integrity and HOST in semen treated with Pentoxyphylline, Theophylline or

Table 1. Effect of antioxidant additives on seminal attributes (%) after various days of cryopreservation.

Seminal attribute (%)	Period	Group I (Control)	Group II (Pentoxiphylline 3.6 mM)	Group III (Theophylline 10 mM)	Group IV (Theobromine 10 mM)	Group V (N-propyl gallate 15 µM)
Individual motility	**0 day**	$39.25^{d}\pm0.21$	$47.64^{a}\pm0.27$	$42.69^{c}\pm0.25$	$43.71^{b}\pm0.25$	$41.17^{d}\pm0.19$
	7 day	$37.05^{e}\pm0.28$	$44.75^{a}\pm0.22$	$40.28^{c}\pm0.22$	$42.27^{b}\pm0.24$	$38.91^{d}\pm0.22$
	30 day	$35.35^{d}\pm0.21$	$41.75^{a}\pm0.27$	$37.45^{c}\pm0.19$	$39.42^{b}\pm0.26$	$37.25^{c}\pm0.62$
Viability	**0 day**	$48.26^{d}\pm0.22$	$52.31^{a}\pm0.29$	$51.37^{b}\pm0.28$	$51.34^{b}\pm0.29$	$50.55^{c}\pm0.31$
	7 day	$46.94^{d}\pm0.16$	$48.88^{a}\pm0.24$	$48.73^{b}\pm0.17$	$48.67^{b}\pm0.23$	$47.46^{c}\pm0.22$
	30 day	$45.21^{d}\pm0.24$	$46.28^{a}\pm0.28$	$45.98^{c}\pm0.29$	$46.10^{b}\pm0.29$	$45.78^{c}\pm0.31$
HOS Response	**0 day**	$40.40^{e}\pm0.48$	$47.39^{a}\pm0.29$	$43.39^{c}\pm0.70$	$45.10^{b}\pm0.41$	$41.84^{d}\pm0.49$
	7 day	$37.79^{e}\pm0.23$	$44.26^{a}\pm0.23$	$40.81^{c}\pm0.33$	$41.55^{b}\pm0.24$	$39.37^{d}\pm0.23$
	30 day	$36.50^{e}\pm0.49$	$41.57^{a}\pm0.30$	$38.26^{c}\pm0.72$	$40.08^{b}\pm0.42$	$37.42^{d}\pm0.50$
Acrosomal integrity	**0 day**	$52.90^{e}\pm0.37$	$60.07^{a}\pm0.22$	$56.42^{c}\pm0.34$	$57.65^{b}\pm0.24$	$53.86^{d}\pm0.42$
	7 day	$48.98^{e}\pm0.37$	$56.46^{a}\pm0.27$	$52.23^{c}\pm0.22$	$53.14^{b}\pm0.25$	$50.42^{d}\pm0.22$
	30 day	$47.10^{e}\pm0.21$	$52.81^{a}\pm0.22$	$49.87^{c}\pm0.34$	$51.48^{b}\pm0.24$	$48.68^{d}\pm0.42$

Means bearing different superscripts within the same row differ significantly ($P<0.05$).

Theobromine during refrigeration at 0, 4, 8, 12 and 24 h. Due to abundant amounts of PUFA in buffalo sperm plasma membrane (Tatham, 2000), they become more prone to damages induced by free radicals leading to oxidative stress (LPO) and consequently impairment of various sperm functions such as sperm motility, integrity of membrane and fertility (Alvarez and Storey, 1989; Aitken *et al*., 1994). Pentoxifylline (PTX) acts as phosphodiesterase inhibitor and prevents cyclic adenosine monophosaphate (cAMP) breakdown (Tash, 1976). In spermatozoa, protein tyrosine phosphorylation play an important role in modulation of capacitation like events and it is regulated by protein kinase (PKA), which is activated by cAMP (Naz and Rajesh, 2004). It is well evident that in human assisted reproductive technology to enhance fertility, PTX is routinely used to increase motility of non motile and ejaculated sperms (Kovacic *et al*., 2006). PTX has been found effective to improve motility of equine epididymal spermatozoa without any deleterious effects on viability and tyrosine phosphorylation (Gausti *et al*., 2017). PTX has been found to enhance the sperm quality of stallion semen on chilled and cryopreserved condition (Goulart *et al*., 2004; Stephens *et al*., 2013). Similarly improvement of post-thaw sperm motility, viability, acrosomal integrity and membrane integrity in PTX treated buffalo semen was observed in our study. Besides PTX, Theophylline and Theobromine also improved the post thaw sperm quality traits. The enhanced semen quality by Theophylline and Theobromine may be due to improved seminal antioxidant profile and reduced production of ROS and oxidative stress.

CONCLUSION

It is concluded that fortification of buffalo semen with pentoxyphylline, Theophylline and Theobromine significantly enhances its sperm motility, live sperm percentage, AI and HOST when compared to control and other methylxanthines during cryopreservation of buffalo semen. Therefore, additives can be incorporated in routine freezing protocol of Murrah buffalo semen to improve quality.

ACKNOWLEDGEMENT

Director and Vice-Chancellor of ICAR-National Dairy Research Institute, Karnal has been highly acknowledged for providing the research facilities and financial assistance and senior research fellowship for Ph.D. program to the first author awarded by Indian Council of Agricultural Research.

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