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L-ARGININE AS SEMEN ADDITIVE TO IMPROVE POOR MOTILITY OF SPERMS CRYOPRESERVED IN LIQUID NITROGEN FOR HOLSTEIN BULLS BORN IN IRAQ

Hajer Kadhem Shahad[1], Souhayla Oneeis Hussain[1] and Jawad Kadhim Arrak[2]
[1]Department of Surgery and Obstetrics, College of Veterinary Medicine,University of Baghdad, Iraq
[2]Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Baghdad, Iraq

ABSTRACT

In an attempt to find a suitable media including L-Arginine as semen diluent to improve semen quality during frozen storage in liquid nitrogen up to 48 hr., this study was conducted. A total of 32 ejaculate collected by artificial vagina from. Nine Holstein Friesian bulls born in Iraq which have same ages, the ejaculates which have individual motility more than 40% dilution with tris and the diluted semen was divided into four aliquots including control and three experimental groups. Each aliquot was further diluted with an equal volume of a Tris based extender without addition (control) or containing 0.005 mM/ml (T1), 0.006 mM/ml (T2) and 0.007 mM/ml (T3) L-Arginine. All diluted semen cooled to 5 C° and evaluation (individual motility%, dead%, abnormality% and acrosome defect%), filling in mini straws and frozen in liquid nitrogen, after 48hr of storage, thawing frozen semen in 37 C° for 30 sec and evaluated same physical properties which done after dilution semen.

Results revealed that individual motility percentage in T1 was significant (P<0.05) compared with the control and other treatment T 2and T 3, but dead and abnormality sperms percentage decreased significant (P<0.05) in treated T1 compared with control, T2 and T3. Moreover all treatment (T1, T2 and T3) showed a significant decrease (P<0.05) in acrosome defect compared with control. In addition the present study show that freezing step caused significantly increase (P<0.05) in dead%, abnormality% and acrosome defect%. In conclusion: Based on this study it was concluded that addition of 0.005 mM/ml to the extender improved the motility of spermatozoa% , but decrease dead%, abnormality% and acrosome defect% compared to the controls and others concentrations during cooling and post freezing in liquid nitrogen storage.

Keyword: Poor motility, caffeine, Holstein bulls, Iraq.

Introduction

Semen cryopreservation is a unique technique for preserving genetic diversity, especially endangered species (Bhakat *et al*., 2011). The most widely used technique for artificial breeding of cattle and buffalo requires a perfect medium to extend and preserve the semen ejaculates from elite bulls for the sake of exploitation of superior quality male germplasma to the maximum possible extent. Various components which combine to make semen extender are such that they possess all those properties which protract the longevity of spermatozoa in extended form during harsh ambient conditions and cryopreservation. A plenty of insults faced by spermatozoa during storage envisaged osmotic changes, pH fluctuations, energy depletion during metabolism, cold shock and cryo-damages during freezing-thawing procedures. During cryopreservation, cholesterol to phospholipid ratio of sperm bio-membranes gets disturbed mainly due to cholesterol efflux and generation of numerous reactive oxygen species (ROS). All these disturbances directly compromise spermatozoa fertility. Therefore, a combination of good quality semen extender and additives must be used in such a way that fertility of spermatozoa can be retained outstandingly during semen preservation (Choudhary *et al*., 2018) Though, oxidative stress and cold shock along with increasing cAMP permeability cause impairment in post-thaw spermatozoa motility and lifespan (Graham *et al*., 2011). Oxidative damage result in a decrease in intracellular ATP levels which in turn decreases sperm motility and also induces lipid peroxidation in polyunsaturated fatty acids rich plasma membrane of spermatozoa (Alahamar, 2018). Such events have been related with increased permeability of plasma membrane, enzyme deactivation and production of metabolic end product that highly toxic to spermatozoa (Spermicidal. High concentration of poly unsaturated fatty acids in ram sperm membranes and limited enzymatic antioxidant defense system [such as: glutathione peroxidase, superoxide dismutase, and catalase make them more susceptible to oxidative stress damages induced by reactive oxygen species (ROS) especially during freeze-thawing (Asadi *et al*., 2017). Accordingly, a pile of researches focused on counteracting the reverse effects of ROS on sperm fertility using different types of antioxidants (Agarwal, 2017; Anel-Lopezi (2017). Arginine; Is α-amino acid that is used in biosynthesis of protein, it contains an α-amino group, an α-carboxylic acid group and a side chain consisting of 3-carbon aliphatic straight chain ending in guanidino group, L-Arginine is an amino acid that can stimulate the motility of mammalian

spermatozoa under in vitro conditions and play an important role in the defense of cellular immunity (Srivastava *et al*., 2006). L-Arginin protects spermatozoa against lipid peroxidation by increasing the production of nitric oxide and deactivating free radicals. Nitric oxide has been shown to be an antioxidant and beneficial to reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2) Al-Ebady, *et al*., 2012).

Materials & Methods

Animals and semen collection: Thirty two poor ejaculates (individual motility 35-40%) were collected twice weekly using the artificial vagina, from eight Holstein bulls born in Iraq. The bulls utilized in this study aged (3-4 year) live in same environment in Artificial insemination center west Iraq (Abu Ghareeb). The ejaculates were transferred to the laboratory and immersed in a water bath (34˚C) until semen evaluation.

Semen processing: individual motility: Estimated by mixing one drop of raw semen with two drops of 2.9% sodium citrate solution on a warm slide at 37˚C, then the mixture covered by cover slide and examined under light microscope at 40x magnification, motility was scored on the basis of the percentage of spermatozoa with normal forward progressive movement, whereas those showing circling movements or those oscillating at one place were regarded as immotile. The score was calculated as (Chemineau *et al*., 1991). Dead: dead percentage estimated by differential staining technique using Eosin-Nigrosin stain (Douglas & Kenneth, 2013). The composition of the stain included Eosin-Y. 1.67 gm and 10 gm of Nigrosin in 100 ml of 2.9 % Sodium citrate buffer, the stain was matured and then used, The smears were prepared induplicate after mixing a small drop of neat semen with four drops of stain on a clean grease free microscopic slide at 370C. Two clean slides were prepared, one of which was used to prepared thin smear for the purpose of estimating the dead sperm percentage and the other for the abnormality. Two Hundred spermatozoa were counted under the objective (40X) of a phase contrast microscope for estimating the percentage of live (unstained) spermatozoa, the pinkish (eosinophilic) and partially stained spermatozoa were classified as dead (Hafez & Hafez, 2000). Abnormality: The other slide that was attended by the smear was counted 200 sperm under oil immersion of a phase contrast microscope for estimating the percentage of abnormalities (Evans & Maxwell, 1987). Acrosoma integrity percentage by using Giemsa staining as described by Chowdhury *et al*. (2014).

Experimental diluters:

Each ejaculate were collected from eight bulls split into four parts and diluted to concentration 30×10^6 sperm /ml as follows:

1- Part 1 : Diluted in EYTG (Control)

2. Part 2 : Diluted in EYTG + 0.005 ml/mol caffeine (**T 1**)

3. Part 3 : Diluted in EYTG + 0.006 ml/mol caffeine (**T 2**)

4. Part 4 : Diluted in EYTG + 0.007 ml/mol caffeine (**T 3**)

Semen diluter was cooled to 5ºC and evaluation was done including individual motility%, dead%, abnormality% and acrosome defect%, after that all semen diluters equilibrated in an equilibration chamber at 5°C for 4 h before filling in 0.25ml French straws. The straws were placed on steel racks and held in liquid nitrogen at -120°C for 9 min. Frozen straws were then immediately immersed in liquid nitrogen (-196°C) and stored for 48 hrs. until further assessment. At the time of analysis, two straws of semen from each treatment were thawed at 37°C for 30s to perform the post thawing semen quality such as individual motility%, dead%, abnormality% and acrosome defect%.

Statistical analysis: The experiment was conducted. Results are quoted as Mean ± SE. Statistical analyses were carried out using the General Linear Model procedures (GLM) of SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Comparisons between values were analyzed by Duncan's multiple range test following an F-test in ANOVA (Duncan. 1955). Significance was set at ($P<0.05$).

Results

Data for influence of caffeine on frozen-thawed bull semen in TRIS diluter are presented in Table 1.

Effect different concentration of L-Arginine on some semen properties:

Individual motility sperm%:

It is clear from Table 1. After cooling diluted semen with addition L-Arginine at a concentration T1 (47.39±2.11) caused a significant ($P<0.05$) increase in the individual motility percentage of sperm compared to the concentrations T2 (35.00±2.70) and T3 (28.86±2.61) and control (31.56±1.87). Also T2 give a significant ($P<0.05$) increase in the individual motility percentage compared to the control treatment and T1, while no significant differences appeared between control and addition Caffeine at a concentration T1. As shown in Table 1 during After freezing, the addition of caffeine by concentration T3 (50.00±2.79) led to a significant ($P<0.05$) increase in individual motility for sperm compared to the T1(27.19±2.23)., T2 (32.50±2.46) and control (30.87±1.39), moreover the addition of L-Arginine at concentration T2 significantly ($P<0.05$) outperformed the control, but no significant differences emerged between the T3 and T2, as well as between T1 and control (27.18±1.53). It was also noted that the overall mean individual motility of sperms in T1increase significant ($P<0.05$) compared to the T2 and T3 and the control, (Table 1).

Dead sperm %

It is clear from the results proven in Table 1 that in the cooling step, there was a significant ($P<0.05$) decrease in the percentage of dead sperm in the T1(15.59±0.45) and T2(17.30±0.71) compared to the control (19.86±0.42), and this was also observed in post- freezing, but only the T2(23.44±1.02) and T3(26.89±1.05) treatments compare with T1(19.05±0.65), and control (30.65±1.11), but no significant differences appeared between the T1,T2 and T3 and between T1 and control during cooling step, but in post-freezing the differences were no significant between T1 and T2 and between T3 and control, (Table 1).

Table 1 : Effect of adding different concentration of L-Arginine to tris diluent on individual motility percentage of Holstein bulls born in Iraq (Mean ±SE)

Physical characters	Treatment	After cooling	Post thawing
Individual Motility %	**Control**	30.87±1.39 **Ca**	19.82±1.34 **Cb**
	T1-0.005 mM	47.39±2.11 **Aa**	40.45±2.28 **Ab**
	T2-0.006 mM	35.00±2.70 **Ba**	27.37±2.71 **BCb**
	T3-0.007 mM	28.86±2.61 **Ca**	19.00±1.91 **Cb**
Physical characters	**Treatment**	**After cooling**	**Post thawing**
Dead %	**Control**	19.86±0.42 **Ab**	30.65±1.11 **Aa**
	T1-0.005 mM	15.59±0.45 **B** a	19.05±0.65 **Ca**
	T2-0.006 mM	17.30±0.71 **ABb**	23.44±1.02 **BCa**
	T3-0.007 mM	17.94±0.59 **ABb**	26.89±1.05 **Aba**

Within row different small letters for each parameter means significant at (p<0.05).
Within Column different large letters for each parameter differed significantly (p<0.05).

Abnormality sperm%

The results shown in Table 2 revealed that after cooling the diluted semen and with addition of L-Arginine, there was an improvement in the quality of semen and through an achievement was significant (P<0.05) decrease in the abnormality sperm percentage compared to the diluted semen without caffeine , as it was noticed that T1(11.75±0.72) was the sperm abnormalities in it significantly (P<0.05) less than T2(14.67±0.77), while no significant differences appeared between control and T3, nor between T1 and T2. It is also evident from Table 2 that in post-freezing, treatment T1 (14.07±0.79), T2(18.19±0.54) and T3 (21.73±1.01) showed a relatively significant decrease (P<0.05) in abnormalities percentage than control (26.27±0.88), and T2 and T3 recorded less significantly (P<0.05) abnormality sperm percentage from T1, while no significant differences appeared between T1 and T2. In Table 2, it was noted that sperm abnormality in the three treatments were significantly (P<0.05) less than control, and also significant (P<0.05) differences appeared between T1 and T3while no significant differences appeared between T1 and T2on the one hand and between T2 and T3 of on the other hand.

Acrosome defect sperm

By observing Table 2 regarding the effect of adding L-Arginine to a tris diluent on the percentage of acrosoma defects of sperm, it was observed that adding caffeine T1, T2 and T3

Table 2 : Effect of adding different concentration of L-Arginine to tris diluent on sperm abnormality and acrosome defect percentage of Holstein bulls born in Iraq (Mean ±SE)

Physical characters	Treatment	After cooling	Post thawing
Abnormality %	**Control**	20.2±0.4**Ab**	26.27±0.88**Aa**
	T1-0.005 mM	11.75±0.72**Ca**	14.07±0.79**Ca**
	T2-0.006 mM	14.67±0.77 **BCa**	18.19±0.54**Ba**
	T3-0.007 mM	16.00±0.90 **Bb**	21.73±1.01 **Aba**

Physical characters	Treatment	After cooling	Post thawing
Acrosome defect %	**Control**	5.50±0.74 **Ab**	19.76±1.17 **Aa**
	T1-0.005 mM	2.07±0.39 **Ab**	10.56±0.76 **Ca**
	T2-0.006 mM	3.13±0.59 **Ab**	14.56±0.71 **Ba**
	T3-0.007 mM	3.69±0.47 **Ab**	17.43±1.08 **Aba**

Within row different small letters for each parameter means significant at (p<0.05).
Within Column different large letters for each parameter differed significantly (p<0.05).

after cooling T1 show less acrosome defect (2.07±0.39) in a significant (P<0.05) way compared to control (tris diluent only), but the differences significant after thawing between T1,T2 and T3, as it becomes clear From Table (2).The the percentage of acrosoma defects was significantly (P<0.05) lower in the T1(10.56±0.76), T2(14.56±0.71) and T3(17.43±1.08) compared to the control (19.76±1.17), whereas no significant differences appeared between the three concentrations T1,T2 and T3 during after cooling or post- freezing, as well as the overall mean.

Discussion

Effect different concentration of L-Arginine on some physical properties of bull semen:

Improvement of semen quality for storage and artificial insemination was achieved by enhancing the activity of sperms; our study was carried out to investigate the effects of different concentrations of L-Arginine on movement of bovine frozen semen. The results showed that L-Arginine increased bovine sperm motility depending on the concentration applied. The positive effects of L-Arginine on sperm motility were also demonstrated .The tested concentrations of L-Arginine improved the progressive movement of sperms when added to the semen in vitro. However, the beneficial effect of L-Arginine was observed upon using 0.005 mM and 0.006 or 0.007 mM, respectively. It has been reported that intracellular calcium and immediate hyper activation were increased by the addition of L-Arginine to sperm (Chen, *et al*., 2018).

The degree of cryo-damage also depends on several factors (Watson, 2000) which limit the survival of spermatozoa during incubation. Under the best experimental

conditions about half of the population of motile sperm survives the freeze thaw process (Sanchez-Partida *et al.*, 1999; Watson, 2000). In the present study it was observed that controlled rate of cooling and freezing resulted in significantly higher sperm total and progressive motility, compared to uncontrolled rate of cooling and freezing. The fact that progressive motility is more affected by the freezing process than individual motility implies that these parameters measure different aspects of cell physiology and in particular, that the physiological basis for the progressive motility parameter is more sensitive to cryobiological damage (Anel *et al.*, 2003). The controlled-rate cooling protocol, besides providing complete automation in the cryopreservation process, might also protect spermatozoa against some adverse effect caused by minor fluctuation in temperature imposed by the transfer of cooled straws from cold cabinet to cell freezer as done in the uncontrolled cooling rate ram semen freezing protocol. Apart from identifying motile and static spermatozoa CASA can also categorize spermatozoa on the basis of velocity of each motile sperm, measure the mean sperm velocity and related sperm track dimensions (Joshi *et al.*, 2003). The measurement of sperm velocity has been considered as an indirect indicator of mitochondrial function in spermatozoa. During cryopreservation spermatozoal mitochondria undergo damages (Gillan *et al.*, 2004; Peris *et al.*, 2004). The freezing process negatively affects ($P < 0.05$) the sperm parameters (individual motility, dead and abnormality), agreement with (Üstuner *et al.*, 2015). The kidding rate after artificial insemination with frozen and thawed semen is poorer than with fresh or chilled semen (Batista *et al.*, 2009), but most properly freezing and thawing procedures had negative effects on motility and acrosome integrity (Üstuner *et al.*, 2015). Hussain *et al.* (2016) reported that significant decrease in individual motility and increase in dead and abnormalities percentage for both poor and good ejaculate during different steps, dilution, cooling and freezing of bull semen, this might be attributed to the fact that lactic acid which produced as an end product of sperm metabolism, resulting in harmful lowering of PH which exerts toxic effect on sperm cell (Ball & Peter, 2004). The considerably reduced values for sperm motility, viability, morphology, and plasma membrane/ acrosome integrity observed after cryopreservation of semen over fresh or pre-freeze stage (Chaudhari *et al.*, 2015). On individual motility, the current results in (Table 1) shows that the best concentration of L-Arginine (0.005 molar) is better than (0.006 and 0.006) which give significant increasing in individual motility in comparison with control samples, Al-Arifi (2005) and Al-Ebady (2010), whose reported that extender supplement with (0.005 molar) results in greater sperm motility in buffalo and bull sperm, but (Foote, 1964), who mentioned that the decrease PH of sperm motility may reduce sperm metabolism and anaerobic heat production by spermatozoa which is associated with longer motility during storage, (Uysal, 2007) who referred in his study that high doses of L-Arginine may causes toxic product that give low motion also, (Chen *et al.*, 2018) shows that L-Arginine have effect on spermatogenesis process and sperm motility (positive relationship).

Dead percentage in (tab. 1) showed many variation in the values of dead during treated with L-Arginine during cooling and cryopreservation, in all period (0.005 molar) shows best one on significant decreasing dead sperm when compared with other concentration (high significant) and control samples(no significant), Moncada and Higges (1993) and Pacher *et al.*, (2007), reported that L-Arginine protects against lipid peroxidation by increasing nitric oxides which is a free radical has actualy been shown to be beneficial antioxidants agents (ROS), also (Al-Arifi, 2005) reveal as above on bull semen that kept at 5°C for 5 days by using catalase enzymes decomposition H_2O_2, which cause lipid peroxidation of sperm membrane during storage. Abnormalities in (tab. 2), best concentration that gave low significant abnormalities was (0.005 molar) ($p< 0.05$) in comparison with control and other concentrations, (Al-Ebady, 2010) was revealed that (0.005 and 0.006) molar shown decreasing values in spermatozoa abnormalities percentages, but (0.007 and 0.008) in the same study was disagreed with us, which leaded high significant values ($p< 0.05$).

In Acrosome defects properties as in dead values (tab. 2), also (0.005 molar) give low significant acrosome defect in compared with other different concentrations of L-Arginine which obtained high significant acrosome defect during cooling and cryopreservation periods, due to increase level of superoxide radical leads to increases membrane fluidity of sperm membrane which play important role in sperm maturation in mice (Kumar, 1993), in ram (Voglamyar, 1984) and in bull (Al-Ebady, 2010), so this membrane fluidity by the controlled peroxidation of membrane phospholipid by reactive oxygen species (Jain *et al.*, 1993, Belen Herro *et al.*, 2000), similar with (Al-Arifi, 2005) in bull sperm and (Medan *et al.*, 2008) in camel, by using catalase enzyme as antioxidants decrease lipid peroxidation of sperm membranes during storage.

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