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EFFECT OF ADDITION OF CYSTEINE AND TRYPTOPHAN TO DILUTED AND COOLED SEMEN OF AWASSI RAMS

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Abstract

The aim of this study was to evaluate the effects of different concentrations of cysteine and tryptophan on individual motility, dead/alive and sperm abnormalities after diluted and cooled of Awassi ram semen. Ejaculates were collected weekly using electro-ejaculator from four Awassi rams. Each ejaculate was divided into 5 equal parts and was diluted in a Tris-based extender, included (control, basic diluents), (basic diluents containing 5 mM cysteine), (basic diluents containing 10 mM cysteine), (basic diluents containing 5 mM tryptophan) and (basic diluents containing 10 mM tryptophan) diluted and cooled semen was evaluated for the percentages of sperm individual motility, dead/alive and abnormalities at 0, 24, 48 and 72h. Results show that different concentrations of cysteine added to Awassi ram semen showed a significant decrease ($P < 0.05$) in individual sperm motility, increases dead, and abnormality compared with control during different storage. While, during different storage periods, all the quality parameters in diluted semen with concentrations of tryptophan decreased significantly. In conclusion, the findings of this study show that different cysteine concentrations had not shown the desired protective effects for 72h on semen parameters of Awassi rams. Nevertheless, some causes may have an effect on the results.

Key words: Awassi ram, cooled semen, cysteine, tryptophan.

Introduction

The advantages of artificial insemination (AI) in animal production are many. The key benefit is really the transmission of genetic material and the control of diseases, as a result of which males distinguish superior genetics (Bailey *et al.,* 2003) to enhance herd productivity (Mittal *et al.,* 2019). Therefore, for the effective use of artificial insemination techniques in the sheep, investigation into methods of dilution of rams semen within the short time following collection (Salamon and Maxwell, 2000) to preserve sperm against damage when cryopreservation (Akcay *et al.,* 2012; Amidi *et al.,* 2016) reflected in ability to fertility (Ntemka *et al.,* 2018). Moreover, the importance of extenders in providing the best possible sperm environment through storage (Paulenz *et al.,* 2002; Soltanpour and Moghaddam, 2014; Maksimovic *et al.,* 2018). Other factors are also extended to support preserved sperm during cooling (Albiaty *et al.,* 2016; Varisli *et al.,* 2018).

Successful cooled ram semen depends on maintaining spermatozoa's membrane integrity, motility and fertilizing capacity (Paulenz *et al.,* 2002). The sperm cells are, however, sensitive to damage by reactive oxygen species (ROS) (Ashrafi *et al.,* 2011) As well as the relatively high level of unsaturated fatty acids in the sperm membrane phospholipids (Lenzi *et al.,* 2000). Furthermore, normal cellular metabolism can generate reactive oxygen species and react with biomolecules such as protein, lipid, and DNA (Manisha *et al.,* 2017) Leads to loss of membrane integrity and allows cellular damage (Jat, 2016). Antioxidants, on the other hand, play an important role in reducing oxidative damage to sperm (Agarwal *et al.,* 2005) through motility protection of the sperm and stability of the membrane (Tvrda *et al.,* 2016) In processing and storing semen (Tvrda *et al.,* 2018). Therefore, improve semen during processing and storage with the use of dilute additions. Cysteine is an important sulfur source and an essential precursor in the processing of antioxidant glutathione that protects cells from toxins such as free radicals (Piste, 2013). Therefore, cysteine offered substantial support during storage of liquid to the ram sperm parameters (Gungor *et al.,* 2017) and improved

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post-thaw motility and enhanced buffalo bull semen membrane and acrosome integrity (El-Sheshtawy *et al.,* 2008). Tryptophan, on the other hand, is aromatic amino acid that adds low concentrations (El-Sheshtawy *et al.,* 2012) therefore the toxicity generally increases with increasing concentrations (Macmilan *et al.,* 1972). The main objective of the current study would be to assess the efficiency of cysteine and tryptophan after dilution and cooling to protect semen characteristics when applied to Awassi rams.

Materials and Methods

Animals and semen collection

The present study was conducted at farm and laboratory belonging to the College of Veterinary Medicine/ University of Fallujah, during the period from 15th February to April 2019. The rams were fed concentrated meal supplemented with lucerne hay and fresh drinking water was provided. Semen samples from four Awassi rams (3 years of age) were used in the study. Ejaculates were collected weekly from each ram using electro- ejaculator. Immediately after collection, the ejaculate was incubated in a water bath at 37^0C, until microscopic sperm quality assessments were performed in the laboratory. The semen samples were evaluated for volume was measured in a conical tube graduated at 0.1 ml intervals, pH measured directly using indicator papers range 5.6 - 8.0 (Madaus GmbH, Koeln, Germany), color, mass and individual motility (Soltanpour and Moghaddam, 2014). The percentage of dead and abnormal sperm (%) were assessed by means of the eosin-nigrosin staining, the sperm concentration was determined by means of a haemocytometer (Salamon and Maxwell, 1995).

Semen processing

One ejaculate of each ram was divided into 5 equal parts and were diluted 1:10 fold with a Tris-based extender according to the concentration (Tris =2.42g, fructose =1g, citric acid =1.34g, egg yolk =20 ml, glycerol = 6.4, penicillin (1000 unit/ ml) streptomycin (1000µg/ml) and distilled water to make the volume 100 ml. Therefore, control and 4 parts-containing extenders were prepared for semen dilution. Groups were; (Control, basic diluents), (basic diluents containing 5 mM Cysteine), (basic diluents containing 10 mM Cysteine), (basic diluents containing 5 mM Tryptophan) and (basic diluents containing 10 mM Tryptophan). Then diluted semen cooled gradually by addition a piece of ice till it reaches 5^0C and maintained at 4^0C in a refrigerator. After cooling semen divided into 5 parts and adding to it as in diluted semen. In addition, semen parameters were measured after cooling at 0, 24, 48 and 72 h.

Statistical Analysis

Statistical analysis was performed with the SPSS Statistics 24.0 (2016). Statistical significance was declared at $P<0.05$.

Results and Discussion

In the present study, the parameters of fresh Awassi rams semen summarized in table 1.

Table 1: The parameters of fresh Awassi rams semen used in the study (Mean ± S.E).

Parameters	Means ± S.E
Volume ml	1.05 ± 0.08
pH	6.80±0.03
Color	Creamy
Mass motility %	77.72 ± 1.23
Individual motility %	82.72 ± 1.23
Concentration × 10^9	227.72 ± 6.88
Dead sperm %	20 ± 1.20
Abnormaliy sperm %	15.27 ± 1.45

Throughout this study, the effect of different cysteine and tryptophan concentrations on percentages of individual motility, dead/live and Awassi ram semen abnormalities would be studied. With the addition of these concentrations to the extender, Awassi ram spermatozoa stored at 4^0C for 72h and evaluated the effect of these concentrations to support sperm viability during cooling for 72h.

The cooled diluted combined with cysteine (5 and 10 mM) and tryptophan (5 and 10 mM) refers to the different percentage of individual motility compared to control in cooled semen (P < 0,05; table 2) during the period 0-24 – 48 and 72h. Adding tryptophan (10Mm) at different periods revealed a lower percentage of individual motility compared to other groups. Contrast, cysteine (5Mm) had a higher percentage of individual motility ($P < 0.05$) relative to groups (cysteine 10Mm and tryptophan 5 and 10 mM) at different periods. Changes in the percentage of individual motility were shown in table 2. comparing similar findings between cysteine 5 and 10 mM at 72h with different tryptophan concentrations. However, the percentage of individual motility at different cysteine and tryptophan concentrations were lower ($P < 0.05$) than control table 2. The biological function of adding amino acids belonging to different cysteine (non-aromated amino acids) and tryptophan (aromated amino acids) to semen extenders affecting the quality of semen (El-Sheshtawy *et al.,* 2012) on the percentages of individual motility of Awassi ram semen. Nevertheless, cysteine 5mM has a major effect on the percentage of individual sperm motility

during 72 h. These results were in agreement with the findings of Khalili *et al.,* (2010) and Memon *et al.,* (2011). Cysteine is an important sulfur source and an essential precursor in the processing of antioxidant glutathione that protects cells from toxins such as free radicals (Piste, 2013) to inhibit oxidative stress or decrease cell damage (Kim *et al.,* 2020). Maia *et al.,* (2010) reported that antioxidants could use to control oxidative stress on ram spermatozoa in the cryopreservation process. On the others hand, Lone *et al.,* (2018) reported that changed antioxidant and oxidant balance might be responsible for difference in the quality of semen during various preservation stages. The addition of cysteine hydrochloride to the semen extender, however, established good functional parameters (Perumal *et al.,* 2012) and increased survival of sperm (Uysal *et al.,* 2007). Furthermore, progressive sperm motility, viability, and membrane integrity with reduced sperm / acrosomal abnormalities were increased (varghese *et al.,* 2015).

The results of the study indicated that, compared to tryptophan 5 and 10mM, the addition of cysteine 10mM to extender semen improved individual motility through 72 h. This result was in disagreement with the findings Khalili *et al.,* (2010). The authors suggested that adding 10 mM of cysteine to the control extender significantly enhanced sperm motility. On the other hand, for various storage periods, the concentration of tryptophan 5 and 10mM seemed to be decreased individual motility. Macmillan *et al.,* (1972) pointed out that aromatic amino acids produced hydrogen peroxide. Bansal and Bilaspuri (2011) reported that hydrogen peroxide was considered a non-radicals influencing sperm activities leading toward motility inhibition. El-Sheshtawy *et al.,* (2012) reported that L-tryptophan (0.5 and 5 mM) tends to cause toxic effects in the female genital tract during the transportation of sperm that reduces the rate of conception. Moreover, catalase-tryptophan combination improves semen quality without improving conception rate (El-Sheshtawy *et al.,* 2013). These evidences had been in agreement with our results with tryptophan 5 and 10 mM that reduces percentages of individual motility and increases sperm dead and abnormalities during different storage.

In the present study, the percentage of dead sperm was higher in tryptophan (10 mM) ($P<0.05$) at all periods than in groups table 3. However, the percentage of dead sperm were lower ($P<0.05$) in cysteine (5mM) in different periods. On the other hand, the findings showed the percentage of dead cysteine sperm (10mM) similar with cysteine (5mM), roughly. However, the percentage of dead sperm in tryptophan (5 and 10 mM) has been increased significantly ($P<0.05$) compared to cysteine (5 mM). Based on the fact that the percentage of dead sperm at different cysteine and tryptophan concentrations was higher ($P < 0.05$) than in control table 3.

Table 4 showed the effects of different concentrations of cysteine and tryptophan in the diluent on the percentage of ram sperm abnormality for periods 0 – 72h. Nevertheless, in all periods, tryptophan (10 mM) was higher percentage of sperm abnormality ($P < 0.05$) compared to groups. On the other hand, cysteine (5mM) showed significantly lower percentage of sperm abnormality ($P < 0.05$) than tryptophan (5 and 10mM) at 72h, whereas cysteine (10mM) showed comparable findings at 48 and 72h with cysteine (5mM). Nevertheless, the percentage of sperm abnormality in both different cysteine and tryptophan concentrations were higher ($P < 0.05$) than control table 4.

Our results indicated that adding different cysteine and tryptophan concentrations that decreased leading in individual motility, increases sperm dead and abnormalities in different storage compared to the findings El-Sheshtawy *et al.,* (2012); El-Sheshtawy *et al.,* (2013).

It appears that different cysteine

Table 2: Individual motility % of Awassi rams sperm addition with different concentrations of cysteine and tryptophan for different storage times at 4^0C (mean ± S.E).

Groups		0h	24h	48h	72h
Control		80.0± 0.95a	76.36± 1.18a	70.45± 1.05a	67.27± 1.40a
Cysteine	5mM	75.90±1.13b	69.09±2.31b	62.72±2.17b	57.72±1.70b
Cysteine	10mM	75.45±1.05bc	67.72±2.17bc	59.09±2.11bc	53.63±2.03b
Tryptophan	5mM	72.72±0.78cd	63.18±2.16cd	55.45±2.07c	47.72±1.94c
Tryptophan	10mM	71.81±1.01d	59.09±1.76d	48.18±2.45d	41.36±2.34d

The values mean ± S.E a, b, c, d different superscripts within the same column demonstrate significant differences ($P < 0.05$).

Table 3: Dead % of Awassi rams sperm addition with different concentrations of cysteine and tryptophan for different storage times at 4^0C (mean ± S.E).

Groups		0h	24h	48h	72h
Control		20.54±0.98b	24.45±1.31d	28.81±1.45d	32.81±1.50d
Cysteine	5mM	23.90±1.59ab	29.90±1.86c	34.63±1.72c	40.72±2.07c
Cysteine	10mM	23.72±1.07ab	29.90±1.16c	37.45±1.44bc	42.90±1.61bc
Tryptophan	5mM	26.27±1.39a	35.18±1.40b	41.27±1.03b	46.09±0.99b
Tryptophan	10mM	26.45±1.42a	40.27±1.89a	49.81±2.45a	55.81±2.06a

The values mean ± S.E a, b, c, d different superscripts within the same column demonstrate significant differences ($P < 0.05$).

Table 4: Abnormalities % of Awassi rams sperm addition with different concentrations of cysteine and tryptophan for different storage times at 4^0C (mean ± S.E).

Groups		0h	24h	48h	72h
Control		17.27±1.42b	20.63±1.29c	25.72±1.10c	29.81±1.82d
Cysteine	5mM	20.18±1.45ab	27.18±2.38b	33.36±2.39b	38.00±2.10c
Cysteine	10mM	20.00±1.15ab	28.09±1.92b	37.18±2.05b	41.45±2.30bc
Tryptophan	5mM	22.36±1.23a	31.90±1.70ab	39.63±1.76b	45.63±1.79b
Tryptophan	10mM	22.54±1.37a	36.72±1.81a	47.27±2.88a	53.45±2.59a

The values mean ± S.E a, b, c, d different superscripts within the same column demonstrate significant differences ($P < 0.05$).

concentrations had not shown the desired protective effects for 72h on semen parameters of Awassi rams. Nevertheless, some causes may have an effect on results. Several studies revealed that cysteine had a negative effect on vital sperm. Chatiza *et al.,* (2018) observed that cysteine supplementation was unsuccessful at 17°C to focus on improving semen liquid preservation longevity and survivability. Severo *et al.,* (2011) reported that increased percentage of estrus returns due to cysteine affect the viability of the sperm. Pinto *et al.,* (2017) reported that the addition of n-acetylcysteine did not enhance the parameters of sperm motility at any of the time points or dosages tested. Consequently, storage of bull epididymis up to 96 hours did not significantly affect parameters of sperm. On the other hand, Pilane *et al.,* (2019) pointed out that unimproved South African buck semen exhibits robust resistance to influences of ROS on sperm parameters.

Previous studies have found that indicated variations in sperm characteristics between artificial vagina (AV) and electro ejaculator (EE) collected ejaculates, leading more successful results when using AV. Bopape *et al.,* (2015) who found that positive results semen parameters when collecting AV semen compared to EE. Jiménez-Rabadán *et al.,* (2012) observed that following cryopreservation, sperm quality is higher when AV obtains ejaculates. Marco-Jimenez *et al.,* (2008) reported that ejaculates obtained with EE alter the sodium concentration, change two protein concentrations and induce one protein loss in seminal plasma. Matthews *et al.,* (2003) reported that there have been no differences in the sperm morphology between the two methods of collecting semen. (Malejane *et al.,* 2014) reported that the AV collection method produced better overall quality semen than the EE collection method. However, these study results are consistent with findings that demonstrated the effect of EE technique on semen parameters when cysteine would be add to the extender compared with the control.

Based on the results obtained from this study Ramukhithi *et al.,* (2011) reported that after freezing and thawing, South African indigenous goat fresh semen collected with electro-ejaculator had an effect on semen pH towards acidic effect that affected sperm motility. Our results, however, showed semen pH to be 6.8 and not altered in the current study. Therefore, suggest excluding the factor. Shannon and Curson, (1981) reported that the percentage of dead spermatozoa between bulls had variations. The parameters of fresh Awassi rams semen were within natural range in the present study, and the difference between the rams seemed to be limited. However, the percentage of dead sperm increased with different cysteine and tryptophan concentrations compared with control during periods 0 -72h.

Conclusion

It can be concluded that the findings of this study show that different cysteine concentrations had not shown the desired protective effects for 72h on semen parameters of Awassi rams.

Nevertheless, some causes may have an effect on the results.

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