Research article

DOI: 10.48165/ijar.2023.44.02.12

The Indian Journal of Animal Reproduction

The official journal of the Indian Society
for Study of Animal Reproduction

ACS Publisher
www.acspublisher.com

Year 2023, Volume-44, Issue-2 (December)

ISSN 0970-2997 ***(Print)*** *ISSN 2583-7583* ***(Online)***

Effect of Adenosine 5′ Triphosphate (ATP) and Incubation Time on Frozen Thawed Buffalo Bull Spermatozoa

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ABSTRACT

The present study was aimed to determine the effect of ATP dose rate on seminal attributes of frozen-thawed buffalo bull's semen. For this purpose, 20 frozen semen straws of four different buffalo bulls were obtained from a private semen production unit (Al-Hiawan Sires, Sahiwal). The experiment was replicated four times. In each replicate, four frozen straws of the same bulls were thawed and pooled. After evaluating the motility, the pooled frozen-thawed sample was aliquoted into three separate tubes containing 0 (control), 1-, or 2-mM concentrations of ATP. Afterward, each aliquot was incubated at 37°C for 4 hrs. The sperm motility, viability, and membrane integrity were observed at various time points (10 min., 1hr, 2hr, and 4hr) during the incubation period of four hrs. It was observed that progressive motility and most of the motion kinematic parameters were greater ($P<0.05$) in control. A significant ($P<0.05$) interaction was observed between ATP dose and incubation time. The progressive motility, total motility, VCL, VAP, ALH, and BCF decreased ($P<0.05$) both by incorporation of ATP (1mM or 2mM) and increasing incubation time (from 10 min to 4hrs) which indicate the ATP and incubation time of 4 hours negatively affect the motion kinetics of buffalo bull semen. In conclusion, it was witnessed that the motility and plasma membrane integrity of sperm declined by the supplementation of ATP in frozen-thawed buffalo bull semen.

Key words: Buffalo bull, ATP, Sperm, Post-thaw.

How to cite :- Hanif, M., Ahmad, E., Naseer, Z., Akhtar, M. S., Ahmad, T., Hussain, T., & Murtaza, S. (2023). Effect of Adenosine 5′ Triphosphate (ATP) and Incubation Time on Frozen Thawed Buffalo Bull Spermatozoa. *The Indian Journal of Animal Reproduction, 44*(2), 64–68. 10.48165/ijar.2023.44.02.12

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Received 30-11-2023; Accepted 18-12-2023

INTRODUCTION

It is well understood that using sperm from superior bulls through artificial insemination at a large scale could be the only option to improve the genetic potential of livestock. However, the artificial insemination remained unsuccessful in breed improvement of buffaloes in Pakistan. The low conception rate in buffaloes with artificial insemination by using frozen-thawed semen discourages the farmer from getting the full benefit of this biotechnology for breed improvement. The reason for the decreased conception rate might be the shorter life span of frozen-thawed sperm in the female's reproductive tract after insemination. It is well documented that spermatozoa endure various stresses during glycerolization, equilibration, cooling, freezing, and thawing process. It sperm loses its motility due to the depletion of ATP contents during the freezing and thawing processes. The sperm motility is affected by many intrinsic and extrinsic factors which include temperature, moisture, pH, and changes in incubation timing. Intracellular adenosine 5'-triphosphate (ATP) is the primary energy source for sperm to accomplish motility inside the female reproductive tract, hyperactivation, and oocyte penetration (Peña *et al.,* 2009). The depletion of ATP during cryofreezing and post thaw may lead to decrease in flagellar wave, which has been recommended as an indicator of energy preserved in living cells.(Atkinson and Walton, 1967). The decrease in AMP (adenosine 3'5'-cyclic monophosphate) cause reduction in motility (Tash and Mann, 1973). The cyclic spermatic AMP would be deeply involved in spermatic function control (Tash and Means, 1988), contributing to ATP formation and increasing sperm motility and vigor. Intracellular ATP produced in mitochondria is the major source of energy for sperm. Therefore, the intactness of mitochondrial membrane must be ensured to keep the sperm motile after freezing and thawing procedure (Luria *et al.,* 2002).

The previous study in cat sperm revealed that the ATP supplementation in freezing media has markedly promoted the intracellular sperm ATP level as well as post- thaw sperm characteristics (Thuwanut *et al.,* 2015). In this background the objective of the present study was to determine the effect of ATP on motility, viability, and membrane integrity of frozen-thawed buffalo bull's sperm during the incubation period of 4 hrs at 37°C.

MATERIALS AND METHODS

Experimental Design:

The experiment was replicated four times. In each replicate four frozen straws of the same bull were thawed at 37 °C. The motility of each thawed sample was assessed separately. Then the samples with ≥ 40 % progressive motility were pooled and split into three equal aliquots. The aliquots were treated with ATP 0 (control), 1, or 2mM concentrations. Afterward samples were incubated at 37°C for 4 hrs. The sperm motility, viability, and membrane integrity were observed at various time points (10 min., 1hrs, 2hrs, 3hrs, and 4hrs) during the incubation period of four hrs.

Motility assessment:

The percentage of sperm motility was assessed by computer-assisted sperm analyzer (CASA) equipped with a heated stage adjusted at 37°C. Briefly, a drop (5µL) of semen was placed on a pre-warmed glass slide (37°C), covered with glass slip (18 × 18 mm) and observed by CASA at ×100 magnification (-ve phase contrast). The motion kinematics such as progressive motility (PM, %), total motility (TM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), oscillation index (WOB), the amplitude of lateral displacement (ALH, µm) and beat cross frequency (BCF, Hz); and were observed.

Viability assessment:

The sperm viability was assessed by using Eosin-Nigrosine staining. Briefly, a portion (one drop) of Eosin-Nigrosine stain was first taken on a pre-warmed glass-slide and mixed with. a drop of pooled semen. Afterward, a uniform smear was prepared from this mixture, air-dried, and observed under a bright-field microscope at 400x magnification. At least 100 sperm were counted. The sperm with unstained heads were considered alive, whereas the sperm with purple-stained heads were considered dead.

Plasma membrane integrity assessment:

The plasma membrane integrity was observed by using a hypo-osmotic swelling test (HOST) Briefly, 25 µL of semen sample was mixed in 475 µL of hypo-osmotic solution (100 mOsm/L). The samples were then placed in a water bath at 37°C for 15 minutes. Afterward, a drop of semen was placed on a glass slide and a uniform smear was prepared. After air drying the smear, a total of 100 sperm were counted under phase contrast microscope at 400x magnification. The sperm cells that appeared with coiled tails were counted as intact spermatozoa whereas, sperm cells that appeared in straight tails were counted as non-intact spermatozoa.

Table 1: The effects of ATP dose rate and incubation time (4 hrs) on motion kinetics of frozen thawed buffalo bull sperm. The values are presented as Mean ± SD.

ATP Dose	Incubation time	PM (%)	TM (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Control	10 min	65.1±7.2	95.8±2.6	74.7±2.8	39.7±8.4	52.9±5.5	45.0±8.5	63.3±5.8	66.1±6.9	2.8±0.4	7.1±0.7
1.mM		46.9±13.2	86.4±6.9	66.3±11.4	36.7±10.6	47.7±10.8	45.0±11.9	63.5±8.4	65.1±8.6	2.4±0.4	6.7±0.3
2.mM		50.5±17.6	89.5±5.9	57.4±13.3	34.2±10.5	42.2±12.0	51.3±11.2	69.7±10.2	69.5±5.8	2.2±0.3	6.8±0.6
Control	1 hr	53.0±4.8	87.8±6.2	77.8±20.7	43.2±27.5	56.1±26.0	44.1±19.0	62.2±15.0	64.3±13.3	2.7±0.1	7.1±1.3
1.mM		43.9±2.2	86.3±6.7	60.3±	31.1±9.7	41.0±8.9	42.0±7.9	62.7±6.2	62.2±5.9	2.4±0.02	6.4±0.7
2.mM		32.7±9.1	72.5±17.0	56.5±6.4	30.2±6.1	39.6±5.4	45.1±10.0	65.1±7.9	64.4±7.1	2.2±0.1	6.5±0.7
Control	2 hr	54.2±10.1	82.6±1.6	82.5±17.6	39.1±10.7	56.5±16.2	41.9±6.9	62.1±4.8	63.2±5.3	2.8±0.3	8.1±1.3
1.mM		30.4±11.1	64.2±13.9	59.3±12.1	30.6±10.2	40.4±11.5	41.9±3.8	64.0±1.8	60.8±4.2	2.3±0.2	6.6±1.2
2.mM		32.4±4.3	79.6±6.4	50.5±6.3	24.5±6.5	32.9±6.4	42.3±7.8	64.8±6.3	62.0±5.4	2.1±0.1	6.1±1.0
Control	3 hr	42.7±12.1	81.4±13.0	64.5±9.4	25.5±4.7	38.5±7.7	34.1±3.3	57.3±2.2	55.7±3.5	2.6±0.2	6.6±0.9
1.mM		30.5±5.8	67.4±9.6	53.5±1.8	25.6±2.6	34.3±3.1	42.5±4.8	65.5±3.0	61.4±3.9	2.2±0.01	6.5±1.1
2.mM		32.1±13.5	73.4±15.2	46.7±4.4	21.4±3.8	28.9±4.2	42.4±6.6	65.3±4.9	61.2±5.0	2.1±0.1	5.6±0.6
Control	4 hr	40.8±11.9	80.5±21.9	61.1±7.1	25.1±2.4	37.0±3.2	34.5±1.7	57.8±1.3	55.8±0.5	2.5±0.3	7.0±0.4
1.mM		32.7±22.3	59.5±30.6	49.7±5.9	25.1±7.7	32.7±5.7	44.9±14.6	67.8±11.8	61.2±10.2	2.0±0.3	6.2±1.6
2.mM		21.9±7.8	66.2±10.0	38.2±2.9	16.5±3.2	22.8±2.8	39.6±10.9	63.8±8.7	58.3±8.2	2.1±0.2	4.6±0.4
P-value		0.004	0.078	0. 000	0.132	0.015	0.869	0.892	0.621	0. 000	0.049

The P-value <0.05 values in same column indicate the significant interaction between ATP dose rate and incubation time. Progressive motility (PM), Total motility (TM), Curvilinear Velocity (VCL), Straight Line Velocity (VSL), Average Path Velocity (VAP), Linearity (LIN,), Straightness (STR), Oscillation index (WOB), Amplitude of Lateral Head displacement (ALH) and Beat Cross Frequency (BCF).

Semen Samples

Frozen semen samples (n=20) of four different buffalo from a private semen production unit of Punjab (Al-Haiwan Sires, Sahiwal) were obtained and transported to the laboratory in a liquid nitrogen container.

Chemicals

The chemicals used in this study were: Adenosine 5′-Triphosphate (ATP), Eosin-Nigrosine stain, Phosphate buffer saline (PBS), Fructose, and Sodium citrate. All of these chemicals were of lab-grade quality and were purchased from Sigma Aldrich (CAS34369-07-8) (St. Louis, MO, USA).

Statistical analysis

The data obtained in the study was statistically analyzed by using SPSS statistical software. The GLM procedure was applied to determine the effect of ATP dose rate × incubation time interaction on motion kinematics, plasma membrane integrity and viability parameters. One-way ANOVA was applied to determine the effect of various dose rates of ATP (0mM, 1mM and 2mM) on motility kinematics, plasma membrane integrity and viability parameters. The significance level was adjusted to $P < 0.05$. The data are presented as mean ± SD.

RESULTS AND DISCUSSION

There was a significant ($P<0.05$) interaction between ATP dose rate and incubation time. The progressive motility, total motility, VCL, VAP, ALH and BCF decreased ($P<0.05$) after incorporation of ATP (1 or 2mM) and by increasing the incubation time (from 10 min to 4hrs); indicating that the interaction of ATP and incubation time has negative effect on the motion kinematics of buffalo bull's sperm (Table 1).

The supplementation of ATP did not ($P>0.05$) improve the viability and plasma membrane integrity of sperm and by increasing the incubation time, the plasma membrane integrity decreased ($P<0.05$). Sperm viability remained unaffected by increasing incubation time. Similarly, no interaction ($P>0.05$) was found between ATP dose rate and incubation times in terms of sperm plasma membrane integrity and viability (Table 2). The present study is the first ever report to the best of our knowledge which demonstrates the effect of extracellular treatment on frozen-thawed buffalo bull sperm. The progressive motility, total motility, VCL, VAP, ALH and BCF decreased both by incorporation of ATP (1mM or 2mM) and increasing incubation time (from 10 min to 4hrs) which indicates the ATP and incubation time of 4 hrs negatively affect the motion kinematics of buffalo bull sperm. The ATP supplementation did not improve the plasma membrane integrity and viability of sperm. However, by increasing the incubation time, the plasma membrane integrity was decreased. Whereas the sperm viability remained unaffected by increasing incubation time. In the previous study the cryo-survival of rat epididymal sperm preserved in raffinose-modified krebs-Ringer bicarbonate-egg yolk extender supplemented with various energy-yielding substrates (ATP, lactate, glucose and pyruvate) was observed and it was reported that the motility of cryo-survived sperm decreased when incubated at 37°C for few minutes. Furthermore, the sperm frozen and thawed in the extender supplemented with 1.85 mM ATP exhibited considerably higher motility and viability than those of sperm frozen and thawed in ATP-free extender (Yamashiro *et al.,* 2010).

Exogenous ATP in the freezing medium may be responsible for the generation of multiple metabolic signals that appear to be related to the sperm motility through a rise in calcium levels (Kinukawa *et al.,* 2006; Luria *et al.,* 2002). The effect of ATP treatment on mammalian sperm has been examined previously. (Foresta *et al.,* 1993) showing that ATP increases in human and bovine sperm, correspondingly. In a previous study, five different concentrations of ATP (0.0, 0.5, 0.75, 1, and 1.5%) were added in diluent and resulted in increased sperm motility and protected the sperm membrane integrity in ram (Bezerra *et al.,* 2019). Similarly, Edwards et al., 2007 described that ATP enhanced the cryo-survival rate when it was used in culture media during in vitro fertilization when human sperm are treated with ATP.

However, our results regarding impact of ATP on buffalo bull sperm are totally in disagreement with the previous reports in different species. Our research work in buffalo bull semen, decreased sperm motility, viability, and membrane integrity were observed at various time points (10 min., 1hr, 2hr, 3hr and 4hr) during the incubation period of four hrs. Not all the studies are in disagreement with our findings. A previous report demonstrated a remarkably interfered acrosome integrity when frozen thawed cat sperm were incubated with 1 mM or 2.5 mM ATP for the period of 6 hrs ATPe after 6 hours and it was suggested that ATP might constitute a new therapeutic procedure to increase sperm subjective, progressive motility and to enhance the IVF outcome in domestic cats without any deleterious effects on sperm quality (Thuwanut *et al.,* 2015).

Table 2: The effect of ATP dose rate and incubation time (4 hrs) on plasma membrane integrity and viability of frozen thawed buffalo bull sperm. The values are presented as Mean ± SD.

ATP Dose	Incubation time	Plasma membrane integrity	Sperm viability
Control	10 min	76.6±3.2	71.0±3.6
1.mM		65.6±8.5	60.6±6.1
2.mM		70.0±4.5	60.0±15.1
Control	1 hr	74.6±7.0	65.3±7.0
1.mM		70.3±1.1	60.3±11.0
2.mM		73.0±6.1	65.6±1.1
Control	2 hr	67.0±4.5	69.3±3.5
1.mM		66.6±3.7	59.6±6.3
2.mM		64.3±2.3	69.3±7.7
Control	3 hr	64.6±0.5	66.6±5.6
1.mM		69.3±2.5	67.6±3.5
2.mM		66.6±5.1	70.0±8.1
Control	4 hr	71.3±3.7	64.3±3.2
1.mM		66.0±3.0	69.3±5.8
2.mM		65.0±7.0	70.3±2.3
P-value		0.06	0.438

The P-value <0.05 values in same column indicate the significant interaction between ATP dose rate and incubation time.

On the basis of the studies discussed above it might be speculated that pre-freezing supplementation of sperm with ATP may enhance sperm cryo-survival by improving the metabolic capacity of sperm before freezing, however, in our study ATP was added after thawing which could not show beneficial impact.

CONCLUSIONS

Current study demonstrated that when frozen-thawed samples of buffalo are treated with different concentrations of ATP (0,1,2 mM) and then incubated at 37°C for four hours, the motility and viability and integrity of plasma membrane were not improved by supplementation of ATP. Instead, the sperm quality was negatively affected by ATP supplementation. Therefore, on the basis of this study, it could be suggested that extracellular ATP should not be incorporated in frozen-thawed buffalo semen.

ACKNOWLEDGEMENTS

This study was the masters project of Misbah Shaheen under the supervision of Dr Ejaz Ahmad and conducted at Semen Analysis and Cryopreservation Laboratory**,** Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan. The authors wish to thank the staff of the laboratory for their cooperation.

CONFLICT OF INTEREST

None.

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