

## EFFECT OF LEVEL AND TIME OF L-ARGININE ADDITION TO SEMEN EXTENDER ON THE FREEZABILITY AND FERTILIZING POTENTIALS OF BUFFALO SPERMATOZOA

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### ABSTRACT

The objective of this study was to investigate the profit of L-arginine addition to the freezing extender on freezability, lipid peroxidation, and fertilizing potentials of frozen-thawed buffalo spermatozoa. Semen was collected with artificial vagina from five adult fertile bulls and diluted with Tris-base extender containing different L-arginine concentrations (0, 0.5, 1, 5 and 10 mM) added during the dilution or after equilibration for 2 h. Diluted semen was cooled to 4°C throughout one hour, equilibrated for 2 h and then frozen in 0.25 ml straws, prior to be stored in liquid nitrogen. Cryopreserved spermatozoa were assessed for post-thawing sperm motility, viability index, acrosomal integrity, lipid peroxidation and fertility rate. The current results clearly indicated that adding 0.5 mM L- arginine to the freezing extender after the equilibration period significantly ( $P < 0.05$ ) improved post-thawing motility, viability index and maintain acrosomal integrity ( $61.66 \pm 10.14\%$ ,  $130.83 \pm 9.62$  and  $19.33 \pm 4.82\%$ , respectively) compared with the control semen ( $33.33 \pm 4.40\%$ ,  $88.33 \pm 11.03$  and  $33.67 \pm 3.28\%$ , respectively). Moreover, addition of 0.5 mM L- arginine to the freezing extender after equilibration period significantly diminished ( $P < 0.05$ ) lipid peroxidation ( $11.33 \pm 3.28$  nmol/ $10^9$ ) compared with the control ( $26.67 \pm 3.18$  nmol/ $10^9$ ). All of these previously enhanced semen characteristics were reflected positively on its fertilizing potentials. The present results revealed that addition of 0.5 mM L- arginine to the freezing extender after 2 h equilibration (short time exposure) might improve semen quality, preserve the fertilizing potentials and reduce cryodamage of the buffalo spermatozoa.

*Keywords: L-arginine, buffalo, spermatozoa, cryopreservation, lipid peroxidation*

### INTRODUCTION

Artificial insemination (AI) technology has been developed in Egypt

with excellent results, especially in dairy cows. However, the application of this technology in the buffalo is still minimal. The application of AI has been shown to have the ability to distribute genes from fabulous genetic males for improving productive performance (El-Sheshtawy *et al.*, 2015). Cryopreservation induces adverse effects on the spermatozoa that may

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result in decreasing the percentage of intact spermatozoa, viability rate, structural integrity, or loss of motility and fertilizing capacity (Medeiros *et al.*, 2002). Mammalian spermatozoa have very specific lipid composition and a high ratio of polyunsaturated fatty acids. Oxidative stress can cause lipid peroxidation of the sperm membrane which may cause severe functional disorders of sperm (Sanocka and Kurpysz, 2004). These peroxides constitute a potential hazard to the structural and functional integrity of spermatozoa, lessening the motility and metabolic activity of cells that are aging either within the reproductive tract *in vivo* or during storage *in vitro* (Mann and Lutwak-Mann, 1981).

L-arginine (2-Amino-5-guanidinopentanoic acid) plays a key role in modulating host defenses and cellular immunity. It actively participates in sperm formation (Adman, 1970). L-arginine plays an important role in stimulating sperm motility in rabbits (Radany *et al.*, 1981), humans (Aydin *et al.*, 1995) and goats (Patel *et al.*, 1998) under *in vitro* conditions. A deficiency in L-arginine causes derangement of sperm metabolism leading to decrease in motility and loss of spermatogenesis (Srivastava *et al.*, 2006). L-arginine prevents membrane lipid peroxidation in spermatozoa under different peroxidation conditions (Srivastava *et al.*, 2006). It has been proposed that the beneficial effects of L-arginine are linked to nitric oxide (NO) (Chemineau *et al.*, 1991). NO is a short-lived free radical, synthesized in many mammalian cell types by a class of NADPH dependent enzymes called nitric oxide synthases (NOS). These enzymes catalyze the conversion of L-arginine to L-citrulline and NO (Moncada *et al.*, 1992). It has shown that L-arginine reported to have a positive effect on improving the rate of glycolysis, resulting in higher rates of ATP and lactate generation in spermatozoa (Aydin *et al.*, 1995). It participates in synthesis of some hormones (insulin,

prolactin, glucagon, growth hormone) as well as biosynthesis of creatine, agmatine, glutamate, proline and polyamines and in the structure of anti-diuretic hormone (ADH) (Soeters *et al.*, 2002).

Therefore, this study was conducted to determine the optimal level and time of adding L-arginine to buffalo semen extender to obtain high freezability and fertilizing ability after cryopreservation.

## MATERIALS AND METHODS

### Farm management and semen collection

This study was carried out at the Artificial Insemination and Embryo Transfer Department, Animal Reproduction Research Institute, and Animal Production Research Institute. Five sexually mature healthy buffalo bulls, ranging 3-4 years old age and 420-450 kg live body weight. Bulls were housed individually in pens and kept at the Animal Reproduction Research Institute farm (Cairo, Egypt) and fed on balanced ration.

Two consecutive ejaculates were collected from each bull weekly for successive six weeks using an artificial vagina (42°C). Immediately after collection, ejaculates with at least 70% motility and >85% normal sperm morphology were pooled to eliminate individual differences. The pooled semen was split into five equal aliquots for processing according to treatments.

### Sperm processing and stress treatment before cryopreservation

Aliquots of ejaculate were diluted at room temperature with Tris-based extender (Tris-egg yolk extender (20% EY) containing 20 ml of egg yolk, 3.025g Tris (hydroxyl methyl amino methane), 1.675 g citric acid, 0.75 g glucose, 7ml glycerol, 0.25 gm lincomycin, 0.005 gm streptomycin and completed with up to 100 ml bidistilled

water) in the extender we use glycerol as cryoprotectant with concentrate 7% add to extender at room temperature 37 °C before cooling. The extender contained different levels of L-arginine (L-Arginine 99 %, Jahangir villa, Mumbai -400005 India) (0, 0.5, 1, 5 and 10 mM) according to (AL-Ebady *et al.*, 2012; Keya *et al.*, 2017 and Abd-Allah *et al.*, 2019). Semen was diluted at a rate of 1:8, and the diluted semen with each L-arginine level and control group were subjected to gradual cooling from 37 °C to 5 °C throughout 60 min in a cold cabinet and equilibrated at 4°C for 2 h. The cooled semen samples were subsequently loaded into 0.25 ml French straws (IMV, L'Aigle, France), and sealed thermally. These straws were placed horizontally 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.

#### **Experiment design:**

1 -Tris-based extender was supplemented with different levels of L-arginine (0. 0.5, 1, 5 and 10 mM) at 37°C, then the diluted semen was subjected to gradual cooling from 37 °C to 5 °C throughout 60 min in a cold cabinet. The cooled semen was equilibrated at 4 °C for 2 h. Frozen semen was thawed at 37 °C for 30 s to study the effect of adding different L-arginine levels to Tris-based extender during dilution process on freezability of buffalo spermatozoa in post-thawed semen.

2 - Tris-based extender extender was supplemented with different levels of L arginine (0, 0.5, 1, 5 and 10 mM) for 2h equilibration at 4°C, then the equilibrated semen was frozen.

#### **Post-thawing evaluation of sperm parameters**

Post-thawing progressive sperm motility: viability index and acrosomal integrity were assessed according to Badr *et al.* (2010).

#### **Lipid peroxidation of post-thawed semen**

The concentrations of Malondialdehyde (MDA), as a marker of LPO in the post-thawed semen supplemented with L-arginine at dilution or after equilibration, were measured according to the method of Kumaresan *et al.* (2009). The MDA concentrations were read at 532 nm and expressed in nmol/10<sup>9</sup>.

#### **Fertility trial**

A total of 128 buffalo cows synchronized to estrus (Hoque *et al.*, 2011) were used in fertility trails. In brief, buffalo cows were i.m. injected with 5 ml Receptal/ buffalo on day 0, 2 ml Estrumate /buffalo on day 9, and 5 ml Receptal/ buffalo on day 9: and artificially inseminated on day 10 by using frozen-thawed straw from control and the best semen diluted with L- arginine. Each female was inseminated with a single straw 12 h after start of estrous behavior, using recto-vaginal technique and the universal insemination gun. The frozen-thawed spermatozoa were deposited in the uterine body. Conception rate was confirmed by rectal palpation at least 45 days after insemination.

#### **Statistical analysis**

All data were analyzed by using Costat Computer Program (1986), Version 3.03 copyright Cottort Software, using one way ANOVA analysis. The results were expressed as means ±SE. The significant differences among mean of treatments were tested using Duncan's multiple range test (Duncan, 1955), when the F-value reveals significant differences at P < 0.05.

## RESULTS

### Effect of adding L-arginine at dilution on sperm freezability

Results presented in Table 1 revealed that, addition of L-arginine only at a level of 1mM to the freezing extender resulted in

significant ( $P<0.05$ ) improvement in percentage of progressive motility and viability index of cryopreserved buffalo semen as compared to control. It is of interest to observe that the most motile spermatozoa were in circular movement.

**Table 1:** Freezability of buffalo spermatozoa as affected by L- arginine addition to semen extender at dilution

Treatment	Progressive motility (%)	Viability index	Acrosomal abnormality (%)
Control	33.33±7.27 <sup>b</sup>	69.17±23.22 <sup>ab</sup>	29.33±4.49
L-arginine (0.5 mM)	48.33±4.41 <sup>ab</sup>	86.67±11.68 <sup>ab</sup>	28.66±5.93
L-arginine (1 mM)	60.00±8.67 <sup>a</sup>	106.67±13.65 <sup>a</sup>	22.33±2.91
L-arginine (5 mM)	38.33±6.02 <sup>b</sup>	71.67±13.03 <sup>ab</sup>	32.33±3.84
L-arginine (10 mM)	28.33. ±4.41 <sup>b</sup>	44.17±7.13 <sup>c</sup>	37.33±3.18

Values with different superscripts within the same column differ significantly at  $P<0.05$ .

Additionally, the present results showed that there was no significant difference in the percentage of acrosomal integrity between L-arginine concentrations and control semen when L-arginine was added to the freezing extender from the beginning of dilution at 37°C.

### Effect of adding L-arginine post-equilibration on sperm freezability

Data in Table 2 indicated that, L-arginine addition to the freezing extender post-equilibration at a level of 0.5 mM appeared to have an quick effect on the quality of

buffalo semen freezability, in terms of significant ( $P<0.05$ ) augment in buffalo semen post-thawing progressive motility, viability index and reducing acrosomal integrity compared with the control semen. On the other hand, increasing level of L-arginine to 10 mM had a drastic effect ( $P<0.05$ ) on post-thawing sperm motility, and the acrosomal integrity of the cryopreserved buffalo spermatozoa did not differ than in control group. However, viability index was significantly ( $p<0.05$ ) lower than in control group by increase L-arginine level to 10mM.

**Table 2:** Freezability of buffalo spermatozoa as affected by L- arginine addition to semen extender after 2 h of equilibration.

Treatment	Progressive motility (%)	Viability index	Acrosomal abnormality (%)
Control	33.33±4.40 <sup>bc</sup>	88.33±11.03 <sup>b</sup>	33.67±3.28 <sup>a</sup>
L-arginine (0.5 mM)	61.66±10.14 <sup>a</sup>	130.83±9.62 <sup>a</sup>	19.33±4.82 <sup>b</sup>
L-arginine (1 mM)	48.33±7.27 <sup>ab</sup>	105.83±11.22 <sup>ab</sup>	26.33±4.34 <sup>ab</sup>
L-arginine (5 mM)	38.33±7.27 <sup>abc</sup>	79.16±16.87 <sup>bc</sup>	28.33±2.73 <sup>ab</sup>
L-arginine (10 mM)	23.33. ±6.01 <sup>c</sup>	49.83±3.83 <sup>c</sup>	35.00±3.06 <sup>a</sup>

Values with different superscripts within the same column differ significantly at P < 0.05.

#### Effect of L- arginine addition to semen extender on lipid peroxidation of the cryopreserved buffalo spermatozoa.

Data regarding the effect of L- arginine addition to the freezing extender (at dilution or post-equilibration) on malondialdehyde (MDA) as a lipid peroxidation marker in cryopreserved buffalo spermatozoa (Table 3) revealed that adding L-arginine at a level

of 1 mM at dilution and 0.5 mM after 2 h of equilibration significantly (P<0.05) diminished level of MDA in post-thawed semen to the minimum values as compared to their controls. Moreover, the current data indicated that increasing level of L- arginine to the semen extender to 10 mM had a severe effect on lipid peroxidation of the frozen-thawed buffalo spermatozoa.

**Table 3:** Malondialdehyde level as affected by time and level of L-arginine addition to semen extender of cryopreserved buffalo semen.

Treatment	Malondialdehyde (nmol/10 <sup>9</sup> sperm cells)	
	L-arginine added at dilution	L-arginine added 2 h post-equilibration
Control	23.66±4.41 <sup>ab</sup>	26.67±3.18 <sup>ab</sup>
L -arginine (0.5 mM)	17.67±3.18 <sup>bc</sup>	11.33±3.28 <sup>c</sup>
L -arginine (1 mM)	11.67±3.67 <sup>c</sup>	19.67±2.34 <sup>bc</sup>
L –arginine (5 mM)	20.33±2.34 <sup>abc</sup>	25.33±3.39 <sup>ab</sup>
L –arginine (10 mM)	29.00±2.09 <sup>a</sup>	31.67±2.60 <sup>a</sup>

Values with different superscript in the same column are significantly different at P<0.05.

### Fertility trail

The concentration of 1 mM of L-arginine was used in a fertility experiment because it was the highest treatment in the progressive motility after dilution and before equilibration (Table 1). Also, Treatment of 1 mM L- arginine is considered the second best treatment after equilibration and dissolution after a 0.5 mM of l arginine, so it was used in the fertility experiment to decide which the best treatments that can be used in diluents.

Conception rate was significantly ( $P<0.05$ ) higher following artificial insemination of the synchronized buffaloes with frozen-

thawed semen supplemented with 0.5 mM L-arginine after 2 h of equilibration ( $68.33\pm 2.55\%$ ) compared with the control semen ( $29.72\pm 6.58\%$ ). However, conception rate was insignificantly higher for buffaloes inseminated by semen supplemented with 1 mM L-arginine added from the beginning of dilution and the control semen ( $44.44\pm 12.11$  vs  $29.7\pm 6.58\%$ , respectively, Table 4) and insignificantly lower than semen supplemented with 0.5mM L-arginine after 2h of equipratiotion ( $68.33\pm 2.55\%$ , table 4).

**Table 4:** Conception rate of buffalo cows following artificial insemination with semen extended with L-arginine.

Treatment	Inseminated buffaloes (n)	Conceived buffaloes (n)	Conception rate
Control	42	14	$29.7\pm 6.58$ b
L-arnginine (1 mM) at dilution	39	18	$44.4\pm 12.11$ ab
L-arnginine (0.5 mM) post-equilibration	47	32	$68.3\pm 2.55$ a

Values with different superscript in the same column are significantly different at  $P<0.05$ .

## DISCUSSION

The present study indicated that, supplementation of the freezing extender with L- arginine was significantly amplified the characteristics of the cryopreserved buffalo spermatozoa, such as progressive motility, viability, acrosomal integrity, reduced lipid peroxidation and preserve the fertilizing potential of the cryopreserved spermatozoa, depending on the dose and time of addition to the semen extender. These results are in line with the findings of several authors (Al-Ebady *et al.*, 2012; Abd-Allah *et al.*, 2019; Susilowati *et al.*,

2019). The previous authors reported positive effects of L-arginine on motility, antioxidant activity and membrane integrity of mammalian spermatozoa.

The current data revealed that the effect of L- arginine on post-thawed semen quality and fertilizing potential differed significantly according to the time of adding L-arginine to the freezing extender. Addition of L-arginine during the diluation at  $37^{\circ}\text{C}$  had no effect on semen viability or acrosomal integrity, but there were positive significant effect when add L-arginine with a concentration 1 mmol/ml on sperm

viability. These results are in agreement with the result of O'Flaherty *et al.* (2004) and Öztürk *et al.* (2017), who found that the percentage of acrosome integrity and viability of spermatozoa did not varied significantly with addition of L-arginine to semen extender. These results may be due to L-arginine enhances c-GMP synthesis thus leading to an enhancement of the metabolic rate. It also enhances the Ca level in the mitochondria, generating a higher ATP level. These two effects lead to over increased sperm motility (Revelli *et al.*, 2001) and play an important role in sperm hyperactivation and acrosomal reaction *in vitro* (Sliwa and Stochmal 2000). These results are equivalent with the current study that recorded increased circular movement of spermatozoa treated with L-arginine from the beginning of dilution before freezing. Circular motility of the cryopreserved spermatozoa indicates exposure of spermatozoa for premature capacitation, hyperactivity and acrosome reaction (El-Shahat *et al.* (2016). Induction of hyperactivity and acrosomal reaction prior to freezing may be the main cause of lowered sperm viability with L-arginine addition for long time, from the beginning of dilution before freezing, before freezing recorded in the present study.

Meanwhile, addition of 0.5 mM of L-arginine after 2 h equilibration and just before filling of straws, short time exposure, improved semen motility, viability, and fertilizing ability, but maintained the acrosomal integrity compared to the control semen.

The positive effect of L-arginine on the post-thawed buffalo semen may be attributed to increase 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 generating a higher adenosine triphosphate (ATP) (Sengoku *et al.*, 1998; Revelli *et al.*, 2001 and Maidin.

*et al.*, 2014). Moreover, it has been reported that L-arginine administrated rabbits and male goats have significant increase in sperm motility, which is thought to be associated with an increase in the rate of glycolysis, resulting in higher rates of ATP and lactate generation in spermatozoa (Schachter *et al.*, 1973; Zini *et al.*, 1995; Patel *et al.*, 1998).

Additionally, nitric oxide is a reactive nitrogen species (RNS) that can act in inter and intracellular signaling, and as an antioxidant (Pacher *et al.*, 2007). L-arginine acts as an antioxidant by absorption of oxygen for cell metabolism and inactivating superoxide (O<sub>2</sub>) anions (Brown *et al.*, 1999), that cause peroxidative damage to membrane phospholipids. The membrane lipids of spermatozoa are highly vulnerable to the action of peroxidizing agents. The peroxides comprise a potential risk to the structural and functional integrity of spermatozoa, reducing the motility and metabolic activity of sperm cells (Lipton, 1999). Intact plasma membrane has a negative correlation with lipid peroxidation and is positively correlated with the viability and motility of spermatozoa (Chen *et al.*, 2018).

L-arginine prevents membrane lipid peroxidation in spermatozoa under different peroxidation conditions (Srivastava *et al.*, 2006). It has been proposed that the beneficial effects of L-arginine are linked to nitric oxide (Palmer *et al.*, 1988), thereby decreasing lipid peroxidation (Srivastava *et al.*, 2006) and maintaining membrane integrity (Aitken and Baker, 2013).

Lipid peroxidation stimulates mitochondria to produce excessive cytochrome- c that will cause apoptosis (Andrew *et al.*, 2002), which cause a rapid decrease of sperm motility (Peterson *et al.*, 2010). There was a correlation between apoptosis, and each of increased DNA fragmentation, and the

lower fertilization rates of *in vitro* fertilization (Manente *et al.*, 2015). These reports may emphasize the current result of improved the conception rate of cow buffaloes that inseminated with L-arginine supplemented semen.

On the other hand, the current results indicated that, regardless the time of addition, high L-arginine concentration has a drastic effect on frozen-thawed semen quality, probably by excessive generation of NO, can cause sperm toxicity as well as reduce sperm motility by contributing to the formation of peroxynitrite, a highly toxic anion of peroxidation (Levonen, *et al.*, 2001). These results are in parallel with the results reported by El-Shahat *et al.* (2016) and Kaya *et al.* (2017). The authors found that high L-arginine concentration reduces sperm motility and adversely affects the quality of spermatozoa, that may be due to increase of L-arginine will be increase Nitric oxide (NO) concentration which have negative effect on sperm motility and are associated with increase sperm toxicity (Rosselli *et al.*, 1995).

## CONCLUSION

The emerged results of this study clearly demonstrate that supplementation of semen extender with 0.5 mM of L-arginine to the freezing extender after 2 h equilibration (short time exposure) have valuable effects on the quality and the fertilizing potentials of the cryopreserved buffalo spermatozoa. These constructive effects appeared due to the improvement of the antioxidant activities and the diminishing rates of lipid peroxidation of the cryopreserved spermatozoa.

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## تأثير مستوى وزمن إضافة الارجنين لمخففات السائل المنوي على قابلية التجميد وإخصاب الحيوانات المنوية للجاموس

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الهدف من هذه الدراسة هو معرفة فائدة اضافة الارجنين الى المخفف المستخدم فى تجميد السائل المنوى وتأثيره على القابلية للتجميد واكسدة الدهون وكفاءة الاخصاب للحيامن بعد الاذابة.

تم تجميع السائل المنوى من عدد خمس طلائق بالغة مخصصة باستخدام مهبل صناعى وتم التخفيف باستخدام مخفف التريس بحيث يحتوى على تركيزات مختلفة من الارجنين (٠) و (٠,٥) و (١) و (٥) و (١٠) مل مول/مل) تم اضافتها اثناء التخفيف أو بعد ساعتين من الموازنة. السائل المنوى المخفف تم تبريد على درجة حرارة 5 درجة مئوية لمدة ساعة ثم فترة موازنة ساعتين على درجة ٤ مئوية ثم تجميدها فى قصيبات ٠,٢٥ مل قبل الحفظ فى النتروجين السائل. يتم تقدير عملية الانضاج للحيوانات المنوية بعد عملية الاذابة حيث يتم تقدير الحركة الامامية و معدل الحيوية للحيامن المجمده وسلامة الاكروسوم واكسدة الدهون ومعدل الخصوبة.

النتائج الحالية بينت بوضوح أن اضافة ٠,٥ مل مول من الارجنين لمخفف السائل المنوى بعدة فترة الموازنة اظهرت تحسن معنوى ( $P<0.05$ ) للحركة ودليل الحيوية بعد الاذابة كما حافظ على سلامة الاكروسوم ( $61,66 \pm 10,14\%$  و  $130,83 \pm 9,62\%$  و  $19,33 \pm 4,82\%$  على التوالى) بالمقارنة بالمجموعة الغير معاملة ( $33,33 \pm 4,40\%$  و  $88,33 \pm 11,03\%$  و  $33,67 \pm 3,28\%$  على التوالى). علاوة على ذلك فإن اضافة الارجنين بتركيز ٠,٥ مل مول الى مخفف السائل المنوى قبل التجميد وخلال فترة الموازنة قل بشكل معنوى ( $p<0.05$ ) من اكسدة الدهون ( $11,33 \pm 3,28$  نانومول / ١٠<sup>٩</sup>) بالمقارنة بالمجموعة الغير معاملة ( $26,67 \pm 3,18$  نانومول / ١٠<sup>٩</sup>). من كل ما سبق فإن تحسين خصائص السائل المنوى بعد الاذابة ينعكس بالايجاب على القدرة الاخصابية للحيامن الموجودة بهذا السائل المنوى.

النتائج الحالية أوضحت أن اضافة ٠,٥ مل مول من الارجنين الى مخففات السائل المنوى قبل التجميد وبعد مرحلة الموازنة بساعتين (فترة قصيرة من التعرض للموازنة) ربما يحسن من صفات السائل المنوى اثناء الحفظ وكذلك يحسن من كفاءة عملية الاخصاب لهذا السائل المنوى كما يقلل من تلف الحيامن اثناء الاسالة فى السائل المنوى لطلائق الجاموس.