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Research Article

ENRICHMENT OF SEMEN EXTENDER WITH ADENOSINE CYCLIC 3', 5'-MONOPHOSPHATE AS A TOOL TO ENHANCE BUFFALO SPERM QUALITY

SUKHBIR SINGH, MEENAKSHI VIRMANI*, PRADEEP SINGH and R.K. MALIK
Department of Veterinary Physiology and Biochemistry, College of Veterinary Sciences,
Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125004, Haryana, India

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ABSTRACT

The present study was designed to explore the effect of Adenosine cyclic 3', 5'-monophosphate (cAMP) on survivability and acrosomal integrity of frozen-thawed semen of mature Murrah buffalo-bulls. A total of 30 semen ejaculates from six Murrah buffalo bulls were used and each sample was further divided into 5 groups. Group C was kept as control, containing semen mixed with TEG extender only and no cAMP was mixed, whereas groups C1, C2, C3 and C4 contained semen mixed with TEG extender supplemented with cAMP to make the concentrations of 2.5, 5.0, 7.5 and 10.0 mM, respectively. The frozen straws were thawed and semen was incubated at 37º C for 3 hours. Evaluation of semen sample was done for progressive sperm motility after each hour of incubation and for other seminal parameters after 1 and 3 hours of incubation. On analysis, it was found that addition of cAMP at 5mM concentration in C2 group significantly maintained higher sperm motility, sperm livability and sperm with percent intact acrosomes as well as significantly decreased abnormal spermatozoa. Thus, it can be concluded that addition of cAMP at a concentration of 5 mM can be used to enhance the quality of Murrah buffalo-bull semen for further use in artificial insemination.

Keywords: Abnormal spermatozoa, cAMP, intact acrosomes, live spermatozoa, progressive sperm motility

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Buffalo plays an essential role in milk production in India and has been referred as unique domesticated animal due to its ability to adapt in the harsh tropical surroundings, resistance to diseases and competence to convert poor quality roughage to milk. Most of the research investigations have been targeted to problems related to female animals. The male has received restricted scientific and medical attention. Inferior seminal quality in buffalo bulls in terms of low post-thaw survival of buffalo spermatozoa has been reported (Anzar *et al.*, 2010) that has placed limitations on wide and successful use of tools for genetic improvement like artificial insemination and increasing calf crop in this species. Incorporation of various additives in the extender for improving the keeping quality, freezability, fertility and motility of preserved buffalo-bull spermatozoa has been the subject of study in the last few years.

cAMP signaling pathway has been related with the regulation of sperm motility in numerous animal species including mammals (Tash and Bracho, 1994). It appears that energy is generated utilizing cAMP inside the sperm that is required for sperm motility. Intracellular cAMP level of sperm is responsible for hyper activated motility, a movement pattern of mammalian spermatozoa characterized by asymmetrical, high amplitude beats of flagellum and capacitation. cAMP activates cAMP dependent protein kinase and catalyzes the phosphorylation of sperm proteins, thereby stimulating the sperm motility as well as oxygen intake of ejaculated bovine spermatozoa (Miro-Moran *et al.*, 2012 and Marechal *et al.*, 2017). Thus, to ensure sperm fertilizing ability, maintenance of higher cAMP levels is necessary.

Buffalo seminal plasma is poor in cAMP and assumed to have conceited inhibitors of spermatozoal motility (Singh and Raina, 2000). From the literature, it seems that very little work has been accomplished on addition of cAMP in buffalo semen. Therefore, it was considered worth to take up studies on addition of cAMP in semen of buffalo-bulls and study the usefulness of this motility stimulator in maintaining the survivability of buffalo spermatozoa.

MATERIALS AND METHODS

Semen collection and processing: A total of 30 semen ejaculates were collected from six mature buffalo bulls with the help of sterilized artificial vagina twice weekly. The semen samples were assessed for various seminal parameters *viz*., volume, sperm concentration and percentage of motile spermatozoa. Sperm motility was subjectively assessed under a phase-contrast microscope equipped with a warm stage (37º C) at 200X magnification and only ejaculates with ≥ 70 % sperm motility was used for cryopreservation. Semen was diluted with TEG extender to make the sperm concentration 80 million/ml and divided into five groups *viz*. Group C, C1, C2, C3 and C4. Group C was kept as control and groups C1, C2, C3 and C4 were supplemented with increasing concentrations of cAMP (2.5, 5.0, 7.5 and 10.0mM, respectively) in order to standardize suitable concentration required for optimum

*Corresponding author: virmanim2003@yahoo.com

activity without any ill-effect on semen quality. The semen samples of all the groups were cryo preserved. After 24 hours, the straws of frozen semen were thawed in water bath at 37ºC for 30 seconds. Thawed semen was transferred to eppendorf tubes kept in a water bath maintained at 37ºC for three hours. Semen from each tube was evaluated for progressive sperm motility after each hour of incubation for three hours and for live and dead spermatozoa, abnormal sperms and spermatozoa acrosomal integrity after 1 and 3 hours of incubation.

Spermatozoa motility: Percentage of motility was evaluated under a phase-contrast microscope at 200X as previously described by Asr *et al.* (2011). Progressive motility was estimated on a continuous scale of 0 to 100%.

Spermatozoa viability: A 50 µl drop of the thawed semen mixed with 50 µl of eosin-nigrosin stain (composed of 5% w/v nigrosine, 0.6% w/v eosine yellow and 3% sodium citrate dihydrate, filtered and pH adjusted to 7.0) was placed on a pre-warmed (37°C) glass slide (Virmani *et al.*, 2020). A thin uniform smear was prepared with the help of another microscopic slide. The smear was air dried and evaluated under a phase contrast microscope (X1000). The heads of two hundred spermatozoa were screened to find out viable spermatozoa (%). The spermatozoa with stained or partially stained heads were considered as dead spermatozoa. Whereas, the spermatozoa with unstained heads were counted as viable spermatozoa.

Spermatozoa acrosomal integrity: A thin smear of extended semen was prepared on a clean and sterilized glass slide. The smear was dried on warm stage (37º C) for 2-3 minutes and fixed in neutral formalin saline solution for 15 minutes followed by rinsing slowly with running tap water. The prepared smear was stained for 40 minutes in the working Giemsa stain solution (Singh *et al.*, 2020). Abnormalities in spermatozoa such as ruffled, swollen or absent acrosome were counted as damaged/abnormal apical ridge.

Statistical analysis: The means and standard errors, multivariate analysis of variance, Duncan's Multiple Range Test for means and linear product moment of biological parameters were calculated, using SPSS/PC student ware computer software (Norusis, 1988).

RESULTS AND DISCUSSION

The results of present investigation are summarized in Table 1. There was relative increase in the mean progressive sperm motility (Mean±SE) in samples containing cAMP as compared to control semen samples. However, this increase was not linear and the increase in sperm motility in the samples containing cAMP at a concentration of 5 mM (group C2) was significantly higher ($P<0.05$) in comparison to groups containing other concentrations of cAMP as well as control samples up to 3 hours of incubation. The findings of present study is in corroboration with the observations of Maxwell *et al.* (1995). They reported better motility of spermatozoa with lower concentration of cAMP in semen upto 6 hours of thawing in ram semen. cAMP, whether added directly in the semen or its enhancement through addition of caffeine, plays an imperative role in preservation of the quality of frozen buffalo spermatozoa (Singh and Raina, 2000). Vijayaraghavan and Hoskin (1986) also reported stimulation of motility of mature sperm and induction of motility in immature caput sperm at relatively low concentrations (20-300 µM) under appropriate conditions. However, Gil *et al.* (2010) reported reduction of post thaw motility of boar spermatozoa at higher concentrations of pentoxiphylline which might be due to elevated cAMP concentration that in turn interferes with the activity of cAMP associated ion channels in the plasma membrane.

The percent live spermatozoa (Mean±SE) in Murrah buffalo bull semen were observed to be significantly higher in group C1 and C2 i.e. with the addition of cAMP at a concentration of 2.5 and 5.0 mM in semen, respectively after first hour of incubation at 37ºC, in comparison to control and other supplemented groups. However, after third hour of incubation, percent live sperms were significantly ($P<0.05$) higher in the C2 semen samples as compared to control and other groups. More percent live spermatozoa at a concentration of 5mM cAMP might be due to the reason that cAMP works in a dose dependent manner and converts inactive form of glycogen phosphorylase into its active form through breaking down glycogen to simple sugar that might be accountable for the longer preservation of buffalo semen (Lehninger, 1987). The results are in accordance with the results of the studies reported by Pereira *et al.* (2019) in boar semen, De Turner *et al.* (1978) in human semen and Lardy *et al.* (1971) in fresh and frozen thawed semen of bulls.

The mean of percent abnormal spermatozoa in Murrah buffalo bull semen were significantly lower ($P<0.05$) in cAMP supplemented groups as compared to control. This is probably due to the reason that cAMP imparts protective effect on the sperm membrane, however no report is available in buffalo bull semen to validate this finding. There was significant increase in the number of spermatozoa with intact acrosome on supplementation of semen samples with cAMP. Highest intact acrosomes (67.20±1.09) were obtained in C2 group containing 5mM cAMP followed by C1, C3, C4 and C groups, respectively.

Table 1. Effect of different concentrations of cAMP on seminal parameters of frozen-thawed semen of Murrah buffalo-bulls after incubation at 37° C

Groups → Seminal Attributes ↓	Incubation time	C	C1	C2	C3	C4
Progressive motility (%)	I hr	$31.66^{d}\pm1.42$	$38.00^{ab}\pm0.92$	$41.00^{a}\pm1.00$	$36.33^{bc}\pm0.98$	$33.66^{cd}\pm1.12$
	II hr	$18.83^{d}\pm1.39$	$30.00^{b}\pm0.98$	$33.83^{a}\pm1.03$	$26.16^{c}\pm0.94$	$24.00^{c}\pm0.91$
	III hr	$8.33^{d}\pm0.55$	$17.33^{ab}\pm1,16$	$19.66^{a}\pm1.12$	$15.50^{bc}\pm1.15$	$13.66^{c}\pm1.14$
Viability (%)	I hr	$53.10^{c}\pm1.24$	$62.50^{a}\pm1.14$	$65.70^{a}\pm1.36$	$58.60^{b}\pm1.27$	$54.80^{c}\pm1.40$
	III hr	$22.76^{d}\pm1.00$	$37.63^{b}\pm1.33$	$41.33^{a}\pm1.28$	$31.33^{c}\pm1.44$	$29.40^{c}\pm1.39$
Abnormality (%)	I hr	$15.10^{a}\pm0.50$	$13.40^{b}\pm0.48$	$11.43^{c}\pm0.44$	$12.26^{bc}\pm0.43$	$12.33^{bc}\pm0.42$
	III hr	$14.93^{a}\pm0.40$	$13.20^{b}\pm0.42$	$11.23^{d}\pm0.43$	$11.66^{cd}\pm0.45$	$12.50^{bc}\pm0.53$
Acrosomal Integrity (%)	I hr	$67.23^{d}\pm0.90$	$78.30^{b}\pm0.81$	$81.80^{a}\pm0.87$	$73.40^{c}\pm0.95$	$69.33^{d}\pm1.11$
	III hr	$47.56^{d}\pm1.73$	$62.16^{b}\pm1.23$	$67.20^{a}\pm1.09$	$55.56^{c}\pm1.49$	$50.33^{d}\pm1.51$

a, b, c, d Means in a row with different superscripts are significantly different (P<0.05).

Table 2. Correlation coefficient among different seminal attributes of Murrah buffalo-bull frozen thawed semen

Seminal Attributes	Viability	Abnormality	Acrosomal Integrity
Progressive motility	0.91**	-0.09NS	0.68**
Viability		-0.10NS	0.72**
Abnormality			-0.09NS

** Significant at p<0.01; * Significant at p<0.05; NS- Non-Significant

The results are in agreement with Singh and Raina (2000) who reported that buffalo semen samples containing 4mM cAMP showed least damaged acrosomes during freezing as compared to semen samples containing other additives *viz*. cattle seminal plasma and caffeine. cAMP possesses a role in sperm kinematics as well as in the acrosome reaction as a second messenger system (Esteves *et al.*, 2007), thereby plays a significant role in preservation and maintaining the quality of buffalo semen. Maxwell *et al.* (1995) also observed significantly higher spermatozoa with intact acrosome in post thaw ram semen treated with cAMP.

Correlations among different seminal parameters of Murrah buffalo-bulls semen after addition of cAMP are presented in table 2. It was found that progressive sperm motility was highly significantly and positively correlated with live sperms (P<0.01) and sperms with intact acrosomes (P<0.01). Both the progressive sperm motility and live sperms were non-significantly and negatively correlated with abnormal sperms. Live spermatozoa were highly significantly and positively correlated with intact acrosomes. Abnormal sperms were non-significantly and negatively correlated with intact acrosomes. The sperm plasma membrane and acrosome integrity are affected as a result of cryopreservation, further causing disturbed regulation of Ca level in the cells, which may be detrimental to sperm motility (Aitken *et al.*, 1983). Increased cAMP concentration might cause efflux of calcium through the cAMP associated outwardly directed Ca pump and the mechanism might have worked more efficiently in the diluted semen, since the Tris egg yolk glycerol extender does not contain calcium and the steep concentration gradient might have resulted in Ca efflux (Peterson *et al.*, 1979).

CONCLUSION

Results of the present investigation concluded that addition of cAMP at concentration of 5mM maintains higher sperm motility, livability and more spermatozoa with intact acrosomes up to three hours of incubation of frozen-thawed semen of Murrah buffalo-bulls. However, further studies on in vivo validation of supplemented semen are warranted to substantiate the findings related to improvement in quality of sperms.

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