EFFECT OF CYSTEINE ON SOME CHILLED SEMEN CHARACTERISTICS OF AWASSI RAMS

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ABSTRACT

This study was carried out at the Ruminant Research Department, Al-Zaafarani, Baghdad to evaluate the effects of cysteine added to the citrate–egg yolk extender and preserved in refrigerator on some semen characteristics of Awassi rams and the deterioration occurred by semen processing and preservation. Semen samples were pooled and divided into seven equal aliquots including; control (c), 0.0 mM of cysteine, and T1 to T6 groups of 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mM of cysteine were added to the extender. Semen quality was assessed by; pH; sperm progressive motility; abnormalities; dead and acrosomal defect percentages. The results showed that pH did not differ significantly, while progressive motility, dead, abnormal, and acrosomal defect percentages were significantly different ($p<0.05$) and revealed how cysteine additives could develop chilled semen quality when preserved in 5°c. Low and moderate levels of cysteine in the extender (2.5-10 mM) were not improved acrosome integrity although motility and other semen criteria were increased. Extension, cooling, and preservation periods had a deteriorative effects on all studied characteristics. The study concluded that it's very important to add cysteine in the range of 7.5-10.0 mM to the extender to keep good semen quality as long as possible for the chilled preservation of Awassi rams semen.

INTRODUCTION

Semen preservation of mammals was applied either with liquid semen for short periods by decreasing metabolism in chilled semen technique, or cryopreserved for long periods by completely blocking this activity in liquid nitrogen. In both cases, sperm motility was declined with great changes in sperm membranes structures lead to decreased artificial insemination fertility (24). Sperm life in the ejaculate was found to be as short as many hours and to prolong their life it is necessary to dilute with suitable buffer to keep their fertilizing capability when preserved in low temperatures (33). Whatever the nature of the extender, motility and sperm membrane integrity were thought to undergo deterioration due to the process of diluting, cooling and storage (4, 13). The irreversible changes which take place in the membranes is an expected results of lipid peroxidation and reactive oxygen species(ROS) production (16) which destroy the spermatozoa by oxidative stress (6) specially when accumulated in large quantities causing unbalance between ROS production by metabolism and it's scavenging with the aid of special defense system presented as antioxidants.

Antioxidants were naturally exists in the semen of mammals in two categories; enzymatic (catalase, reduced glutathione, glutathione peroxidase, superoxide dismutase) and non enzymatic (cysteine, ascorbate, taurine, hypotaurine, vitamine E) (5, 28). In rams, where spermatozoa membrane rich in polyunsaturated fatty acid (PUFA) (21) and possible exposure to dilution, cooling, freezing and thawing (25) may induced acceleration of ROS over-production and accumulation and then to decrease motility, viability and structural damage of the cell (10, 17), and fertility decrease (12).

In the last decade, it is well known that all commercial extenders of modern generation like AndroMed®, Triladyl® and Bioxcell® were supplemented with antioxidants, but the manufacturers companies hidden the quantity and quality used, saving their secrets for profit purpose. In fact, thiol compound found in cysteine, glutathione and thioglycole was considered as important antioxidant supplements to protect the sperm against oxidation stress and hydrogen peroxide production (7).

Cysteine is an amino acid of low molecular weight having good capacity to penetrate sperm membranes easily; also, it is a precursor for intracellular glutathione (18) in addition to direct scavenge of ROS and free radicals (34). Consequently, many studies were conducted by supplying it in the semen extenders of bull, buffalo, ram, and buck successfully (9, 19, 29, 34), these studies reported an interesting protection role of sperm motility, membrane integrity, low acrosome defect and reducing abnormal sperm percentages. Up to our knowledge, there are insufficient studies on Awassi ram semen and lack in the information of using antioxidants to prolong sperm life in the chilled semen techniques. Therefore, this study was proposed to investigate the capability of different levels of cysteine in the extender to improve Awassi semen quality preserved in refrigerator and the deterioration occurred by semen processing and preservation.

MATERIALS AND METHODS

This study was conducted on three Awassi ram aged 2.5 - 5.0 years and body condition score of 2-3 in Ruminant Research Department / Alzaafarani , Baghdad during the period from March 13 to April 24/2016. Semen was collected once a week by electro ejaculator directly transferred to the laboratory for evaluation and subsequent preservation. Raw and preserved semen characteristics were evaluated in conventional procedures; volume, by graduated cylinder; appearance, 1–5 scale (4); pH, pH- litmus paper; mass motility, 0 – 5 scale under x40 magnification (11); progressive motility, as percentage of forward motile sperm under x400 magnification (31); sperm concentration, with the aid of special dense meter (591 B); dead and abnormal spermatozoa percentages by eosin-negrosin stains (27); acrosome defect percentage by eosin – fast green fast stains (36). Ejaculates were ignored when having less than 3 degree mass motility, 70% progressive motility, 3 degree for appearance, 0.3 ml volume, and $1x10^9$/ml of concentration.

Semen specimens were pooled together and subdivided into seven equal aliquots, then diluted to 1: 4 (semen : extender) by citrate - egg yolk extender (2.37 gm Tri sodium citrate; 0.5 gm glucose; 100 ml distill water; 20% egg yolk). Cysteine was added in the levels of 0.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, mM as control C and treatments T1 – T6 respectively. Antibiotic was supplied in the rate of 1mg Streptomycin +100000 IU Benzathin penicillin. Diluted semen then was cooled to 5°c by programmable refrigerated incubator within two hours. Evaluation for chilled semen treatments were repeated daily for three consecutive days.

Data were analyzed statistically in general linear model to compare means by ANOVA one way analysis and significant of means at $p< 0.05$ of Duncan multiple range test were applied by SPSS software (32).

RESULTS AND DISCUSSION

Some of the natural properties for Awassi raw semen have been shown in Table (1); it's in the normal range of Awassi breed (4).

Table 1. Some fresh semen characteristics of Awassi ram.

Ram No.	Ejaculate Volume (ml)	App. [1]	Mass mot. (%)	Progr. mot. (%)	pH	Conc. (billion)	Dead sp. (%)	Abn. Sp. (%)	Acro. Defect (%)
185	1.73	4	3.83	86.66	7.2	2.36	13.04	10.33	3.53
179	1.65	4	2.75	82.50	7.0	2.13	15.50	7.50	4.82
195	1.20	4	2.00	73.41	7.0	2.67	18.14	8.12	5.61

[1]: appearance scale: 1, watery; 2, cloudy; 3, milky; 4, creamy; 5, thick creamy.

In Table (2), general semen characteristics for different levels of adding cysteine to the extender were revealed. Treatment means were differed significantly ($p< 0.05$) for progressive motility, dead, abnormal sperm (%) and acrosome defect (%) while was non significantly for pH, these results indicated how cysteine additives could develop chilled semen quality when preserved in 5°c. Treatments T_3 and T_4 (7.5, 10 mM cysteine) clearly had the highest means in all studied parameter over the other treatments including control, this was in agreement with (34) whom insured that 10 mM of cysteine in the extender was sufficient to make high significant differences in sperm motility, total abnormality, viability, and acrosome integrity, while (18) have shown that 5mM was the critical level of adding cysteine to make differences in sperm motility compared to control. However, (30) illustrated that 10 mM of cysteine addition improved significantly all the studied criteria in comparison with 5mM. Moreover, very low level of cysteine (1 mM) was found to have beneficial effect in protecting ram spermatozoa against cryo- injuries (20). In freeze- thaw buffalo semen, it was demonstrated that 5and 7.5 mM cysteine caused significant increase in sperm motility and membrane integrity while significant decrease in ROS accumulation in comparison to the control (33).

Table 2: Effects of cysteine addition on some preserved semen characteristics of Awassi ram (independent of processing and preservation periods).

Trait / Cysteine	pH	Progressive Motility (%)	Dead spermatozoa (%)	Abnormal Spermatozoa (%)	Acrosome Defect (%)
Control C (0.0mM)	6.43 a ±0.04	55.62 c ±2.62	28.65 a ±1.94	18.25 a ±1.10	7.55 b ±0.78
T1 (2.5mM)	6.39 a ±0.06	57.50 c ±1.98	27.60 a ±1.83	15.45 ab ±1.04	9.95 ab ±0.96
T2 (5mM)	6.39 a ±0.12	60.62 ab ±2.85	26.65 a ±2.96	13.90 bc ±0.89	9.00 ab ±0.92
T3 (7.5mM)	6.40 a ±0.09	63.75 a ±2.34	20.50 b ±2.06	11.20 cd ±1.82	7.95 ab ±0.59
T4 (10mM)	6.40 a ±0.07	61.00 a ±3.02	20.90 b ±0.98	9.50 d ±1.39	9.75 ab ±0.88
T5 (12.5mM)	6.39 a ±0.06	59.62 b ±1.79	27.25 a ±1.56	9.60 d ±1.98	10.05 a ±0.73
T6 (15mM)	6.38 a ±0.04	58.75 b ±1.54	20.45 b ±1.38	9.10 d ±1.96	10.15 a ±0.95

Means with different superscripts within each column are significantly different ($P < 0.05$).

There is no doubt that cysteine addition to the citrate- egg yolk extender leads to high sperm functions, specially the motility, this could be due to the capabilities of cysteine in keeping motility and metabolic roles of the sperm (1), it was thought that the abundance presents of poly unsaturated fatty acids (PUFA) in the spermatozoa membrane make it in critical situation when the peroxidation of lipids take place in fast and hug rhythms causing changes of membrane

flexibility and ionic efflux, a way in which spermatozoa membrane lost shape and composition, leads to low motility (12, 15). Also, high PUFA contents over the saturated ones and decrease the ratio of cholesterol/phospholipids of spermatozoa membrane (21) make the membranes favorable targets for destroying upon throw chilling and freezing process. These anomalies in cooperation with lost function and membrane components directed the sperm cell gradually to death (14). All these theories may be true for control group in the present study which is disappeared or significantly declined in cysteine groups.

Characteristics mean of some cysteine treatments (Table 2) did not differ significantly from the highest ones (T3, T4), percentages of sperm motility in T2, dead sperm in T6, and abnormal sperm in T5 and T6 were found very close to corresponding values in T3 and T4, only acrosome defect % in control group was found to be the best (statistically) in comparison with all others. These fluctuation patterns may due to lost componential and functional balance in the sperm membrane which is expected as the lipids were oxidized, additionally, increasing cysteine level in extender into maximum (12.5 and 15 mM) was not always synchronize with improving preserved semen characteristics. Moreover, (23) revealed that using high levels of antioxidants in semen extender may exert deteriorating effect on the functional integrity of the axonemal and mitochondrial structures, as both associated with the motility. On the other hand, (34) reported a decrease in ram sperm abnormalities (frozen- thawed semen) when cysteine was added up to 15mM, a fact was shown in current study but with chilled preserved semen.

Low and moderate levels of cysteine in the extender (2.5-10 mM) were not improved acrosome integrity although motility and other semen criteria were increased, these results were in line with the finding of (20) with 1 and 2 mM supplementation of cysteine which increased motility but keep acrosome integrity as compared to the control.

Means of Awassi ram semen characteristics during series handling and processing to prepare it for chilled preservation were presented in Table (3), sperm motility and pH were significantly declined while viable, abnormal and acrosome defect percentages were significantly increased with the stages above. Generally, these results indicated that deterioration take place with preservation and storages activities, the acceptable explanation is that viable sperm have to pass different metabolisms to keep their life, natural exclusions of these oxidation activities is ROS which is believed to be harmful to sperm functions if there were no scavenging system present, but increasingly overproduction and accumulation by time of preservation may destroy sperm, with such situation, antioxidant could play control roles specially when it was in harmony with extender used. It was thought, that main causes responsible for sperm destroy while chilled or cryopreserved is cold shock, osmotic and oxidation stress (2, 3, 35), In the present study, the later factor may be as important as the preservation conducted under 5˚C and was in agreement with the finding of Ball, et. al. (13).

It is interesting to note that progressive motility for all treatments except T6 was exceeded control group (un- tabulated data), independently of preservation periods, it was 31.87, 37.50, 45.62, 43.12, 35.62, 35.00, and 26.25% for C, T1 to T6 respectively in the third day of storage, clearly indicated to the benefit effect of the presents of cysteine in extender as a thiol group donor, and

then thiol throw intracellular pathway inverted to glutathione which easily penetrate sperm membrane and scavenging ROS (8, 26, 33) and improving semen quality while preserved (29).

Table 3. Effects of preservation stages on some chilled semen characteristics of Awassi rams (independent of cysteine treatments).

Trait / Preservation stages	pH	Progressive Motility (%)	Dead spermatozoa (%)	Abnormal Spermatozoa (%)	Acrosome Defect (%)
Fresh semen	7.06 a ±0.02	80.85 a ±2.08	15.56 c ±1.04	8.65 c ±1.08	4.65 c ±1.05
After dilution	6.56 a ±0.03	76.42 b ±1.14	16.25 c ±0.54	9.28 c ±1.01	6.32 c ±0.55
After cooling	6.52 a ±0.06	72.67 c ±1.89	17.60 bc ±0.96	10.53 c ±1.82	7.60 bc ±0.89
Day 1 preservation	6.35 b ±0.03	57.05 d ±1.05	19.07 b ±0.74	12.10 bc ±1.09	9.07 b ±0.64
Day 2 preservation	6.30 bc ±0.09	55.00 d ±2.54	21.00 a ±0.98	13.89 ab ±1.28	11.00 a ±1.09
Day 3 preservation	6.25 c ±0.06	36.42 e ±2.96	21.64 a ±1.12	16.28 a ±1.60	12.00 a ±1.61

Means with different superscripts within column are significantly different (P < 0.05).

Generally, sperm motility in the present study significantly decreased throw preservation in 5˚C, a trend confirmed by (4) and (22).

In conclusion, it is necessary to add 7.5 - 10 mM amino acid cysteine to the citrate egg yolk extender to preserve Awassi ram semen in 5˚C., and semen quality were continually deteriorated as the storage period prolonged, but the deterioration were less affected when cysteine was added. More studies are needed to identify other antioxidants to support Awassi rams semen preservation.

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تأثير السستين في بعض صفات السائل المنوي المبرد لكباش العواسي

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الملخص

أُجريت الدراسة على السائل المنوي لثلاثة كباش عواسي في قسم بحوث المجترات / دائرة البحوث الزراعية في الزعفرانية – جنوب بغداد لاجل تقويم تأثير إضافة السستين الى مخفف السترات – صفار البيض والحفظ لثلاثة ايام متتالية في التبريد بالثلاجة في بعض صفات السائل المنوي، وتأثير عمليات التخفيف والتبريد والحفظ في خفض نوعية السائل المنوي. جمع السائل المنوي للكباش مع بعضه ثم قسم الى سبعة أجزاء متساوية وتضمنت مجموعة السيطرة (c) إضافة **0.0** ملي مول والمعاملات من **T1** الى **T6** إضافة **2.5**، **5.0**، **7.5**، **10.0**، **12.5** و**15.0** ملي مول سستين. تمت دراسة وتحديد نوعية السائل المنوي المحفوظ بالإعتماد على درجة الحموضه **pH** والنسبة المئوية لكل من الحركة الأمامية والنطف الميتة والمشوهة وتشوهات الاكروسوم. أظهرت النتائج ان درجة **pH** لم تتأثر معنوياً في حين تأرت الصفات الأخرى معنويا **($p< 0.05$)** في اضافة السستين الى مخفف السترات – صفار البيض، وظهر التحسن في معظم الصفات عند إضافة مستويات مختلفه من السستين. المستويات الواطئة والمتوسطة من **(2.5– 10 mM)** لم تؤد الى تحسن في سلامه الاكروسوم على الرغم من انها حسنت الحركه الأماميه وبقية الصفات المدروسة . أظهرت عمليات التخفيف والتبريد والحفظ بالثلاجة تاثيراً سيئاً في كل الصفات المدروسة مع مرور الوقت وتدرج الفعاليات . خلصت الدراسة الى أهمية إضافة السستين بين **7.5 – 10.0** ملي مول الى المخفف للحفاظ على نوعية جيدة للسائل المنوي المبرد لكباش العواسي ولأطول مدة ممكنه.

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