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EFFECT OF DIFFERENT L-ARGININE CONCENTRATIONS ON IN VITRO BUFFALO SEMEN FERTILIZING POTENTIALS

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ABSTRRACT

Sperm capacitation and acrosome reaction are essential for fertilization. L-Arginine plays an important role in the physiology of spermatozoa and has been shown to enhance their metabolism and maintain their motility. This study investigated the effect of L-arginine on cryopresreved buffalo sperm capacitation, acrosome reaction and the in vitro fertilizing potentials of the cryopresreved buffalo spermatozoa. Frozen-thawed buffalo spermatozoa have been incubated in TALP medium with different concentrations of L-arginine (5, 10 and 20 mM) and the percentages of capacitated and acrosome reacted spermatozoa have been determined by silver nitrate stain. L-arginine induced both capacitation and acrosome reaction. The optimum concentration that induced capacitation and improved in vitro fertilizing potentials appeared to be 10 mM. The data presented suggested the beneficial effect of L- arginine in cryopresreved buffalo sperm capacitation and acrosome reaction.

Key words: *L-Arginine; Nitric oxide; Sperm capacitation; Acrosome reaction; In Vitro Fertilizing Potentials.*

INTRODUCTION

Although the IVEP efficiency has greatly increased in recent years in buffalo, the cleavage rate is still lower than most domestic species, suggesting that in vitro fertilization (IVF) is a critical step (Gasparrini, 2007). Many factors are known to affect IVF efficiency, such as sperm quality, bull, environment, appropriate time of insemination, as well as an appropriate capacitation of frozen-thawed sperm.

Mammalian spermatozoa must undergo a series of membranous and metabolic changes before they can fertilize the egg. These physiological changes represent a complex process called capacitation (Visconti *et al.,* 2002). Various in vitro studies have indicated that the process of capacitation is biochemical in nature. Capacitation regulates transient changes in the sperm motility pattern, termed hyperactivation, and enables the exocytotic event of acrosome reaction, an essential event for oocyte fertilization (Yanagimachi, 2003). Though the capacitation is achieved synergistically and efficiently in the female reproductive tract, it can also be accomplished in vitro in various well-defined media for several species of mammals. Parrish *et al.* (1988) demonstrated that the glycosaminoglycan heparin capacitates bovine sperm in vitro. So heparin has been the most widely capacitating agent used in most domestic species (Bergqvist *et al.,* 2007 and Roy and Atreja, 2008). Recently, various substances are used in a trial to achieve in vitro sperm capacitation; one of them is L-arginine.

L-arginine is an amino acid which takes part in sperm formation and has been found to be a basic component of the nucleoprotein of spermatozoa of various species (Adnan, 1970). There is correlation between L-arginine deficiency and loss of spermatogenesis as well as decrease in sperm motility (Polakoski *et al.,* 1976 and Jungling and Bunge, 1976). Moreover, It plays a vital role in the sperm metabolic activity inside reproductive tract or throughout storage in vitro conditions in rabbits (Radany *et al.,* 1981, Mann and Lutwak-mann, 1981 and Patel *et al.,* 1998). L-arginine produces nitric oxide by the action of nitric oxide syntheses enzyme (NOS) (Palmer *et al.,* 1988). Several researches indicated that incubation of spermatozoa with NO donors increases intracellular cyclic guanosine

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monophosphate (cGMP) levels of bull (Zamir *et al.*, 1995) and human spermatozoa (Revelli *et al.*, 2001). Moreover, NO is a highly reactive oxygen species (ROS) and has a protective effect against lipid peroxidation on the sperm cells (Srivastava *et al.*, 2000). However, higher concentrations of l-arginine can have adverse effect on motility and fertility of human (Rosselli *et al.*, 1995) and rat (Ratnasooriya and Dharmasiri 2001) spermatozoa.

Therefore, this study was conducted as an attempt to investigate the effect of L- arginine on capacitation, acrosome reaction and in vitro fertilizing potentials of the cryopresreved buffalo spermatozoa and to explore the best time and L-arginine concentration which gives optimum sperm stimulation.

MATERIALS AND METHODS

Materials

All chemicals used were of the highest commercially available purity. l-Arginine, fructose, glycine, glycerol, bovine serum albumin (BSA, fraction V), HEPES and Heparin (purified from porcine intestinal mucosa) were purchased from Sigma Co. (St. Louis, MO, USA).

Semen Collection

Semen samples were collected randomly from five fertile Egyptian buffalo bulls (aged 3 to 5 y) kept at the Animal Reproduction Research Institute farm (Cairo, Egypt). Two consecutive ejaculates were collected from each bull weekly for successive six weeks using artificial vagina. The collected semen samples from all bulls were pooled to eliminate variability between the evaluated samples. The semen samples were assessed for volume, sperm concentration, and percentage of motile spermatozoa. The ejaculates with at least 70 % motility, $800x10^6$ sperm cells/ml and >85% normal sperm morphology (all bulls were previously evaluated by eocin-nigrocin staining before the study) were used for the present study. All experiments were done with at least 3 replicates for each group.

Semen Processing

After semen evaluation, fresh semen samples were pooled and diluted at 30°C with Tris-based extender to obtain 120×10^6 sperm/ml. Semen was cooled from 37 to 5°C throughout 60 min in a cold cabinet. The cooled semen was loaded into 0.25 ml polyvinyl chloride straws (IMV, L'Aigle, France), horizontally placed in a refrigerator and kept at 4°C for 1 h. These straws were then placed 6 cm above the liquid nitrogen surface where the temperature was approximately −120°C. After 15 min, they were immersed directly into liquid nitrogen (−196°C) for storage. The straws were stored at least for 24 hour before evaluation. Frozen semen straws were thawed in water bath at 37°C for 30 sec. Post-thawing sperm motility; viability and acrosomal integrity were assessed according to Badr *et al.* (2010).

Sample preparation

Frozen semen samples, over 3 replicates, were thawed in a water bath for 30 sec. at 37 °C. It was then equilibrated for 5 min in Tyrode's albumin lactate pyruvate (TALP) medium followed by centrifugation at 600g for 5 min to separate the buffer (Parrish *et al.*, 1988). The obtained pellet was washed in the same medium and centrifuged as stated above. The final pellet was resuspended in TALP medium in absence (control group) or presence of different concentrations of l-arginine (5, 10 and 20 mM). Aliquots for different treatments were incubated in a controlled gas atmosphere of 5% CO_2 at 38.5°C in humidified air for 0, 30, 45 and 60 min. After each incubation time, the semen assessments were repeated. Finally, frozen-thawed buffalo spermatozoa were used to fertilize buffalo oocytes in vitro to evaluate penetration and cleavage rate, as well as the percentage of fast developing embryos at 48 hrs post insemination.

Analysis of acrosomal membrane integrity

Acrosome integrity was assessed using silver nitrate stain in a procedure slightly modified (methods of washing + counting 300 sperm not 100 only) from the method described by El- Amrawi and Nemetallah (1991) and Chinoy *et al.* (1992). The sperm suspension was spread over slides and dried at room temperature. The preparations were fixed in ethyl alcohol 70% and 95%, 2 minutes for each then stained with the solution of 1% silver nitrate ($AgNO_3$) for 2 hours in an incubator at 65°C, in complete humidity. After the preparations turned gold in colour, the chemical reaction was interrupted and the preparation rinsed several times with distilled water. The preparations were dried at room temperature. The dyed preparations were analyzed for acrosomal integrity using the Olympus BX50 light microscope with a 100-fold magnification. The percentage of acrosome-intact spermatozoa was counted in at least 300 sperm cells per slide.

Assessment of fertilizing ability in vitro

Buffalo ovaries were recovered from a local abattoir and transported to the laboratory in physiological saline at 35°C. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2-8 mm in diameter and only those with uniform cytoplasm and multilayered cumulus cells were selected, washed twice in the aspiration medium and once in the maturation medium, TCM-199. Groups of 15 COCs were matured in 50 µL of IVM medium, covered with mineral oil, for 24 hrs at 38.5 °C and 5% CO_2 in air. In vitro matured COCs were washed and transferred into 75 µl of IVF medium, F-TALP medium covered with mineral oil. Buffalo frozen sperm from the five bulls were processed as described above and after

centrifugation the pellet was resuspended to a final concentration of 2 x 10^6 sperm/mL in the IVF medium. Gametes were co - incubated for 6 hrs at 38.5 °C, in 5% CO_2 in air. After 6 hours some inseminated oocytes were fixed for 24-48 h in ethanol: acetic acid (3:1 v/v), then stained with 1% Orcein dissolved in 45% acetic acid to evaluate the fertilization rate according to Martino *et al.* (1994). Oocytes were evaluated for normal fertilization on the basis of oocyte that had set of male and female pronuclei in the ooplasm were considered to be fertilized normally, while the penetration rate was evaluated by the presence of sperm tail only in the ooplasm. The other presumptive zygotes were vortexed for 2 min to remove cumulus cells in Hepes-TCM-199 washed twice in the same medium and cultured in synthetic oviductal fluid medium (SOFaa) (SOF with 1 mM glutamine, 1% MEM nonessential amino acids and 1% MEM essential amino acids) according to Badr (2009) and covered with mineral oil for 7- days at 38.5°C in an atmosphere of 5% CO_2 in air with maximum humidity. The cleaved oocytes were recorded after 48 h of insemination and those developed to the morula and blastocyst stages were recorded at 5-7 days post-insemination according to Totey *et al.* (1992).

Statistical analysis

All data were analyzed by using Costat Computer Program (1986), Version 3.03 copyright Cottort Software, and were compared by the least significant difference least (LSD) at 5% levels of probability. The results were expressed as means ±SE. The mean values of the percentages of motile sperm, acrosome intact sperm and embryo development were compared using Duncan's multiple range test by one-way ANOVA procedure, when the F-value was significant ($P < 0.05$). Sperm fertilizing potentials and zygotes developmental competences were assessed using Chi-square analysis.

RESULTS

Data presented in table 1 showed the effect of l-arginine on sperm progressive motility. The sperm motility was measured at zero time, after 30, 45 and 60 min of incubation. It was observed that at lower concentrations (5 or10 mM), l-arginine maintained the sperm motility compared to the control. However, at higher concentrations motility was significantly decreased. It was observed that motility of the control sample (incubated for 60 min) was maintained with time compared to that observed at zero time. Whereas, the motility was affected significantly at high concentration of l-arginine compared to control.

Table 1: Effect of l -arginine on percent of sperm progressive motility of buffalo spermatozoa.

Concentrations	0 h	30 m	45 m	1h
Control	75±5.00[a]	75±2.89[a]	70±2.89 [a]	51.67±4.40 [a]
L-arginine 5mM	80±2.89[a]	71.67±4.41 [a]	61.67±4.41[ab]	40±5.78 [ab]
L-arginine 10 mM	81.67±1.67[a]	75±2.89 [a]	70±5.78 [a]	40±2.89 [ab]
L-arginine 20 mM	75±5.78 [a]	55±5.78 [b]	50±5.78 [b]	25±7.64 [b]

Values with different superscript letters in the same columns are significantly different at ($P<0.05$).

Data regarding the effect of l -Arginine on sperm capacitation and acrosome reaction in buffalo spermatozoa are presented in table 2. In order to know the effect of l-arginine on capacitation, cells were incubated for 60 min in TALP medium with different concentrations of l-arginine. Sperm cells incubated in TALP medium alone were used for comparison. It was observed that L-arginine promoted capacitation at all concentrations (used in the present experiment) compared to the control sample. The current results indicated that the percentage of spermatozoa with reacted acrosomes increased when cells were treated with l-arginine as compared to the control. 10 mM l-arginine resulted in highly significant ($P< 0.01$) sperm capacitation compared with the control group (86.00 VS 51.67%).The extent of capacitated spermatozoa is about two fold higher (54.66% at 10 mM l-arginine concentration) compared with the control sample (27.67%) after 45 min of sperm incubation.

Table 2: Effect of l -Arginine on sperm capacitation and acrosome reaction in buffalo spermatozoa.

Concentrations	0 h	30 m	45 m	1h
Control	7.33±1.45 [a]	21.66±5.24[b]	27.67±6.49 [b]	51.67±4.41 [c]
L-arginine 5mM	10.67±2.96 [a]	28.00±3.51[ab]	42.00±4.16 [ab]	71.67±6.01 [ab]
L-arginine10 mM	9.67±2.60 [a]	37.33±3.38[a]	54.66±2.90 [a]	86.00±4.35 [a]
L-arginine 20 mM	13.33±2.02 [a]	41.00±5.29[a]	51.00±4.58 [a]	61.66±5.82 [bc]

Values with different superscript letters in the same columns are significantly different at (P<0.05).

Data concerning the effect of replenishing of in vitro fertilization media with 10 mM L-arginine, best concentration that induced sperm capacitation, on the in vitro fertilizing potentials and embryo development are presented in tables 3 and 4. The current results revealed that, addition of 10 mM L-arginine to the IVF media had a positive effect (P < 0.05) on the in vitro fertilization and embryo developmental rates compared with the control semen. Addition of 10 mM L-arginine to the IVF media resulted in enhanced proportion of in vitro fertilized oocytes, morula and blastocyst development (60.93, 30.56, and 22.22%, respectively) compared with the control (41.07, 10.81and 5.41%, respectively).

Table 3: Effect of l -Arginine on in vitro fertilizing potentials of buffalo oocytes.

Concentrations	No of oocytes	penetration	Fertilization
Control	56	32 (57.14) a	23 (41.07) a
L-arginine 10Mm	64	44(68.75) a	39 (60.93) b

Values with different superscript letters in the same columns are significantly different at (P<0.05).

Table 4: Effect of l -Arginine on in vitro embryo development of buffalo oocytes.

Concentrations	No of oocytes	Cleavage rate	Morula	Blastocyst
Control	37	16 (43.24) a	4 (10.81)a	2 (5.41) a
L-arginine 10mM	36	21 (58.33)a	11 (30.56) b	8 (22.22) b

Values with different superscript letters in the same columns are significantly different at (P<0.05).

DISCUSSION

The current results clearly suggest a positive effect of l-arginine on sperm capacitation, acrosome reaction and in vitro fertilizing potentials in buffalo spermatozoa. These results agree with the previous studies (Thundathil *et al.,* 2003 and O'Flaherty *et al.,* 2004).

The mechanism of l-arginine action is through the production of NO in the presence of NOS (Palmer *et al.,* 1988) in various biological systems including spermatozoa (Meiser and Schulz 2003 and Wang *et al.,* 2006). Nitric oxide plays a decisive role in regulating multiple functions within the male as well as female reproductive systems. This enzyme is found on the acrosome and tail region of non-capacitated spermatozoa (Herrero *et al.,* 1996). At low concentrations, nitric oxide is a molecule of great biological significance and has long been considered to play an important role in sperm physiology such as sperm motility, enhance capacitation, acrosome reaction in human spermatozoa (Zini *et al.,* 1995) and sperm-egg interaction (Revelli *et al.,* 2001).

Concerning sperm motility, the addition of L.arginine in low concentrations enhances sperm motility by increasing production of nitric oxide which enhances the metabolic rate, it also enhances cGMP synthesis, thus leading to increase the calcium level in the mitochondria and improving the rate of glycolysis which elevates the rate of Adenosine-5'-triphosphate

(ATP) and lactate generation in spermatozoa. These two effects lead to increase sperm motility (Patel *et al.,* 1998, Sengoku *et al.,* 1998 and Revelli *et al.,* 2001).

Moreover, NO stimulate hyperactivation motility (Herrero *et al.,* 2000). Sperm hyper activation motility is promoted by exogenous addition of NO which may increase phosphorylation of flagella proteins (Thundathil *et al.,* 2003). Additionally, NO has been demonstrated to trigger tyrosine phosphorylation (Herrero *et al.,* 2000) and double serine/threonine phosphorylation (Thundathil *et al.,* 2003), both events being closely related with capacitation.

Recently, there are many evidences that, L-arginine prevents bilayer phospholipids membrane peroxidation under various peroxidation situations through production of NO mechanism which protects structural and functional integrity of spermatozoa (Srivastava *et al.,* 2000).

In this study, low concentration of NO improves sperm motility however; higher concentrations of L-arginine significantly decreased the progressive motility of buffalo spermatozoa and in vitro fertilizing potentials of buffalo spermatozoa. This finding is supported by the observation made by Roselli *et al.* (1995) and Ratnasooriya and Dharmasiri 2001), where high concentration of nitric oxide decreases motility and induces toxicity in spermatozoa. The reason for the reduced motility may be a significantly reduced cAMP concentration at higher concentrations of NO (Herrero *et al.,* 2000), an important requisite for the motility machinery.

In conclusion ,the present study have made an attempt to indicate the role of L-arginine on buffalo sperm capacitation and acrosome reaction, indispensable processes required for the acquisition of the fertilizing ability of buffalo spermatozoon. The current results suggested that 10 Mm L- arginine could induce in vitro sperm capacitation and acrosome reaction that subsequently enhance the fertilizing potentials of buffalo spermatozoa in vitro. Further studies are being conducting to better understand the participation of L-arginine in the signaling pathways of these processes.

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تاثير تركيزات مختلفه من ل-ارجينين على امكانية الاخصاب المعملى للسائل المنوى فى الجاموس

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تهيئة الحيوانات المنوية ضروري للاخصاب. ل-ارجينين يلعب دورا هاما فى فسيولوجيا الحيوانات المنوية وكذلك لتعزيز عملية التمثيل الغذائى والحفاظ على حركة الحيوانات المنوية. استهدفت هذه الدراسة تاثير ل-رجينين على تهيئة الحيوانت المنوية للجاموس للاخصاب بعد حفظها بالتجميد.تم حفظ الحيوانات المنوية بعد الاساله فى بيئة الTALP مع تركيزات مختلفه من ل-ارجينين (٥ و١٠ و٢٠ مل مول) وقد تم تحديد نسبة الحيوانات المنوية المهيئة باستخدام صبغة نترات الفضة. تبين ان ل- ارجينين قد احدث تهيئة للحيوانات المنوية والتركيز المطلوب لاحداث التهيئة وتحسين الاخصاب المعملى هو ١٠ مل مول. واشارت البيانات ان ل-ارجينين له تاثير مفيد فى تهيئة الحيوانات المنوية للجاموس التى تم حفظها بالتجميد.