Global Veterinaria 2 (4): 146-150, 2008
ISSN 1992-6197

Use of Selected Amino Acids to Improve Buffalo Bull Semen Cryopreservation

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Abstract: This study was designed to evaluate the effects of selected amino acids (glutamine, glycine, alanine and cysteine) on cryopreservation of buffalo bull spermatozoa. Semen was collected at weekly intervals from five mature buffalo-bulls kept at Buffalo Semen Freezing Center, General Organization for Veterinary Services, Ministry of Agriculture, Abbasia, Egypt. Semen was diluted with Tris Citrate Fructose Yolk (TCFY) extender supplemented with different concentrations of amino acids (25, 50 and 100 mM glutamine, glycine, alanine and 5 mM cysteine) then processed for cooling and freezing. Semen quality parameters (subjective motility, membranes and acrosome integrity) were examined after cooling and freeze-thawing. Results demonstrated that both glutamine and glycine treatments showed positive effects on post-thaw motility, membrane and acrosome integrity when added to TCFY extender at 25 mM concentration. The Further increase of these amino acids concentrations to 100 mM caused a significant ($P<0.01$) reduction in sperm motility, membrane and acrosome integrity. Cysteine addition to extender resulted in significant ($P<0.01$) enhancement of post-thawing sperm motility and improvement in membrane and acrosome integrity. In conclusion, the addition of 25 mM glutamine, glycine and 5 mM cysteine in conventional freezing medium enhanced post-thaw motility and improved membrane and acrosome integrity of buffalo bull semen.

Key words: Cryopreservation · buffalo- bull · semen · glutamine · glycine · cysteine

INTRODUCTION

The discovery of the biological role of amino acids in prevention of cell damage during freezing stress come from the observation that a variety of plants are able to accumulate the amino acid proline in response to cold temperature [1] and, it has also been reported that some amino acids protect several type of animal cells against freezing stress including sperm [2-4]. Some amino acids, particularly glutamine and glycine, are formed in substantial amount from glucose within seminiferous tubules [5]. Rete testes fluid has 17 times higher acidic amino acids than seminiferous tubules fluid and glutamate contributes 90% of total amino acids within epididymal fluid [6]. Several amino acids (e.g glutamine, glycine, proline and histidine) has been detected in seminal plasma and used successfully as non permeating cryoprotectants to cryopreserve spermatozoa of many mammalian species including goat, ram and stallion [4,7-9], however, the exact role of these amino acids in sperm physiology was still unclear.

It was observed that addition of amino acids with glycerol improve post-thawing sperm motility in goat [7]. Also, use of glycine betaine and proline could improve motility of frozen-thawed ram sperm in presence of glycerol and egg yolk if used at low concentration [8]. [10] recorded that human and stallion frozen-thawed sperm motility and fertilizing ability were significantly improved when 80 mM glutamine was added to the extender.

The main goal of this study was to asses the optimal concentrations of glutamine, glycine, alanine and cysteine amino acids that could be used in bovine semen extender for preserving buffalo-bull sperm motility during freezing and thawing. This development may eventually be a step to obtain a chemically suitable extender for buffalo bull semen.

MATERIALS AND METHODS

Semen collection and initial evaluation: Five mature buffalo-bulls maintained at Buffalo Semen Freezing Center, General Organization for Veterinary Services,

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Ministry of Agriculture, Abbasia, Egypt, were used as semen donors. Ejaculates were collected using an artificial vagina at weekly intervals for 5 weeks. Semen samples were initially evaluated for subjective sperm motility and sperm concentration. Ejaculates fulfilling minimum standard of sperm motility (70%) and sperm morphology (80%) were processed for freezing. The ejaculates were pooled in order to have sufficient semen for a replicate and to eliminate the bull effect. The semen was given a holding time for 10 minute at 37°C in the water bath before dilution.

Semen processing: The reference cryopreservation extender (control) was Tris-citric acid-fructose egg yolk (TCFY) diluent, prepared according to [11]. The amino acids, glutamine, glycine, alanine, were obtained from Sigma Chemical Co.(USA) and added to the control extender at the concentrations of 25, 50 and 100, while cysteine was added at concentration of 5 mM. [7,26]. Eleven aliquots of semen were diluted at 37°C with each extender in order to provide concentration of 60 million sperm/ml. Extended semen was cooled slowly (approximately for 2 hrs) to 4°C and equilibrated for 4 hrs. Semen was packed into 0.5 ml polyvinyl French straws. After equilibrium periods, the straws were placed horizontally on a rack and frozen in a vapour 4 cm above liquid nitrogen for 10 minutes and were then dipped in liquid nitrogen.

Assessment of semen quality parameters: The assessment was undertaken on neat semen, after cooling and freeze thawing of buffalo bull spermatozoa. Frozen straws were thawed at 37°C/ 1 minute. The parameters studied were subjective sperm motility, sperm membrane integrity and normal intact acrosome percentage.

Sperm motility: Sperm motility % assessed subjectively using microscope set at magnification of 400 and equipped with a heating plate (37°C). Visual motility was assessed microscopically with closed circuit television [12].

Sperm membrane integrity: Sperm membrane integrity % was assessed using the hypo-osmotic swelling test HOS test as outlined by [13]. Two hundreds spermatozoa were assessed and the percentage of spermatozoa with curled tails (swollen/ intact plasma membrane) was calculated.

Acrosome integrity: The acrosome integrity % was evaluated under x 1000, using Giemsa staining, a method adopted by [14].

Statistical analysis: The results expressed as mean± standard errors. The data were processed using statistical analysis program [15], user guide 6.04. The ANOVA was used to study the effect of amino acids at various concentrations. In the statistical models, the effect of amino acids was the main source of variance. In addition, differences among means were compared with LSD test at $P<0.05$.

RESULTS

The effect of adding selected amino acids (glutamine, glycine, alanine and cysteine) to semen extenders used for processing of buffalo-bulls semen on sperm motility, sperm membrane and acrosome integrity after cooling and post-thawing were summarized in Table 1 and 2.

The addition of 25 mM glutamine and glycine to the control freezing extender caused a significant ($P<0.01$) increase in sperm motility, after cooling. However, no further increase could be noticed when these amino acids were added at a concentration of 50 mM. The further increase in glutamine, alanine and glycine concentration to 100 mM caused a significant ($P<0.01$) reduction in sperm motility, membrane and acrosome integrity % as compared with 25 mM concentration (Table 1).

Table 1: Effect of different concentrations of selected amino acids on characteristics of buffalo-bull spermatozoa after cooling (Mean±SE)

		Semen characteristics		
Treatment		Motility	Membrane integrity	Acrosome integrity
Control		60.50±1.74	71.10±1.57	65.30±1.73
Glutamine	25mM	67.00±2.00^{a*}	75.10±2.01^{a}	72.10±2.75^{a*}
	50mM	62.00±1.33^{b}	71.20±1.84^{b}	67.70±2.19^{a}
	100mM	55.00±2.00^{c*}	64.60±1.59^{b}	60.00±1.71^{b}
Glycine	25mM	67.00±1.7^{a*}	72.20±2.87^{a}	66.90±2.64^{a}
	50mM	63.50±1.29^{a}	70.20±1.52^{a}	62.30±1.85^{b}
	100mM	55.00±1.7^{b*}	57.60±1.69^{b*}	60.20±1.05^{b}
Alanine	25mM	58.00±1.30^{a}	69.10±2.82^{a}	61.80±2.80^{a}
	50mM	53.00±1.05^{b*}	60.60±0.85^{b*}	61.70±1.95^{a}
	100mM	52.00±1.12^{b*}	55.60±2.32^{b*}	58.00±2.11^{a*}
Cysteine	5mM	65.00±2.47*	72.80±2.48	69.60±1.33

Within each amino acid, means with different alphabetical superscripts are significantly different at least at $P<0.05$. Within columns, (*) indicates significant difference (at least at $P<0.05$) of a given element from control

Table 2: Effect of different concentrations of selected amino acids on post-thawing characteristics of buffalo bull spermatozoa (Mean±SE)

Treatment		Semen characteristics		
		Motility	Membrane integrity	Acrosome integrity
Control		32.50±1.53	41.10±1.66	50.30±2.89
Glutamine	25mM	41.00±2.56^{a*}	50.20±2.17^{a*}	59.00±1.97^{a}
	50mM	36.00±2.45^{a}	49.40±3.44^{a*}	52.40±1.92^{a}
	100mM	26.50±1.07^{b}	32.20±1.57^{b*}	39.00±1.97^{b*}
Glycine	25mM	42.50±2.59^{a*}	51.30±4.12^{a*}	61.30±2.54^{a*}
	50mM	41.00±3.31^{a*}	47.40±2.95^{a}	57.60±2.78^{a*}
	100mM	33.00±1.86^{b}	33.30±4.45^{b*}	37.00±2.11^{b*}
Alanine	25mM	29.50±1.38	42.90±2.06^{a}	51.00±3.35^{a}
	50mM	31.00±2.08	37.00±2.80^{a}	45.00±2.99^{a}
	100mM	29.50±2.03	30.00±2.01^{b*}	38.30±1.390^{b*}
Cysteine	5mM	42.50±3.96*	53.90±1.98*	58.30±2.99*

Within each amino acid, means with different alphabetical superscripts are significantly different at least at P<0.05. Within columns, (*) indicates significant difference (at least at P<0.05) of a given element from control

Addition of 5 mM cysteine resulted in significant (P<0.01) increase in sperm motility, membrane and acrosome integrity % after cooling. Acrosome integrity % was significantly (P<0.01) improved after cooling when glutamine added to freezing extender at 25 mM concentration (Table, 1).

The addition of 25 mM glutamine to TCFY extender caused a significant (P<0.01) increase in post-thawing sperm motility and membrane integrity % as compared to the control extender. Membrane integrity % was significantly (P<0.01) higher when glutamine added at 50 mM concentration, however, at concentration 100 mM glutamine significantly (P<0.01) reduced the sperm motility, membrane and acrosome integrity % as compared to 25 and 50 mM glutamine concentration. Also, the addition of 25 mM, glycine significantly (P<0.01) increased all studied sperm parameter, but further increase in glycine concentration to 100 mM resulted in significant (P<0.01) reduction in sperm membrane and acrosomal integrity %.

No significant effect was detected for alanine on post-thawing sperm quality parameters at concentration of 25 and 50 mM, but at concentration 100 mM a significant reduction in post-thawing membrane and acrosome integrity occurred.

Cysteine addition to freezing extender caused a significant (P<0.01) improvement in post-thawing sperm motility, membrane and acrosome integrity %.

DISCUSSION

In the present study, additions of selected amino acids (glutamine, glycine and cysteine) to cryopreservation media significantly enhance freezing-thawing sperm motility % when added at a concentration of 25 mM for glutamine and glycine and 5 mM for cysteine with no further significant enhancement of sperm motility could be noticed when glutamine and glycine added at concentrations of 50 mM. The further increase in all amino acids concentrations to 100 mM significantly decreases sperm motility %. These results are similar to those of [3] who showed that glutamine addition to the INRA82 extender significantly increase the motility parameters of stallion spermatozoa after freezing and thawing for concentration of 30 to 80 mM and ineffective when added at a concentration of 120 mM and that 160 mM significantly decreased motility. Also, [8] noticed that 53 mM glycine betaine offered protection for frozen thawed ram spermatozoa, but that higher concentration of the amino acids did not improve the result. [7] reported that lower concentration (20-70 mM) of glycine, aniline and glutamine manifest their cryoprotecting efficacy on goat sperm in the presence of glycerol or dimethyl sulfoxide. [9] suggested that glutamine could have synergistic cryoprotective role with glycerol on cryopreservation of stallion spermatozoa and its penetration to sperm cells is very low, thus glutamine may play a cryoprotective role at extracellular level. [4] showed that glutamine has a cryoprotective effect during the freeze/thaw process of goat sperm. Previous studies reported that the optimal concentrations of glutamine in cryopreservation extender were 25 mM in goat [4], 10 mM in bull [17], 30 mM [3] or 50 mM in stallion [9] and in man [10].

[18] recorded that glycine significantly improved sperm motility in cooled and frozen-thawed striped bass spermatozoa. Addition of glycine to the extender had a positive effect on bovine sperm motility [19] when used short term refrigerated storage. This effect might be due to the fact that glycine was metabolized by bovine spermatozoa [19] and is beneficial to sperm by reducing lactic acid accumulation in extenders [20-21]. The data revealed cysteine significantly improve post-thawing sperm motility, membrane and acrosome integrity. This result was in full agreement with [26]. A recent report by [27] demonstrated that thiol-radicals containing amino acids, such as glutathione, cysteine, N-acetyl-cysteine and 2 mercaptoethanol, prevented hydrogen peroxide-

mediated loss of sperm motility in frozen-thawed bull semen. Both cysteine and N-acetyl-cysteine are precursors of intracellular glutathione biosynthesis [28].

The mechanism of sperm protection by amino acids still unclear and is not completely understood. A variety of hypotheses have been proposed by various authors to explain the protective mechanism of amino acids during the freeze/thaw process. [22] reported that certain amino acids, in combination with glycerol, protected calcium ATPase during the changes of state of the freezing medium. [23] demonstrated that sodium glutamate protected certain enzymes. Amino acids are charged molecules [24], it is possible that they electrostatically interact with the phosphate groups of sperm plasma membrane phospholipids, thereby forming a layer on sperm surface that protects it against thermal shocks. Also, they may contribute to sperm osmolarity [25] and can have a positive role in sperm vitality [19].

The toxicity of higher amino acids concentration during freezing-thawing process has long been recognized in several previous studies in goat buck [4], ram [8], stallions [17, 9] and human spermatozoa [11]. [9] explained the toxic effect of higher amino acids concentrations is mainly due to their osmotic toxicity and hypertonicity.

In conclusion the current results demonstrated that addition of 25 mM of glutamine, glycine and 5 mM cysteine increase the cryoprotecting efficacy of buffalo bull cryopreservation extender and this will help us to define the suitable extender for freezing of buffalo bull sperm. However, further studies still needed to recognize the effect of such addition on *in vitro* and *in vivo* fertility in farm animals.

ACKNOWLEDGMENTS

We acknowledge all staff of Buffalo Semen Freezing Center and Dr. M. El-Husseiny in the General Organization for Veterinary Services, Ministry of agriculture, Egypt, for their kind assistance during this study.

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(Received: 6/2/2008; Accepted: 5/3/2008)