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A Comparative Study of Numerous Antioxidants Supplementation on Several Characteristics for Cooled Storage of Awassi Rams Epididymal Sperms

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THE study was conducted to investigated effect of three amino-acids which were Methionine (0.2 Mm), L-Arginine (0.4 Mm) and D-Alanine (15 Mm) on Awassi Epididymal sperms characteristics after storage at 4°C. Twenty five pairs of testicles (n=25, 50 testis/semen samples) from 25 mature Awassi Rams aged between 1-4 years were used in present study. Epididymal sperms were collecting by slicing and squeeze of epididymis and diluted in egg yolk extender. Four test tubes samples were created, each of which contained an equal amount of penicillin and streptomycin as 100.000 IU and 100 mg, respectively, fructose, one kind of amino acid, sodium citrate, and egg yolk. The fourth test tube left as control group with no adding of amino acid and all test tubes kept in refrigerator temperature at 4°C. The results show that after 24,27 hours of storage, Methionine improve significantly $p<0.05$ sperm individual motility which were 72.6±2.9%, 57.5±3.2%, respectively, sperm live percentage was 73.6±2.3%, 53.2±2.8%, respectively, Sperm abnormalities was 3.0± 0.1, 7.2±0.1%, respectively. In present study, data recorded of sperm characteristics after adding methionine were eminent on that sperms values were recorded after adding arginine and methionine. In conclusion Methionine at dose 0.2 Mm had superior effect on epididymal sperm characteristics after storage at 4°C for 72 hours in cooling method, however; Arginine (0.4 Mm) and Alanine (15 Mm) also act to improve sperm characteristics when compare with control group in Awassi rams epididymal sperms.

Keywords: Epididymal sperms, Methionine, Arginine, Alanine, Ram.

Introduction

Despite the widespread use of mammalian sperms in artificial insemination, whether refrigerated or frozen; However, its quality remains generally low; This is due to its extreme sensitivity to injury during preservation or to dissolving after preservation[1-2]. Moreover, the conditions in the laboratory during preparations for storage or treatment of semen (sperms) may affect their quality and thus affect the process of fertilization of eggs, the first embryonic divisions, the development of the blastomere or the embryonic vesicle [3].

Storing sperm, whether by refrigeration or freezing, exposes the sperm to stimuli at the cell level known as oxidative stress, in addition to other variables that may be either chemical, osmotic pressure differences, thermal or mechanical changes, or the occurrence of poisoning or saturation[4]. Chemicals resulting from sperm metabolism at all stages of semen preservation. The above changes lead to damage to the membrane of the sperm cell, affecting its movement and vitality, and thus reducing the ability to fertilize [5].

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Recently, antioxidants have been used during preservation of animal semen in various storage protocols to compensate for the depletion of endogenous antioxidant concentration in seminal plasma due to dilution, as well as to counteract oxidative stress in vitro and reduce the generation of free radicals (FRs) [6].

Alanine is one of the naturally occurring amino acids. Previous studies indicated its ability to protect ram sperm cells from freezing during cryopreservation [7-8]. Previous reports [9-10] indicated that addition of Alanine to semen diluent improves the process of preserving in male Indian (Murrah), Egyptian and Italian buffaloes preserved using a methods of Freezing.

Methionine is one of the amino acids that have the ability to protect the cell from oxidative damage, as it acts as a precursor amino acid to the amino acid cysteine and is also characterized by its ability to interact with oxidants to form Methionine sulfoxide to keep its damage away from sperm [11]. Methionine is also a precursor to the amino acid glutathione (GSH), which is known to protect the sperm cell from oxidative damage and plays a vital role in detoxification [12]. Methionine protects the sperm cell membrane to promote the intracellular biosynthesis of glutathione in the organism [13]. Respective report indicated that using methionine Improves spermatozoa motility parameters in rams [14] with 0.05, 0.1, 0.2, and 1 mM of concentration for maintains sperm motility, plasma membrane integrity, mitochondrial activity, and total antioxidant capacity and decreases MDA content.

Arginine It an amino acid that protects the cell from oxidative stress as it works to regulate levels of O2-superoxide and hydrogen peroxide [15]. Arginine is considered as initiating amino acid for nitric oxide (NO), which in turn maintains the movement and metabolism of sperm, whether inside the genital tract or during the preservation of semen in the laboratory [16]. Nitric oxide removes reactive oxygen species (ROS) and thus The semen lipid bilayer membrane protects against oxidative stress [17-18]. Nitric oxide supports capacitation and acrosome reaction in rat sperm [19]. A previous paper refers to adding arginine to epididymal sperm(cauda epididymal sperm) may be useful for cryopreservation of semen. [20].

Epididymal sperms represented a cheap source of sperms, but its viability was constricted with time of animal death and time of storage, so the present study was invented to test benefits of adding D-Alanine, L-Arginine and Methionine on several characteristics of Awassi Rams after 24, 27 hours of storage at $4C^0$.

Materiial and Methods

Animal samples and Study area

The study was carried out in the laboratory of Artificial Insemination, College of Veterinary Medicine, University of Mosul, Mosul, Iraq (N: 36° 20’ 24”: E: 043° 07’ 48”) from September 2021 to March 2022. Twenty five pairs of testicles (n=25, 50 testis/semen samples) from 25 mature Awassi Rams aged between 1-4 years, slaughtered at Al-Sadoon Abattoir were gained. The testes were transported in a cold box after washing with normal saline and antibiotics (penicillin-streptomycin) within two hours after slaughtering.

Epididymal Sperms collection

The epididymis were all carefully removed and the cauda epididymis was sliced and squeezed in a Petri plate at room temperature to collect the spermatozoa[21]. The semen was gathered and placed in a 15 ml graduated glass tube. Sperms were observed under a light microscope for individual motility, living sperms, and sperm abnormality percentages. Red eosin 5% and nigrosine10% stains were used to estimate these percentages. All test samples’ sperm parameters were examined before and after dilution, and then at 24, 72 hours after dilution and storage with egg yolks extender.

Sperms individual motility

Sperm individual motility was estimated according to Kundu et al.[20], Briefly, 5 µl of semen was assessed for motion under the microscope (40x) using coverslip and scored into 0-100 grades according to numbers of sperms which have aggressive forward motility[22].

Sperm live percentage and abnormalities

By using the sperms- Eosin-Nigrosin smear staining. in order to create a semen smear, 2 µl of semen and 10 µl of Eosin-Nigrosin stain were combined (10 gm of Nigrosin, 1.7 gm Eosin and 2.9 gm of sodium citrate in100 ml of distilled water). Sperm cells were counted under a microscope(X40) after the slide had dried for two to three minutes as living if the stain was strictly excluded (whitehead) and dead if the stain was eosin(redhead) against a Nigrosin background (400x)., the sperm›s liveness % and anomalies were calculated as same method as described by Jha et al.[23].

Sperm concentration

Sperm concentration was done by using coloro-meter apparatus by take 0.1 ml of semen and mixed with 0.9 ml normal saline, then mixed carefully and take the apparatus value for every sperm sample the applied equation of ram semen as following:

Sperm concentration=[(apparatus read ×2514.1)-537.38] × 10^6 as same way was described by Naoman & Taha [24].

Semen dilution and amino-acids adding

Spermatozoa volume totaling 0.5 ml was taken from the epididymis and diluted to 1 ml with sodium citrate 2.9%., then dividing into four aliquots (0.25 ml for each sample). Sperms were diluted 1:10 using semen as the extender. In general: Four samples (four tubes) were created, each one of them contained an equal amount of penicillin and streptomycin 100.000 IU and 100 mg, respectively, fructose, 2.4g, one kind of amino acid, sodium citrate 2.9%, and egg yolk 10%, , and 100 ml normal saline, respectively [25-26]. One tube was constructed for control values, and rest of pickers were prepared for the three amino acids Arginine (0.4 Mm), Alanine (15 Mm), and Methionine (0.2 Mm). To prevent shocking the sperm, the extender is gradually introduced straight into the semen. All extenders were tested for motility after equilibration in the water bath for 2–5 minutes., live and dead sperm percentage, sperm abnormalities, and considered as zero hours, the samples in test tubes were moved in the refrigerator under 4 ^{0}C and sperms examined after 24h, 27h, as a fixed time under a light microscope.

Statistical analysis

The results of the present study were expressed as mean ± standard error. One-way ANOVA was used to compare data using Sigma Stat (Jandel scientific software V3.1). Duncan›s Multiple Range Test was used to assess if there were any significant variations at $p < 0.05$.

Results

Individual motility

Results of the current study (Table 1), showed that the addition of amino acids led to an improvement in the sperm individual movement. Where the highest value was recorded in samples of Methionine (72.6±2.9), followed by Arginine (66.0±3.3) and Alanine (64.0±3.1), then a group where it recorded the lowest rate (57.0±3.2) with reference to Methionine was higher than significant ($P < 0.05$). of each of Arginine and Alanine after 24 hours of preservation at a refrigeration temperature of 4 °C.

After 27 hours of cooling storage, The results of the study showed that Methionine continued to be superior in its effect on the individual motility of sperm samples kept at refrigeration temperature after 72 hours of preservation, as the highest value was recorded in Methionine at a rate of (57.5±3.2), followed by Arginine (47.0±3.8) and Alanine (47.0±3.8), (45.6±3.2), then the control group (37.2±3.2), with reference to the significant superiority of Methionine, and in sequence Methionine, Alanine, and Arginine, then the control group, respectively.

Live sperm percentages

The amino acid Methionine outperformed the rest of the amino acids and the control group in improving the quality of live sperm, as it recorded the highest value (73.6±2.3), followed by Arginine (70.1±2.8), Alanine (65.8±3.0), then the control group (58.4±3.2), and significantly ($P <0.05$) after keeping the samples for 24 hours and at the refrigeration temperature as shown in table 1.

After 27 hours of storage, Methionine continued to outperform the rest of the amino acids at a rate of (53.2±2.8) over the rest of the amino acids and the control group in a significant way in the characteristics of live sperm after preservation and for a period of 72 hours at the cooling temperature, noting that the lowest results were recorded in the control group (40.6±3.4) with reference to the absence of any significant differences (Figure 4): Between the effect of Alanine (46.8 ± 3.2) and Arginine (49.2 ±3.3), at level of ($P<0.05$).

Sperm abnormalities percentage

The results of the current study in table(1) showed that the addition of amino acids led to a decrease in the percentage of Abnormalities of sperm, as the percentage recorded in all amino acids was less than that of the control group in an insignificant way. Where the lowest value was recorded in the samples to which Methionine was added at a rate of (3.0±0.1), followed by Arginine (4.0±0.1) and Alanine (3.9±0.1), then the control group, where it recorded the highest rate (3.4.0 ± 0.1), respectively after 24 hours of Store at a cooling temperature of 4 °C. It should be noted that the calculated percentage of malformations included primary and secondary malformations of the sperm. Fig. 1, Pictures A,B,C,D.

After 72 hours of storage, The percentage of deformed sperm (Fig. 6) did not record a significant difference ($p < 0.05$) between all samples that had amino acids added, and it remained within its levels close to that of the control group (7.8 ± 0.1), although it was lower in samples that had Methionine added (7.2 ± 0.1).) followed by Arginine (7.7 ± 0.1) and Alanine (8.1 ± 0.1), respectively. It should be noted that the calculated percentage of deformities included primary and secondary deformities of the sperm, and that most of the deformities recorded after preservation were mostly secondary deformities, including free heads, free tails, coiled, twisted, or folded tails.

TABLE 1. Epididymal sperms characteristics after adding D-Alanine, L-Arginine and Methionine

Epididymal sperms characters $2.6\pm 0.2 \times 10^9$ sperm/ml N=25				
Parameters	**Control**	**D-Alanine (15 Mm)**	**L-Arginine (0.4 Mm)**	**Methionine (0.2 Mm)**
Individual motility at 0 h	87.5± 0.2[a]	87.5± 0.2[a]	87.5 ± 0.2[a]	87.5± 0.2[a]
Individual motility after 24 h	57.0±3.2[a]	3.1[b] 64.0±	66.0± 3.3[c]	72.6± 2.9[d]
Individual motility after 72 h	37.2± 3.2[a]	45.6± 3.2[b]	47.0± 3.8[c]	57.5± 3.2[d]
Live sperm percentages at 0 h	82.5± 0.2[a]	82.5± 0.2[a]	82.5± 0.2[a]	82.5± 0.2[a]
Live sperm percentages after 24 h	58.4± 3.2[a]	65.8± 3.0[b]	70.1± 2.8[c]	73.6± 2.3[d]
Live sperm percentages after 72 h	40.6± 3.4[a]	46.8± 3.2[b]	49.2± 3.3[c]	53.2± 2.8[d]
Sperm abnormalities at 0 h	2.0±0.2 [a]	2.0±0.2 [a]	2.0±0.2 [a]	2.0±0.2 [a]
Sperm abnormalities after 24 h	3.4.0± 0.1[a]	3.9± 0.1[b]	4.0± 0.1[b]	3.0± 0.1[ab]
Sperm abnormalities after 72 h	7.8± 0.1[a]	8.1± 0.1[b]	7.7± 0.1[b]	7.2± 0.1[ab]

The different letters in same raw refers to significant differences at p <(0.05)

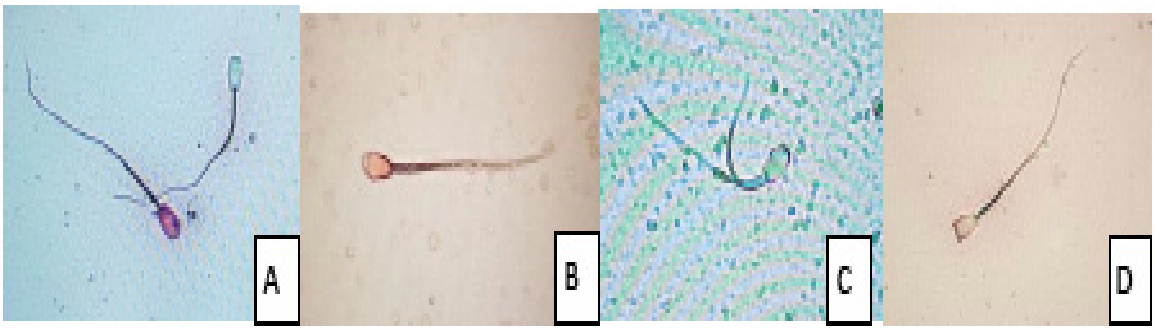

Fig. 1. Pic A. represented two sperm the white sperm head was live and red head sperm was dead. Pic B,C,D. represented some of sperms abnormalities which recorded in present study. All samples Stained with red eosin 5% and Nigrosin 10% blue under a microscope under 40X high power.

Discussion

Data of present study revealed that Methionine at dose 0.2 Mm has superior effect on epididymal sperm characteristics after storage at 4^0C for 72 hours in cooling method, however; Arginine (0.4Mm) and Alanine (15Mm) also act to improve sperm characteristics when compare with control group. Methionine has many advantage during sperm storage as anti-oxidative stress: It is one of the amino acids that have the ability to protect the cell from oxidative damage, as it acts as a precursor amino acid to another the amino acid cysteine and is also characterized by its ability to interact with oxidants to form Methionine sulfoxide to keep its damage away from sperm [11]. Methionine also serves as a precursor to the amino acid glutathione (GSH), which is known to shield sperm cells from oxidative damage and to be essential for the detoxification process. Methionine shields the sperm cell membrane to encourage intracellular glutathione biosynthesis in the middle section [12]. Methionine (0.2Mm) in present study improved sperms individual motility after 24,27 hours of storage at 4C which were 72.6±2.9, 57.5±3.2, respectively; however, these results disagreement to previous report [27] how show that high level of Methionine (2-4Mm) leads to improve sperm individual motility after storage at 4 ^{0}C in Merino Rams semen collected by artificial vagina, this disagreement is not clear but it's may be due to differences in source of semen and species of rams, In our point of view Methionine in low dose improve epididymal semen quality because seminal plasma of ejaculated semen may interfere with Methionine activity, consuming, diluting it or united with its particles, so the ejaculated semen need more dose of Methionine in compare with epididymal sperms, these exploration clear role of Methionine of proving sperm live percentage and reducing sperm abnormalities.

L-Arginine It is an amino acid that guards against oxidative stress by controlling the quantities of O2-superoxide and hydrogen peroxide in the body [14]. Nitric oxide (NO), which keeps sperm moving and functioning properly both inside the vaginal tract and when semen is being preserved in a laboratory, is made from the precursor amino acid arginine [19]. Nitric oxide eliminates reactive oxygen species (ROS), protecting the semen lipid bilayer membrane from oxidative stress [15-16]. In present study Arginine improve sperms individual movement, sperms live percentage and reduce sperms abnormalities when compare with control group, however; the question why its action less than Methionine is not clear, this may be due to that Methionine interfere and re-activated mitochondrial itself while Arginine was not, even though; the present study was in agreement with previous report [28-30] who refers to positive action of Arginine when added to semen of at same dose.

D-Alanine It is one of the naturally amino acids found in seminal plasma. Previous studies indicated its ability to protect sperm cells from oxidative stress rams through cryopreservation [31]. Kamal, et al., 2022 [32] indicated that the addition of Alanine to semen diluent improves sperm preservation process of Sahiwal bull spermatozoa. In present study, Alanine improve sperm individual motility, sperms live percentages and reduce sperm abnormalities, these were in in agreement with number of researchers [31-38] were indicated that addition of Alanine to semen diluents led to an increase in the percentage of sperm with a functional plasma membrane in the semen of rams. but; why Alanine was less than Methionine and Alanine, in our point of view; Methionine act in two ways, the first one was act as stimulator to produce another two amino-acids cysteine and glutathione, the second way was act to activate mitochondria and DNA methylation, this provide continues regeneration of sperm bio-membranes, while Arginine and Methionine act collaterally on free oxygen particles by reduce it or protect sperm membrane from it.

Conclusion

Methionine at dose 0.2 Mm had superior effect on epididymal sperm characteristics after storage at 4C for 72 hours in cooling method when cpomaring with Arginine (0.4Mm) at and Alanine (15Mm) however; Arginine and Alanine also act to improve sperm characteristics when compare with control group in Awassi rams epididymal sperms.

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Conflict of Interest

There was no conflict of interest.

Ethical approve

No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with slaughter house specimens.

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دراسة مقارنة لبعض مضادات الأكسدة على بعض خصائص التخزين المبرد للحيوانات المنوية في الكباش العواسية

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اجريت الدراسة الحالية لمعرفة تأثير ثلاثة أحماض أمينية وهي الميثيونين (٠,٢ ملم) ، إل-أرجينين (٠,٤ ملم) ود-ألانين (١٥ ملم) على خصائص الحيوانات المنوية البربخية في الكباش العواسي بعد التخزين عند درجة حرارة ٤ مئوية. تم استخدام خمسة وعشرين زوجًا من الخصيتين (العدد = ٢٥ ، ٥٠ عينة من الخصية / السائل المنوي) من ٢٥ كباش عواسي ناضجة تتراوح أعمارهم بين ١-٤ سنوات في الدراسة الحالية. تم جمع الحيوانات المنوية البربخية عن طريق تقطيع البربخ وتخفيفها في مخفف صفار البيض. تم تخفيف الحيوانات المنوية بنسبة ١:١٠ ، تم تكوين أربع عينات من أنابيب الاختبار ، كل منها يحتوي على كمية متساوية من البنسلين والستربتومايسين مثل ١٠٠,٠٠٠ وحدة دولية و ١٠٠ مجم ، على التوالي ، الفركتوز، نوع واحد من الأحماض الأمينية ، سترات الصوديوم ، وصفار البيض. وتم ترك أنبوب الاختبار الرابع بدون اضافة كمجموعة تحكم وحفظت جميع أنابيب الاختبار في درجة حرارة الثلاجة عند ٤ درجة مئوية. أظهرت النتائج أنه بعد ٢٤،٢٧ ساعة من التخزين ، تحسن عينات الحيامن المضاف لها الميثيونين بشكل ملحوظ $p < 0.05$ على صفة الحركة الفردية للحيوانات المنوية والتي كانت ٧٢,٦ ± ٢,٩٪ ، ٥٧,٥ ± ٣,٢٪ على التوالي ،. في الدراسة الحالية ، شهدت البيانات المسجلة لخصائص الحيوانات المنوية بعد إضافة المثيونين تحسن في قيمها التي تم تسجيلها تليها قيم المسجلة عند إضافة الأرجينين و الالنين. في الختام كان للميثيونين بجرعة ٠,٢ ملم تأثير متفوق على خصائص الحيوانات المنوية البربخية بعد التخزين عند ٤ درجة مئوية لمدة ٧٢ ساعة في طريقة التبريد. يعمل الأرجينين (٠,٤ ملم) والألانين (١٥ ملم) أيضًا على تحسين خصائص الحيوانات المنوية عند مقارنتها بمجموعة السيطرة في الحيوانات المنوية البربخية للكباش عواسي.

كلمات مفتاحية: الحيامن البربخية، الميثونين، الارجنين، الالنين، الكباش.