Journal of Animal and Poultry Production

Journal homepage: www.japp.mans.edu.eg
Available online at: www.jappmu.journals.ekb.eg

Effects of Supplementing Extenders with L-Arginine on Semen Cryopreservation and Fertility in Buffalo Bulls

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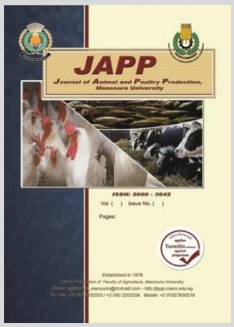

ABSTRACT

Knowledge of influence added more one concentration of L-arginine to enhance the poor quality of buffalo bull spermatozoa considers the main objective in our study. Semen collected from five buffalo bulls once weekly and ejaculates with 45-50% mass motility were pooled. Semen was diluted at 37C° by Tris-egg yolk extender, also separated to five portions, 1st portion without supplement it consider control to other portions and 2nd, 3rd, 4th and 5th were supplemented with 3, 5, 7 and 10 mM L-arginine respectively. Progressive sperm motility, live sperm%, abnormal sperm%, chromatin normality, Conception rate, and integrity of sperm acrosome were determined. Data declared the, extenders with concentrations of 7 and 10 mM L-Arginine was improved all parameters studied than 3 and 5 mM L-arginine extenders and control extender at different cryopreservation processes. A significant difference in chromatin integrity was detected at all treatments. Also, the conception rate was higher in buffalo cows artificially inseminated with 7 and 10 mM L-arginine (67.27 and 71.70 %) compared with control (45.45%). Conclusively, it could be concluded that adding L-arginine with concentrations of 7 or 10mM to Tris-extender improved the cryopreservation and fertility of the poor quality of Buffalo bull spermatozoa.

Keywords: L- Arginine, Semen extender, Semen quality, Chromatin integrity, Buffalo bull

INTRODUCTION

The main factor induced reproductive capacity of males is sperm production,however, consider economically vital kinds, moreover, application of artificial insemination has been exposed to have the aptitude to distribute genes from great genetic males to refining productive recital (El-Sheshtawy *et al.*, 2015). Successful AI relies on successful extender that reservation efficient motion spermatozoa through storing at diverse temperatures may be possible decrease of fertilizing ability of spermatozoa which depended on variable reasons (Berndtson, 2008).

On other, hands structure of extender is one of greatest factors affected cryopreservation consequently, various additives have been incorporated into semen extenders which used for expand sperm fertility and motility (Entesar, 2015). According to Aitken *et al.* (1998) damage of spermatozoa may be reduced with cryopreservation, however, reduction in ratio of whole sperm depended on freezing and thawing of bull semen Woeleder *et al.*(1997). While, some adverse effects on spermatozoa established as a despair in feasibility percentage, structural integrity, depressed motility and conception rates (Medeiros *et al.*, 2002).

Gholami *et al.* (2010) concluded that, underwrites of sperm cryopreservation to development in of reproductive practices also, most influence origin degree is the superiority of semen frozen. The defined of arginine, as an amino acid which, contain D-and L-forms, on other, hands, l-arginine institute for distress reproductive process, while, it have a protective influence on spermatozoa in contradiction of sperm plasma membrane also, improves cell metabolism. Regarding to l-arginine, low concentrations may be increased sperm motility while high dose decreased sperm motility, moreover it, assistances to encourage acrosome response, sperm chemo taxis, and sperm-egg interaction but metabolism of spermatozoa may be increased at add 0.5 mμ concentration ,in the case of highest concentration may reduce quality during storge (Hassanpour *et al.*, 2010 ; Kaya *et al*., 2017and Abd-allah *et al.*, 2019).

Dietary intake of L-arginine helps in improving semen and fertility parameters in rams; Kaya *et al*., (2019) found that 5 mg/ kg dietary L-arginine for 7 weeks improved libido, increased sperm mass activity, increased spermatozoa motility and density, lowered abnormal spermatozoid ratio and preserved spermatozoa membrane integrity. Moreover, Ahangar *et al*., (2017) state that dietary L-arginine enhanced testes weight and semen volume.

Therefore, this study was carried out to enhance the freezability and conception rate of poor buffalo ejaculates after the addition of L-Arginine to semen extender.

MATERIALS AND METHODS

This study was conducted at International livestock management training center (ILMTC), Sakha Station, Animal Production research Institute. 5 sexually mature healthy Buffalo bulls ranged from 3-4 years old age. Also, were kept in separate containers and nourished on

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DOI: 10.21608/jappmu.2020.161209

recommended ration according to Animal Production Research Institute.

Semen sampling

Semen was collected once weekly by artificial vagina for ten weeks. Immediately following every collection, only ejaculates with low motile spermatozoa (estimated 45 to 50% motility) were pooled to eliminate the bull effect, then diluted with Tris extender to contain $80x10^6$ spermatozoa/ml, which formerly take to 37 ºC and divided into five parts formerly exposed progressively cooling decreased of 37 ºC to 4 ºC for 4 h (as equilibration period) at frozen unit previously being positioned to grasses (0.25 ml French straws). Straws were apprehended for ten minutes at surface of nitrogen liquid (vapor,˜-120°C) previously actuality absorbed formerly kept in liquid nitrogen (-196°C).

Preparation of the extenders

Structure of tris-egg yolk extender enclosing 20 ml of egg yolk, 3.025g and tris hydroxyl methyl aminomethane, also contained 1.675 g , 0.75 g, 7 ml, 0.25 gm , 0.005 gm of citric acid, glucose, glycerol, lincomycin, streptomycin respectively, with accomplished up to 100 ml bidistilled water, this extender was acting as a control. L-arginine (99%+PURITY, Bhiwadi- 301019, Rajasthan, India) was added to Tris basic extender (control) at the concentration of 0, 3, 5, 7 and 10 mM.

Semen processing

Determined percentage of progressive motility, live, abnormal and acrosome integrity were appraised post-dilution, post equilibration, and post-frozen-thawed spermatozoa. Progressive sperm motility was estimated according to Melrose and Laing (1970), live sperm and abnormal sperm according to Salisbury *et al.* (1978), while acrosome integrity estimated according to Watson (1975). But Chromatin integrity was performed post-thawing only according to Erenpreisa *et al.* (2003).

Acrosome Integrity

Acrosome integrity was detected with Giemsa stain procedure which labeled by (Watson, 1975). Diluted one drop semen was smeared on warmed slide which dehydrated in existing warm air, moreover, smears fixed with engagement in 10% buffered saline of formal for 15 min, then washed in consecutively tap water. Regarding to smears were air-dried then absorbed in buffered Giemsa solution in a Coplin jar for 90 min, after which they cleaned temporarily in refined water and dried, then examined under light microscope at magnification 100x by using oil immersion lens. Calculated percentage of normal acrosome for about 200 spermatozoa aimlessly nominated from at least four microscopic fields. Finally, acrosome considered normal when stain was clearly and consistently dispersed ended spermatozoa anterior to equatorial segment.

Chromatin integrity by Toluidine blue Test

Staining, after air drying of smears, ten fixed at concentration of fresh 96% ethanol-acetone (1:1) at 4°C for 30 minutes then hydrolyses in 0.1 NHCl at 4°C for 5 min continuously,slides washed thrice in distilled water for 2 minutes ,finally stained with 0.05% TB in 50% McIlvaine buffer (pH=3.5) for 10 minutes at room temperature. The chromatin quality of spermatozoa was determined according to the metachromatic staining of sperm heads with the aid of light microscopy at ×1000 magnification, a stock solution of 1% TB in distilled water and stored at 48C.Finally stock solution can stored at 48C for up one year (Erenpreisa *et al.* 2003).

Fertility trial

A total of 240 Egyptian Buffalo owned by small and medium scale breeding holders in different villages in Kafr-Elsheikh Governorates were artificially fertilized with arbitrary frozen quantities from numerous extenders, also, each female fertilized with single straw 10 h after start of estrous behavior. Moreover, by used technique of recto-vaginal and worldwide fertilization firearm, thawed semen was dropped in uterine body just next to frontal end of cervix and outset percentage were inveterate with rectal palpation as a minimum 60 days later fertilization.

Statistical analysis

Statistics found was imperiled to analysis of variance (ANOVA) one way with using (SAS,2001) after arcsine transformation and to separate significantly means of different were used Duncan Multiple Range Test (Duncan, 1955) . The model that was analyzing data as stated below:

$$Yij= \mu + Ai + eij,$$

Where:

Yij= Dependent variables,
μ= Mean of population,
Ai= Outcome owing to Larginine (i = 0, 3, 5, 7 and 10 mM),
eij= Experimental error.

RESULTS AND DISCUSSION

Results

Semen Characteristics

Sperm Progressive Motility (%):

In spite of non- significantly ($P \geq 0.05$) differences among different L-arginine extenders and control extender in the percentage of sperm. However, data, declared significantly higher progressive motility post-dilution and process, in all arginine extender with compere post- control equilibration and post-thawing processes (Table, 1).

Table 1. Effects of L-Arginine on progressive motility (%) on spermatozoa of Buffalo bull, at different grade of cryopreservation.

Item	L-Arginine treatment (mM)				
	0.0 mM	3 mM	5 mM	7mM	10 mM
Post-dilution	53.2 ±1.3	52.5±0.94	53.7±1.6	53.7±1.6	54.5±1.8
Post-equilibration (at 5 ºC)	41.6^{B}±1.9	49.1^{A}±2.3	49.3^{A}±1.8	50.0^{A}±1.9	51.6^{A}±1.7
Post–thawing	28.1^{C}±1.6	35.6^{B}±2.7	39.2^{AB}±2.8	41.6^{AB}±1.4	43.4^{A}±1.9

A, B, C, the different superscripts with the same row were significantly (P<0.05) among treatments.

Live spermatozoa (%):

There is no significant ($P \geq 0.05$) differences among extenders contain different L-arginine levels and control extender in live spermatozoa post-dilution stage (Table, 2), however, after both equilibration and frozen-thawing processes, the percentages of live spermatozoa was higher significant in both concentration 7 and 10 mM L-arginine extenders (60.6and 62.3% VS 50.7% and 52.6 and 54.5 VS 36.3%, respectively) compared to that in control extender. Besides, the percentages of live spermatozoa were higher in

both 3 and 5 mM L-arginine extender than control extender Post-equilibration and thawing processes but the differences were not highly significant.

Table 2. Effect of L-Arginine concentrations of extender at spermatozoa live (%) of Buffalo bull with different grade of cryopreservation.

Item	L-Arginine treatment (mM)				
	0.0 mM	3 mM	5 mM	7 mM	10 mM
Post-dilution	69.3 ±1.3	68.2±1.2	70.3±1.5	69.6±1.2	70.7±1.1
Post-equilibration (at 5 ºC)	50.7^{B}±1.7	56.4^{AB}±2.0	57.6^{AB}±1.8	60.6^{A}±1.9	62.3^{A}±1.5
Post–thawing	36.3^{B}±1.8	48.6^{AB}±2.1	49.2^{AB}±2.0	52.6^{A}±1.7	54.5^{A}±1.4

A,B,C, different superscript with the same row was significantly (P<0.05) amongst treatments.

Abnormalities of sperm (%):

The percentages of abnormalities were not different among all extenders studied after the dilution process (Table, 3). However, after both equilibration and thawing processes the percentages of sperm abnormalities in both7 and 10 mM L-arginine extenders were lowest (P<0.05) significantly than in control extender. Also, 5 and 3mM L-arginine extenders were lower (P<0.05) significantly than in the control extender.

Table 3. Effect of L-Arginine concentrations on extender at abnormal spermatozoa (%) of Buffalo bull with different grade of cryopreservation.

Item	L-Arginine treatment (mM)				
	0.0 mM	3 mM	5 mM	7 mM	10 mM
Post-dilution	27.4 ±0.9	26.6±0.5	27.7±0.6	26.3±0.7	27.1±0.8
Post-equilibration (at 5 ºC)	38.3^{A}±1.0	34.3^{AB}±0.8	33.2^{AB}±1.1	30.4^{B}±0.9	31.1^{B}±1.2
Post–thawing	45.2^{A}±1.2	43.7^{AB}±1.0	42.6^{AB}±1.3	40.1^{B}±1.1	38.3^{B}±1.4

A,B,C, different superscript with same row were significantly (P<0.05) amongst treatments.

Acrosome Integrity of sperm (%):

Data presented in Table 4 shows changes in values of acrosomal integrity of moderate motile sperms. The data revealed that adding different concentrations of L- arginine increase the percentage of acrosomal intact of sperms when compared with the control during the whole period of preservation, and best concentrations of L- arginine gave higher percentage of acrosomal integrity sperms in all periods of storage was (7 and 10 mM) with highest significantly differences at associated with control.

Table 4. Effects of L-Arginine on acrosome integrity (%) of Buffalo bull spermatozoa, with different grade of cryopreservation.

Item	Treatment				
	Control	L-Arginine			
	EY 20%	3 mM	5 mM	7 mM	10 mM
Post–dilution	63.1^{B}±2.3	72.1^{A}±3.2	77.7^{A}±2.5	77.2^{A}±3.2	78.3^{A}±4.0
Post-equilibration (at 5 ºC)	54.4^{B}±2.4	68.4^{A}±3.5	73.3^{A}±1.5	74.6^{A}±2.2	75.0^{A}±2.5
Post–thawing	46.5^{C}±1.6	58.7^{B}±3.5	65.4^{AB}±2.7	67.8^{A}±3.1	67.9^{A}±2.7

A,B,C, different superscript with same row were significantly (P<0.05) amongst treatments.

Chromatin Integrity (%):

Data declared that, sperm chromatin integrity percentage was highest significant (P<0.05) at all extenders contained L-arginine compared with the control extender (Table, 5). The highest values were recorded with 7 and 10mM L-arginine extenders compared with the other 3 and 5 mM L-arginine extenders and control extender being 38.25 and 39.50% VS 30.75 and 33.81% and 25.50%, correspondingly.

Table 5. Effects of L-Arginine on chromatin normality (%) of Buffalo bull spermatozoa with different grade of cryopreservation.

Treatment (L-arginine mM)	Chromatin normality (%)
0	25.50^{c}±1.65
3	30.75^{bc} ±2.40
5	33.81^{b} ±1.51
7	38.25^{a}±2.15
10	39.50^{a}±1.44

Conception rate:

Data in (Table, 6) detected the, rate of conception was highest at all arginine extenders than with control extender being 56.92, 53.33, 67.27 and 71.7% VS 45.45% for 3, 5, 7 and 10mM arginine extenders VS control extender, respectively.

Table 6. Rate of conception in Buffalo fertilized by frozen semen cryopreserved with different L-Arginine concentrations.

Item	Inseminated females	Conceived females	Rate of conception (%)
Control (20% EY)	55	25	45.45^{c}
3 mM AR	65	37	56.92^{b}
5 mM AR	60	32	53.33^{b}
7 mM AR	55	37	67.27^{a}
10 mM AR	60	43	71.70^{a}
Overall L-Arginine	240	149	62.08

Discussion

The supplementations l-arginine to poor semen quality significances increased sperm appearances as post thawing motility, livability, abnormalities, acrosome, and chromatin integrity. Concerning sperm motility, the addition of L-arginine in 7 and 10mM concentrations enhances sperm motility following equilibration and freezing-thawing processes by increasing production of nitric oxide which enhances the metabolic rate, it also enhances synthesized of cGMP, which due to rise level of calcium at mitochondria and making highest value in ATP, moreover, these effects may lead to escalation in motility of sperm.this finding is nearly similar to that recoded by (Zini *et al*., 1995; Revelli *et al*., 2002).

Data state L-arginine stimulate poor motility of bull spermatozoa, however, it act as adjuvant in bull semen extender for preserve capability of spermatozoa after cryopreservation in nitrogen liquid .this result in agreement with finding of Al-Ebady *et al.* (2012) but disagree with Öztürk *et al.* (2017) and O'Flaherty *et al.* (2004) whom stated supplementation of bovine semen extender with arginine reduced percentages of post-thawed subjective motility, on other hands, low concentrations of L-arginine had slight influence on sperm gesture limits; while highest level of L-arginine significances reduced precise motion

strictures in ram epididymis sperm. Results is nearly similar with Susilowati *et al*. (2019) who detected the added of 4 mM L-arginine in skim milk extender, which it stowed in cool temperature, maintains goat spermatozoa in the greatest quality. Moreover, added of 1 mm L-arginine to Murrah Buffalo semen improved sperm motility after freeze-thawing. This nearly similar to our study, we can concluded added 7 or 10 mM L-arginine to Buffalo bull extenders improved the ratio of acrosome integrity and other 3 or 5 mM L-arginine extenders. On the other hand, Öztürk *et al*. (2017) found that the percentage of acrosome integrity did not vary significantly with the addition of L-arginine to semen extender. Moreover, AL-Ebady *et al*. (2012) found no significant differences in acrosome integrity between control and treated part of semen with L-arginine after freezing, also is agreement with Siddique and Atreja (2013) whom found the added 1 mm L-arginine addition to Murrah Buffalo semen increased sperm motility after freeze-thawing .

Concerning the sperm chromatin integrity, the addition of different L-arginine concentrations (3, 5, 7 and 10 mM) with the semen extenders led to reducing ($P<0.05$) sperm chromatin damaged compared to the control extender. This in agreement with Öztürk *et al*. (2017) whom determined, used L-arginine with semen extender led to decrease damage DNA ($P<0.05$) compared to the control.

. Concerning the conception rate, the use of frozen-thawed semen supplemented with different L-arginine levels led to an increase ($P<0.05$) fertilized Buffalo compared to control.

In conclusion the, addition of L-arginine delivers a defensive influence by improving the spermatological strictures and defensive chromatin integrity. Treatment of poor buffalo spermatozoa with L-arginine at a concentration of 7 mM and 10mM was considered the best concentrations to be used to decrease the percentage of abnormal sperm, increase the progressive motility and the acrosome integrity of poor motility spermatozoa and conception rate. More detailed studies are required to confirm the benefits of using L-arginine in extender of poor quality semen.

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تأثيرأضافة ل- أرجنين كمكمل للمخففات على حفظ السائل المنوي وخصوبته في طلائق الجاموس.

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تُعد معرفة تأثير إضافة الأرجنين بأكثر من تركيز لتعزيز جودة الحيوانات المنوية الفقيرة لطلائق الجاموس الهدف الرئيسي في الدراسة الحالية. تم جمع السائل المنوي من خمس طلائق من الجاموس مرة واحدة أسبوعياً والتى قدرت حركته الكليه من 45-50٪. تم تخفيف السائل المنوي عند 37 درجة مئوية بواسطة مخفف صفارالبيض (الترس) . تم تقسيم العينات إلى خمسة أجزاء ، الجزء الأول بدون إضافة أى مكمل والذى يعتبر جزء التحكم (الكنترول) بالنسبة للأجزاء الأخرى ، باقى الأجزاء الثاني والثالث والرابع والخامس تمت الاضافة بنسبة 3 و 5 و 7 و 10 ملي لترمن الأرجنين على التوالي. تم تحديد الحركة التقدمية ، النسبة المئوية الحية والنسبة المئوية غير الطبيعية للحيوانات المنوية وسلامة الأكروسومات للحيوانات المنوية ،الحالة الطبيعية للكروماتين، معدل الحمل. أوضحت النتائج أن المخفف بتركيزات 7 و 10 ملي للأرجينين قد تحسنت عن جميع المعاملات التي تمت دراستها خلال التركيزات 3 و 5 ملي مولار من مخففات الأرجنين ومخفف الكنترول في عمليات الحفظ بالتبريد المختلفة. تم الكشف عن إختلاف كبير في سلامة الكروماتين في جميع المعاملات. كذلك كان معدل الحمل أعلى في إناث الجاموس الملقحة صناعياً بتركيز 7 و 10 ملي مولار أرجنين (67.27 و 71.70٪) مقارنة مع الكنترول (45.45٪). بشكل قاطع ، يمكن الاستنتاج أن إضافة الارجنين بتركيزات 7 أو 10 mM إلى مخفف الترس أدى إلى تحسن الحفظ بالتبريد وكذا الخصوبة فى عينات السائل المنوى ذو الحيوية الفقيرة لطلائق الجاموس.