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RESEARCH ARTICLE

In Vitro Evaluation and Post-thawing Activation of Najdi, Arabi (Najdi × arabi) Rams Spermatozoa Using Amino Acids and Pentoixfilline Added to Tris Extender

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Abstract

This study was done on pooled semen ejaculates of three groups of rams, Arabi, Najdi and their crosses collected by artificial vagina, semen was evaluated before dilution and after thawing from freezing for one month. Dilution of ram's semen was done by Tris extender as a standard diluents and its semen parameters was evaluated compared with parameter of extenders after addition of glycine and cysteine amino acids. Then all spermatozoa of all diluents were in vitro activated using CO^2 incubator for one hour after addition of pentoxiphilline. Also a new seminal diluents was used which is 5% of beet pulp juice of pH 7. Results revealed that dilution with extender having amino acids have significantly better results than Tris standard extender. Also in vitro activation after addition of pentoxifilline gave same results. It is obvious that glycin extender had the better results followed by cysteine extender then Tris standered extender and lastly the extender of beet pulp juice.

Keywords: *Extender, Rams spermatozoa, Amino acid, Beet pulp.*

Introduction

Cryopreservation induces partially damage to sperm membrances [1,2] which may decrease sperm motility, viability and fertilization rate after artificial insemination [3].Damage during cryopreservation has been attributed to cold shock, ice crystals formation, oxidative stress, cryoprotectant toxicity, osmotic changes and lipid protein reorganization within the cell membrane [4,5].

It is suggested that membrane is thought to be a primary target of chilling or freezing damage in cells [6]. Mammalian sperms characteristically contain high concentration of poly unsaturated fatty acids in their membranes and they lack a significant cytoplasmic component containing antioxidants.

Therefore sperm cells are highly susceptible to lipid peroxidation by free radicals such as H_2O_2 [7]. Which lead to the structural damage of sperm membranes during the freezing-thawing process [8]. It is well known that ram sperms have a higher poly unsaturated/ saturated fatty acid ratio. This can make the ram sperms more sensitive to cold shock and peroxidative damage than those of other species with subsequent loss of membrane integrity of the acrosomal region and impaired cell function [9] .Based on this information the composition of extenders and suitable cryoprotectants seems to play an important role for successful semen cryopreservation [10].

Therefore in recent years different antioxidants have been used to protect spermatozoa from the deleterious effects of cryopreservation and free radicals are eliminated by antioxidant systems [11].Since the discovery of the biological effects of amino acids in prevention of cell damage during freezing stress numerous studies investigated their protective effects on different types animal cells against freezing damage including sperms [12].

Furthermore after the detecting of several amino acids in seminal plasma such as (cysteine, glycine, proline and histidine) they used successfully as cryoprotectants to

cryopreserve spermatozoa of many mammalian species [13] .However by which mechanism these amino acids components protect spermatozoa during freezing-thawing process have not clearly understood and are still unclear; therefore, based on the presented results of these investigations and few studies about the role of amino acids on ram spermatozoa quality after freezing and thawing.

So one of the main aims of this study was to study the effect of certain concentrations of glycine and cysteine on freeze ability of Arabi and Najdi ram spermatozoa in addition to spermatozoa of their crosses compared with standard extender which is the Tris. On the other hand sperm motility stimulant substance was used in the culture media for in vitro sperm activation after thawing from one month freezing .Sperm motility stimulant used was pentoxiphylline which as aphosphodiestrase inhibitor of the methyixantine group. It inhibits the break down at cyclic a denosine monophosphate (cAMP) which is known to play a central role in sperm motility [14].

In general pentoxiphylline has been reported in preserving [15, 16]. Appropriate use at cryoprotectants before and after cryopreservation refers to have the biggest impact on preventing sperms fragmentation thus improving sperm cryosurvival rates. Therefore also the objective of this study is to examine the effects of in vitro addition of pentoxiphylline after thawing of freezing rams spermatozoa diluted with extenders of glycine and cysteine compared with thawied frozen semen with Tris extender.

Material & Methods

This experiment was performed on several semen samples collected once weekly from twelve rams divided as three groups. The first group included four Arabi ram; the second group included four Najdi rams and the third group included four cross rams (Arabi×Najdi).Those rams were found in the animal farm of college of agriculture, university of Basrah. All rams are 3 to 4 years of age and 60-65 kgs body weight each. They are of good size and high tolerance to harsh environment. The animals were kept under natural photoperiod and nutritional levels were adjusted to meet maintenance requirements; they had free access to salt lick and fresh water. Semen was collected by artificial vagina of sheep and goats from all rams of each group and pooled independently and directly evaluated then diluted by three types of extender as follows:

Extender No.1 composed of the following materials:

Tris(hydroxyl methyl amino methane merck.(Germany)3.07 gm, citric acid1.64 gm, fructose 1.26 gm, egg yolk 2.5 ml, gentamycine 0.5 ml g, glycerol 0.5 of 8% and distilled water 100 ml.

Extender No. 2 composed of the following materials:

All material of extender No 1 in addition to 16.89 gm glycine amino acid.

Extender No.3 composed of all materials of extender No.1 in addition to 12.12 gm cysteine amino acid (in concentration of 5 millimole).

Semen Collection and Evaluation

Semen was collected once a week for 8 weeks from the mentioned fertile rams that had been trained to serve an artificial vagina (42-43C°). The prepuce was wiped clean prior to collection to prevent contamination of the semen. Semen was collected in the morning and transported to the laboratory at 37C°within 10-15 minutes and placed in a water bath at 37C°. Ejaculates were evaluated for volume (ml) sperm concentration ($X10^9$ sperm /ml) massive activity; individual motility (% diluted with normal saline) viability % (using eosin nigrosin staining) and morphological normal and at spermatozoa %.

Only semen samples with adequate motility and concentration were used for cryopreservation. To evaluate motility and progressive motility a sample of diluted spermatozoa was placed under a cover slip in the center of pre-warmed (37C°) slide and it was transferred to microscope stage and subjectively assessed (X400 magnification).The rate of motility and progressive motility was determined in percentages. Viability was performed using a modification of the eosin-nigrosin stain procedure described by [17]. A mixture of 10 ml of diluted spermatozoa and 10 ml eosin-nigrosin stain was smeared on a slide and allowed to air dry in a dust free environment.

Two hundred spermatozoa from different microscopic fields were examined under bright-field microscopic using 400X objective and the number of non stained (viable) and stained (non-viable) spermatozoa was counted. The experiment was designed to investigate the influence of the different four mentioned extender on cooled (before freezing) and on frozen thawed spermatozoa of the 3 different ram groups, and then studying the effect of activation of the frozen thawed spermatozoa of all used extenders in-vitro after addition of pentoxiphylen to them. Semen sample were pooled and diluted 1:4 with the prepared extenders.

Then extended semen samples were packaged in 0.5 epindrof tubes. Those tubes were kept at 5 C° for 4 hours for equilibration. After equilibration time, the cooled samples were then held 4-5 cm above the surface at the liquid nitrogen for 10 minutes before being plunged in to liquid nitrogen for storage (-196 C°). After a month of storage the frozen-thawed semen were assessed for progressive motility; viability, morphological abnormalities percentages were calculated. The cryopreservation added to the extenders. The thawing was done after one month of cryopreservation [18].

Preparation of Pentoxifylline

Pentoxifylline was prepared by dissolving 10 mg of PX powder (sigma USA) in 10 ml of phosphate puffer (PBS) (0.1) then stirring until dissolve [19].Mixing 5 ml of Px prepared above with each used extender to obtain 10 ml Px. This concentration was prepared under sterile condition and using Millipore filter.

After one month of cryopreservation-the thawing process was done as mentioned above and each sample after thawing was divided into 2 parts first part activated with the same medium of dilution without pentoxifylline the other part was activated by adding pentoxifylline to the extender medium. The two parts then were kept in CO^2 incubator for one hour at 38 C° and 5% humidity after that evaluation of each sample was done as before freezing.

Statistical Analysis

The data was entered and analyzed using SPSS 16. Version software (statistical package for social science).

The significant difference in mean values of studied parameters was assessed by applying ANOVA and least significant difference (LSD) and p-value of less than 0.05 was considered significant.

Results

Microscopic seminal characteristics in primary evaluation included individual motility percentage, a live sperm percentage, dead sperm percentage and abnormal sperm percentage of each of Arabi, Najdi and their crosses rams were presented in table 1,2 and 3.those results showed that the four characteristics mentioned above were significantly differed between the three extenders used in the three rams groups and it was clear that the best extender which gave better sperms characteristics was extender with glycine followed by extender of cysteine when those two amino acids were added to the Tris buffer extender.

Those three Tables (1,2,3) shows the effects of supplementation of baic tris extender with glycine and cysteine amino acids on cooled rams (Three types) spermatozoa. The results indicated that addition of those two amino acids to the basic Tris extender increased significantly ($p<0.05$) the sperm individual motility, viability and decrease sperm abnormality.

Results in Tables (4.5 and 6) showed the effects of those amino acids on post thaw semen characteristics after one month of freezing. Those results indicates presence of significant differences ($p<0.05$) in assessed parameters by adding glycin and cysteine amino acids to tris extender also results showed their cryoprotection profiles which is clear and well when certain amino acid added to it.

It is also clear that glycin and cysteine extenders significantly ($p<0.05$) decreased percentages of abnormal sperm of all types of rams in this study in frozen-thawed samples. The results of the present study indicated that glycine was more effective for preservation motility than cysteine and tris. Concerning total abnormality all with amino acid treatment level were decreased significantly.

Also results in tables 7;8 and 9 showed a clear significant improvement in active sperm motility of all ram breeds spermatozoa

was notice after in vitro activation either without or with pentoxifilline, whereas results with pentoxifilline was clearly better than without pentoxifilline in all extenders used either of amino acid extenders or tris extender. This improvement due to activation is better than results of before cryopreservation. Activation of cryopreserved semen in vitro caused significant increase in the motility due to treatment with pentoxifilline compared to the results of before cryopreservation and after in vitro activation of control media (tris) extender.

Table 1: Effect of diluents on semen characteristics of Arabi rams before freezing

Semen characteristics				
Diluents	%individual motility	%Alive sperms	%Dead sperms	%Abnormal sperms
Tris	63.58±2.6 A	80.5±1.9 A	9.74±0.1 A	8.4 ±3.2 A
Glycine	63.58±2.6 A	81.4±1.6 B	9.29±0.2 B	8.4 ±0.3 B
Cystine	62.97±1.9 B	80 ±1.8 C	9.84±0.3 C	8.75±0.4 C
LSD	1.5	1.12	0.25	0.32

Table 2: Effect of diluents on semen characteristics of Najdi rams before freezing

Semen characteristics				
Diluents	%individual motility	%Alive sperms	%Dead sperms	%Abnormal sperms
Tris	73.7±1.9 A	86.9±1.7 A	8.23±0.1 A	6.7 ±0.2 A
Glycine	76.1±2 B	88.1±2.1 B	7.9 ±0.2 B	6.1 ±0.3 B
Cystine	74.1±2.5 C	86.4±2.3 C	8.3±0.3 C	6.6 ±0.4 C
LSD	2	1.8	0.7	0.4

Table 3: Effect of diluents on semen characteristics of Cross Arab X Najdi rams before freezing

Semen characteristics				
Diluents	%individual motility	%Alive sperms	%Dead sperms	%Abnormal sperms
Tris	69.14±2.3 A	80.1±2.1 A	9.02±0.2 A	7.57±0.3 A
Glycine	71.82±2.7 B	83.92±2.6 B	8.73±0.1 B	7.32±0.1 B
Cystine	68.35±2.2 C	82.6±2.1 C	9.12±0.3 C	7.67±0.2 C
LSD	2.1	2.2	0.5	0.3

Table 4: Effect of diluents on semen characteristics of Arabi rams after a month of freezing

Semen characteristics				
Diluents	%individual motility	%Alive sperms	%Dead sperms	%Abnormal sperms
Tris	57.18±1.7 A	77.67±1.8 A	10.44±0.5 A	9.82±0.5 A
Glycine	59.82±2.3 B	78.65±2.4 B	10.06±0.5 B	9.44±0.4 B
Cystine	56.99±2.1 C	77.62±2.2 C	10.59±0.3 C	10±0.4 C
LSD	2.1	1.2	0.5	0.4

Table 5: Effect of diluents on semen characteristics of Najdi rams after a month of freezing

Semen characteristics				
Diluents	%individual motility	%Alive sperms	%Dead sperms	%Abnormal sperms
Tris	69.23±3.5 A	84.87±4.6 A	9.13±1.3 A	7.48±1.2 A
Glycine	71.39±3.3 B	86.1 ±3.6 B	7.82±3.2 B	7.02±3.5 B
Cystine	69.02±3.8 C	84.45±3.9 C	9.34±1.1 C	7.52±1.2 C
LSD	1.4	1.2	0.8	0.5

Table 6: Effect of diluents on semen characteristics of Cross (Najdi X Arabi) rams after a month of freezing

Semen characteristics				
Diluents	%individual motility	%Alive sperms	%Dead sperms	%Abnormal sperms
Tris	65.18±2.6 A	80.58±2.7 A	9.73±0.9 A	8.59 ±0.8 A
Glycine	66.55±2.9 B	82.04±2.8 B	9.4 ±0.7 B	8.22 ±0.6 B
Cystine	64.6 ±3.1 C	80.1±3.2 C	9.82±0.4 C	8.67 ±0.7 C
LSD	1.4	1.5	0.3	0.2

Table 7: Activation of Arabi rams Spermatozoa using same diluents with pentoxyphyline after thawing from one month freezing

Percentages of individual sperm motility					
Diluents	Before freezing	After freezing	After activation		LSD
			Without Px	With Px	
Tris	62.6±1.5 Aa	56.5±1.8 Ab	66.1±1.9 Ac	75.2±1.7 Ad	5.5
Glycine	65.9±2.5 Ba	59.1±2.3 Bb	68.9±2.4 Bc	78.3±2.6 Bd	5.2
Cystine	80.1±2.9 Da	56.9±2.8 Cb	65.1±3.1 Cc	75.2±3.4 Cd	5.9
LSD	2.5	2.2	2.5	2.6	

Capital letters clarify the differences that are statically significant at 0.05 levels vertically
Small letters identify the differences which are significant at 0.05 level horizontally

Table 8: Activation of Najadi rams Spermatozoa using same diluents with pentoxyphyline after thawing from one month freezing

Percentages of individual sperm motility					
Diluents	Before freezing	After freezing	After activation		LSD
			Without Px	With Px	
Tris	73.7±1.8 Aa	66.2±1.6 Ab	76.3±1.7 Ac	84.5±1.5 Ad	5.1
Glycine	76.1±2.1 Ba	71.4±2.3 Bb	82.2±2.4 Bc	90.1±2.6 Bd	4.2
Cystine	74.1±2.1 Da	69.1±2.4 Cb	79.2±2.7 Cc	87.2±2.8 Cd	4.3
LSD	2.3	2.7	2.1	2.2	

Capital letters clarify the differences that are statically significant at 0.05 levels vertically
Small letters identify the differences which are significant at 0.05 level horizontally

Table 9: Activation of cross rams Spermatozoa using same diluents with pentoxyphyline after thawing from one month freezing

Percentages of individual sperm motility					
Diluents	Before freezing	After freezing	After activation		LSD
			Without Px	With Px	
Tris	68.14±2.4 Aa	64.18±2.1Ab	72.12±2.6 Ac	82.3±2.3 Ad	3.1
Glycine	71.8±3.1 Ba	66.6±3.2 Bb	77.7±3.4 Bc	86.6±3.6 Bd	3.8
Cystine	68.4±3.7 Ca	64.6±3.5 Cb	73.7±3.7 Cc	83.8±3.4 Cd	3.9
LSD	2.2	1.5	2.6	2.7	

Capital letters clarify the differences that are statically significant at 0.05 levels vertically
Small letters identify the differences which are significant at 0.05 levels horizontally

Discussion

The aim of cryopreservation is to gain a high number of post –thawing survival normal sperms, but there are many factors during cryopreservation process which can affect the post- thawing outcome [20]. Therefore, various methods of cryopreservation including technical aspects of freezing and thawing sperms and of preparing cryopreservation media which have been evaluated for their effects on post thawing sperm quality [21] but currently.

The standard methods for freezing-thawing semen which will optimize sperm recovery have not been firmly established yet. Concerning cryopreservation of rams spermatozoa, various extenders have been described [22] but there are few reports on the effects of the amino acids on the post-thaw motility and viability of ram spermatozoa [23].

Demonstrated that the addition of low concentration of proline and glycine to medium containing egg yolk and glycerol improved the motility of ram spermatozoa [24].Presented the positive effects of cysteine on motility. The results of this study demonstrated that the supplementation of tris extender with glycine and cysteine caused improvements in the frozen the froze-thawed characteristics of spermatozoa such as motility, viability significantly. These observations were in agreement with the finding of [24] for ram spermatozoa and [25] for goat sperms [13].

Concluded that addition of 25 Mm glutamine, glycine and 5 Mm cysteine in conventional freezing medium enhanced post-thaw motility of buffalo bull semen. Furthermore our results indicated that addition of used amino acids caused better results in all three types of ram groups used in this study. These results were in agreement with the finding of [26], the authors reported that 5-15 mm amino acids improved significantly sperm motility of frozen-thawed goat spermatozoa.

However the exact mechanism of sperm protection by amino acids has not been understood and remains unclear. Areport by [27] demonstrated that thiol-radicals containing amino acids as glycine; cysteine

and mercaptoethanol, prevent hydrogen peroxide mediated loss of sperm motility in frozen-thawed bull an a buffalo semen .however, [28, 29] reported that 50 Mm glycine significantly improved sperm motility in cooled and frozen-thawed striped bass spermatozoa, also [30] found that cryopreservation caused significant decrease in progressive motility just like results of this study.

The same observations were reported by [31, 18]. The decrease in certain sperm parameters may result from both freezing and thawing which may inflict irreversible injury on proportion of spermatozoa [32]. Then after lipid peroxidation can lead to a decline in sperm motility, viability [33].

It has been shown that reactive oxygen species (ROS) production impacts membrane fluidity and the recovery of motile, viable spermatozoa after cryopreservation. More over the process of cryopreservation can reduce the antioxidant activity of the semen fluid making spermatozoa more vulnerable to ROS. Induced damage [34].Also results showed that addition of Px to all used extenders in this study especially to media of amino acids gave best results of in vitro sperm activation of cryopreserved rams spermatozoa regarding motility, viability and number of abnormal sperms. This result may be caused by synergism effect of Px with amino acids as earlier mentioned that Px action to inhibit the phosphodiesterase enzyme leading to increase of cAMP which may lead to increase motility of sperm [35]. After activation of cryopreserved sample can be explained by the interaction of Px with the lipid content of the sperm cell membrance which contains a high proportion of polyunsaturated fatty acids.

This interaction of Px with polyunsaturated fatty acids will positively affect to decrease the production of lipid peroxidation which is the source of ROS [36].

The author explained that Px may affect positively on the increase in the motility during activation may lead to decrease in abnormal cells and absence in leukocytes and any harmful cells that may generate ROS can be generated spermatozoa and leukocytes [37] Px has been oxygen free radical scavenging capacity. It has been concluded that post –thaw cAMP concentration in spermatozoa were found to be three times lower than freeze concentration in the same sample.

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