

Effect of Coated Methionine and Lysine and Their Combination on Fertility of Awassi Male Sheep

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Abstract: This study was conducted to determine the effect of Amino acid Methionine and Lysine on the reproductive performance of male Awassi sheep. Twenty Awassi males aged range 10-12 months and average weight $32 \pm 0.5\text{kg}$ were used in this study, during the period from May to August 2022. The animals were randomly divided into four equal groups. The first group was the control group (T1). In this group, sheep were fed on standard diet without any additive. The second treated group (T2) were fed on standard diet supplemented with coated Methionine (1.5g/sheep/day). The third treated group (T3) were fed on standard diet supplemented with coated Lysine (2.5g/sheep/day). The fourth treated group (T4) were fed on standard diet supplemented with coated Methionine and Lysine at the same doses used in T2 and T3. The results showed that there was a significant improvement ($P \leq 0.05$) in sperm characteristics of T4 as compared to other groups. T4 also showed the highest testosterone levels as compared with the other groups. T4 group also showed a well development in the seminiferous tubules, Sertoli cells and Leydig cells as compared with T1. T4 showed the best histological testicular architecture especially Sertoli cells as compared with T1, T2 and T3. It was concluded from this study that coated Methionine and Lysine might improve semen characteristics, testosterone levels and male reproductive performance in Awassi male.

Keywords: Coated lysine, Coated methionine, Semen, Sheep, Testosterone.

Introduction

Amino acids, are essential components to build a protein required for healthy animal development, fertilization, lactation, and semen preservation (Ugur *et al.*, 2020). The ruminants derive their amino acids from dietary protein and microbial protein synthesis in the rumen (Younis & Abdl-Elazem, 2019). Degrade proteins from food, in the rumen, are representing the main source of the amino acids

absorbed in the small intestine of ruminants (Gilbreath *et al.*, 2021). In most diets, microbial proteins synthesis Methionine and Lysin which are classified as the first or second limiting amino acid (Wei *et al.*, 2019). Ruminant animals are lacking of the amino acid methionine because they cannot synthesised it (Alkhashab *et al.*, 2021). Therefore, diet digestible proteins and microbial protein must

provide methionine (Mohany *et al.*, 2021). Coated methionine is used to prevent it from microbial degradation and travels to the small intestine for absorption. Methionine is breakdown in the rumen, however, its bioavailability is limited because of methionine degradation in rumen (Schwab *et al.*, 2001). Therefore, adding rumen protected Methionine(RPM) to ruminant diets as a supplement can boost the flow of nitrogen and amino acids to the small intestine (Donkin *et al.*, 1989), which will enhance growth and nitrogen consumption efficiency and, as a result, ruminant livestock's overall performance (Mavrommatis *et al.*, 2021). Alkhashab *et al.* (2021) which they found that adding protected methionine to diet can effect on ram testicular dimensions and semen qualities in growing Awassi ram lambs.

Nizza *et al.* (2000) discovered that methionine supplementation dramatically increased libido and the motility of rabbit sperm. It has not yet been investigated if a high diet methionine may restore testicular damage. Lack of protein negatively correlates with the effectiveness of utilizing protein and energy, the number of amino acids available for absorption in the intestine, and its physiological effects during the reproductive period, which is represented by the low fertility rate through its impact (Wu, 2010). Hydrolyzed protein, though of its importance in providing the needs of microbiology for the formation of microbial protein, is imbalanced with the non-decomposed part (Wu *et al.*, 2014). In animals, adding amino acids to the diet or administering them enhanced semen traits (such as motility, velocity, morphologically normal sperm, and acrosome integrity) and the outcome of fertilization; Methionine and Lysine have

become more widely used due to their particular importance (Xia *et al.*, 2021). The first amino acids were discovered for manufacture without the need to raise as supplements to relatives in the form of free amino acids after shielding them from Keratin metabolism to enhance the productive performance of animals (Hacham *et al.*, 2007). Lysine is a vital amino acid promote sheep's reproductive performance functionality (Mirzoyan *et al.*, 2006). Supplementation of diet with amino acids like lysine improves sperm quality, increases fertilization capacity (Dong *et al.*, 2016), Amino acids L-lysine help increase sperm count and motility, as well as enhance the strength of ejaculation. Zhang *et al.* (2023) observed that the total motility and progressive motility were significantly increased in 1% Carboxylated ϵ -Poly-L-lysine (CPLL) supplemented on the post-thaw quality of cryopreserved goat sperm compared to control. According to bioinformatics analysis, Lysine is linked to sperm functions like motility, capacitation, acrosome response, and sperm-egg interaction. So, Lysine acetylation is a crucial regulatory mechanism for sperm activities (Sun *et al.*, 2014). Few studies have examined the effects of dietary amino acids on gamete quality and male gonadal development. Therefore, this study was aimed to show the effect of protected Methionine and Lysine on semen traits.

Materials & Methods

The study was conducted in a local field located in the Baybukht region, about 20km from the center of Mosul city. Twenty Awassi sheep average weight of 32 ± 0.5 kg and ages range (10 to 12 months) were used in this study. The study lasted for three months (from beginning of May to the end of August 2022). The

animals were randomly distributed into four equal groups (n=5/group), the first group, (Control T1) sheep fed on standard diet without any addition, the second treated group (T2) were fed on protected Methionine at dose (1.5g/sheep/day), the third treated group (T3) were fed on protected Lysine at dose (2.5g/sheep/day), the fourth treated group (T4) were fed on protected Methionine and Lysine at the same doses used in T2 and T3 in addition to the standard diet. Blood samples were collected from the jugular vein at the end of the experiment (5ml), by 5ml disposable syringe. The blood samples were placed in test tubes free of anticoagulant and left for about 15minut at room temperature (25°C). Serum was collected following centrifugation at 1207g for 15 minute and kept in refrigerators at -20 until analysis. Serum testosterone concentrations were assayed by using enzyme linked immunosorbent assay (ELISA) kit (DiaPlus Inc., USA).

Semen collection: an American-made electro-ejector type electrical stimulation equipment was used in this study (Electrojac 6). The semen was collected from all rams at the end of the research period. Briefly, were lying on one of their sides on the table. Its head and legs were tethered to make control simple. The foreskin hole was cleaned with warm water, the surplus wool was clipped, then dried with cotton, and a tube opening was introduced. This was followed by the cathode probe being put into the rectum after being cleaned and lubricated with glycerol. Through the foreskin's entrance, the tube was inserted. It was held in place for a few seconds as electrical stimulation was used to manipulate the tube throughout the ejaculation process before being removed. By moving the probe to

the ram rectum, the stimulation procedure was repeated many times. After collecting the semen, the tube was sealed with cotton and submerged in water bath for 20min at 37°C to liquefy it for semen analysis (Hafez & Hafez, 2000). After the washing-up, the liquefaction time was recorded, time was noted, and each sample was examined in the laboratory at the Faculty of Veterinary Medicine, University of Mosul. Data of both macroscopic and microscopic semen examinations were recorded. The animals were slaughtered at the end of the experiment, and one testis was taken from each animal for histological study.

Microscopic examinations: Testis were dissected and tissue samples were fixed at 10 % buffered neutral formalin for 48 hrs ,the tissue samples were dehydrated in alcohol and then cleared with xylol and embedded in paraffin. Histologic section was made into 3-4 µm in thickness and stained with hematoxylin and eosin according to (Culling, 1974; Creasy, 2002). In histological sections of the testes, Sertoli and Leydig cells were counted as a mean of five measurements/field (60.08µm²/400X) for 5 fields for each animal in the group (Petersen *et al.*, 2015). Using the software of microscope camera (USB 2.0 digital image camera) (Omax ToupView 9.0-Megapexil China) calibrating to all lenses of Microscope-Olympus-CX31 by the aid of 0.01mm stage micrometer (ESM-11/Japan) using the histomorphometric measurements ruler line in this software by a Pathologist in the Veterinary Medicine diagnostic lab. It provided calibration line measurements (Petersen *et al.*, 1999).

The components of the diet were estimated from the nutritional compounds in the

laboratory according to what was reported (AOAC, 2016), except for the energy, which was calculated mathematically according to (Al-Khawaja *et al.*, 1978).

Statistical analysis: The statistical analysis of the data was carried out according to the completely randomized design (CRD) one-way ANOVA. The significance of the differences between the treatments was tested using Duncan's multiple range test (Duncan, 1955), and used the ready-made statistical analysis

program (SAS, 2001) to analyze the data according to the following mathematical model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Y_{ij} = The observation value j for treatments I .

μ = The general mean of the studied trait.

T_i = Effect of treatments.

e_{ij} = The Experimental error.

Table (1): Ingredients and chemical composition of the standard diet

Feed material	%
Barley	61.5
Wheat bran	22
Yellow corn	5
Soybean meal	5
Hay	5
Urea	0.5
Salt	0.5
Limestone	0.5
Chemical composition	
Dry matter	92.12 %
Organic matter	95.77 %
Crude protein	13.76 %
Crude fat	2.08 %
Raw fiber	7.02 %

Results

The results showed in table (2) there is no significant differences in pH and ejaculate volume between all experimental groups (Table 2). T4 was significantly improved ($P < 0.05$) in sperm concentration as compared to the control and T2 groups. Mass motility showed higher increase in T1 ($P < 0.05$) compared to T1 and T3 but not T2. All treated groups showed a significant increase ($P < 0.05$) in individual motilities compared to the control group. The

ratio of live sperm revealed a significant decrease ($P < 0.05$) in T2 and T4 as compared with the control group, even though there was no significant difference between either of the treated groups and the control group for the proportion of dead sperm. Additionally, compared to the control group, T2, T3, and T4 groups were significantly lower rate of deformed sperm ($P < 0.05$). The T4 had the most significant levels of testosterone compared to T1, T2, and T3. In addition to that T2 and T3 was significantly greater than control group.

Table (2): The Effect of Protected Methionine and Lysine on some reproductive traits (Mean± S.E)

Traits \ Treatments	T1 Control	T2 Protected Methionine	T3 Protected Lysine	T4 Protected Methionine + Lysine
pH	7.025±0.04 a	7.041±0.01a	7.05±0.03a	7.05±0.01a
Ejaculate volume ml	1.125±0.06 a	1.21±0.02a	1.2±0.06 a	1.2±0.04 a
Sperm Concentration × 910 /ejaculate	0.85±0.1b	0.8±0.06b	0.97±0.02 ab	1.122±0.03 a
Sperms Mass Motility Percentage (1 – 5)	60±7.43c	85.83±1.93 ab	77.5±1.68 b	92.5±0.75 a
Sperms Individual Motility Percentage %	40.42±8.29b	68.75±6.91a	62.92±7.14a	77.5±4.37a
Live Sperms %	58.75±8.71b	66.25±6.74a	59.58±6.7ab	76.25±4.73a
Dead Sperms %	41.25±8.41a	33.75±6.74a	40.42±6.7a	23.75±4.73a
Deformed Sperms %	3±0.43a	1.42±0.15b	1.25±0.13b	1.25±0.13b
Testosterone level ng. ml ⁻¹	1.48±0.35c	3.49±0.76b	3.85±0.49b	5.77±0.23a

Different letters horizontally differ significantly at the 5% level

Table (3) showed a significant increase (P<0.05) in the diameters of the seminiferous tubules, Leydig cells in T2, T3 and T4 compared to T1.

However, Sertoli cells was significantly increased in T4 compared to T1, T2, and T3.

Table (3): Measurements of seminiferous tubules diameters (μm) and the mean counts of the Sertoli and Leydig cells (Mean \pm S.E)

Treatment Trait	T1 Control	T2 Protected Methionine	T3 Protected Lysine	T4 Protected Methionine + Lysine	P-value
Seminiferous tubules diameters (μm)	149.4 \pm 12.6b	249.2 \pm 38.4a	268.8 \pm 9.8a	217.2 \pm 4.3a	0.005
Sertoli cells counts	4.4 \pm 0.51b	6.6 \pm 0.92b	6.4 \pm 0.51b	9.2 \pm 1.02a	0.003
Leydig cells counts	12.5 \pm 0.42b	20.6 \pm 1.74a	22.8 \pm 2.13a	26.0 \pm 0.74a	<0.001

Different letters horizontally differ significantly at the 5% level.

The microscopic investigation of testis of the control group showed degeneration and necrosis of spermatogenic cell in in a few seminiferous tubules with presence of normal Sertoli cell and Leydig cell (Figs. 1, 2). Second treated group with protected methionine showed well developed seminiferous tubules and Spermatogonia cells as well as sertoli and Leydig cell (Figs. 3 and 4).

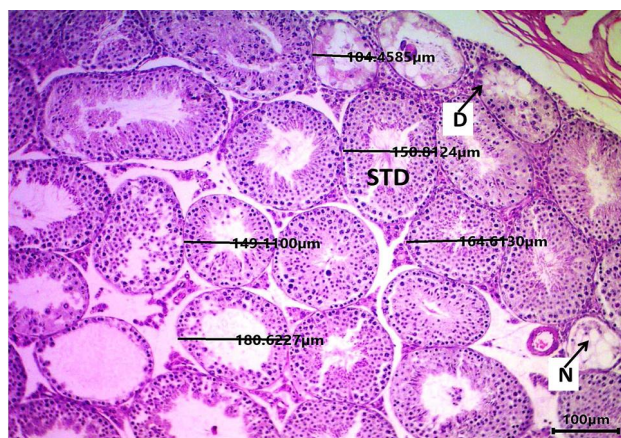


Fig. (1): photomicrograph of sheep testis of the control-treated group showing the measurements of seminiferous tubules diameter (STD) with degeneration (D) and necrosis (depletion) (N) of the spermatogenesis cells in a few seminiferous tubules. Omax Toup View Camera software. H&E stain

The microscopic picture of third treated group with protected lysine showed mild degeneration of spermatogenic cell in few seminiferous tubules with presence of normal Leydig cell and Sertoli cell (Figs. 5 and 6). The fourth treated group with protected methionine and lysine showed very well improvement in the structure of seminiferous tubules and spermatogenic cell, Sertoli cell and Leydig cell (Figs. 7 and 8).

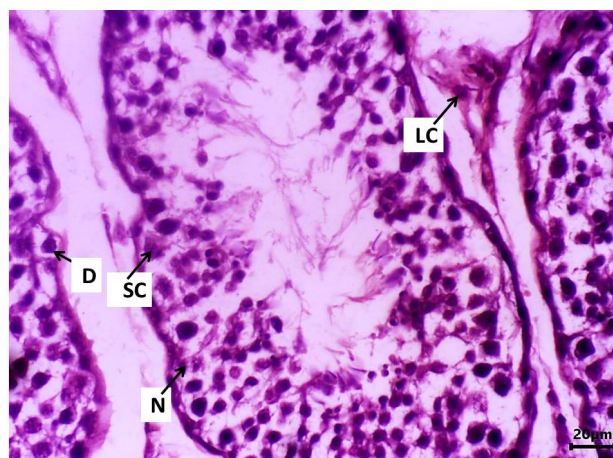


Fig. (2): photomicrograph of sheep testis of the control group showing degeneration (D) and necrosis (N) of the spermatocytes, with the presents of Sertoli cell (S.C.) and Leydig cell (L.C.)

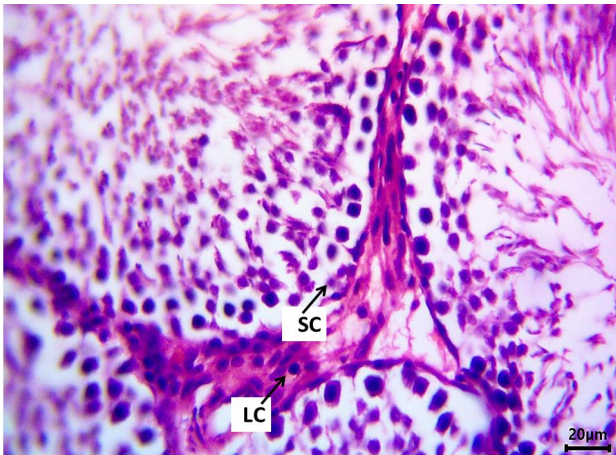


Fig. (3): photomicrograph of sheep testis of the protected Methionine treated group (T2) showing well-development of the spermatogenesis cells with the presents of Sertoli cell (S.C.) and Leydig cell (L.C.). H &E stain



Fig. (5): Photomicrograph of sheep testis of the protected lysine treated group (T3) showing the seminiferous tubule diameter (STD) measurements in well-development. Omax Toup View Camera software. H &E stain

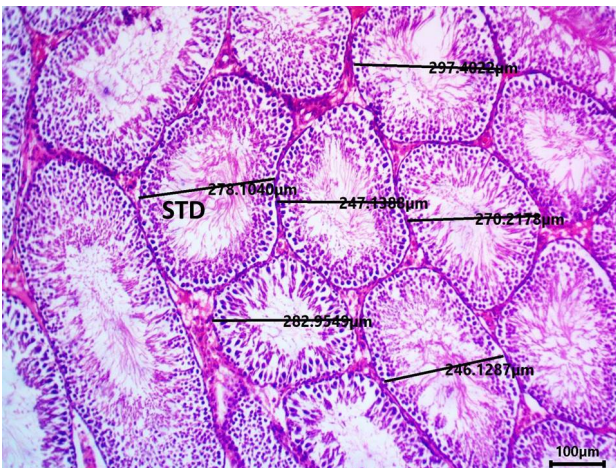


Fig. (4): Photomicrograph of sheep testis of the Methionine treated group (T2) showing the seminiferous tubule diameter (STD) measurements in well-development. Omax Toup View Camera software. H & E stain

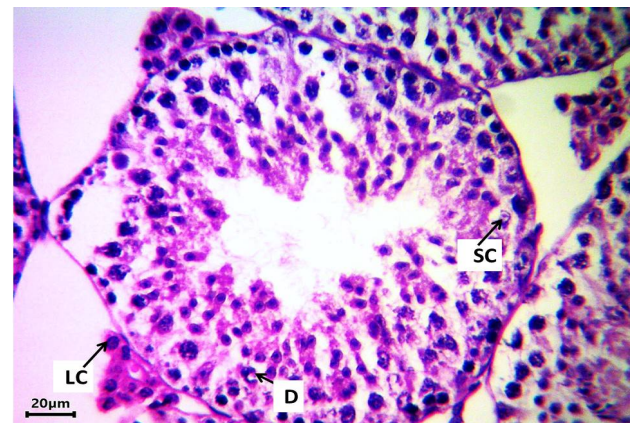


Fig. (6): Photomicrograph of sheep testis of the protected Lysine treated group (T3) showing mild degeneration (D) of the spermatocytes with the presents of Sertoli cell (S.C.) and Leydig cell (L.C.). H & E stain

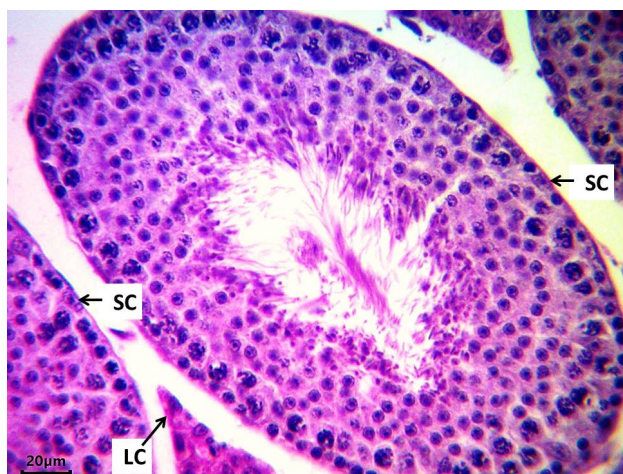


Fig. (7): Photomicrograph of sheep testis of the protected Methionine and Lysine treated group (T4) showing well-development of the spermatogenesis cells with the presents of Sertoli cell (S.C.) and Leydig cell (L.C.). H & E stain

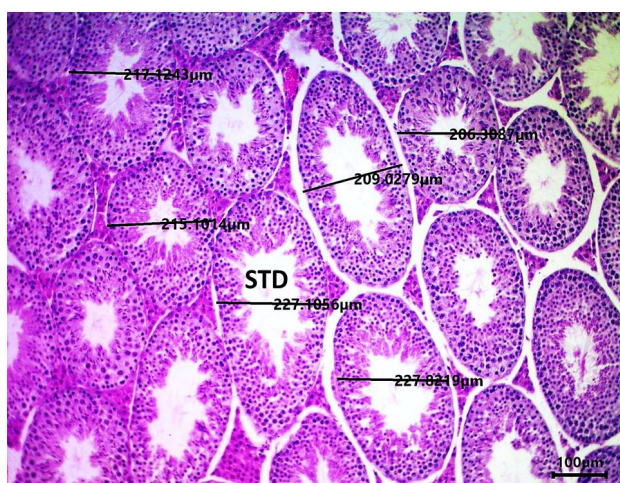


Fig. (8): Photomicrograph of sheep testis of the protected methionine and Lysine treated group (T4) showing the measurements of seminiferous tubule diameter (STD) in well development. Omax Toup View Camera software. H & E.

Discussion

There is growing evidence that amino acids are beneficial for enhancing the reproduction and influence the success of sperm production, promotion of fertilization, and controlling reproductive processes (Kwasek *et al.*, 2014). The protective impact of protected Methionine and Lysine on the testis and epididymis may be

the cause of the semen qualities improvement including a rise in ejaculate volume, sperms concentration, individual and mass motility, and a decrease in the percentage of dead and abnormal sperm (Hong *et al.*, 2016). An essential endogenous antioxidant amino acid in animals are Methionine and Lysine (Lahnsteiner, 2010). Sperm are susceptible to oxidative stress due to insufficient antioxidant protection which makes them highly dependent to the antioxidant machinery in the seminal plasma (Aitken *et al.*, 2012). Semen antioxidant defense systems, present in both sperm and seminal plasma, which can counteract with excessive levels of reactive oxygen species (ROS). Under physiological conditions, there is a balance between ROS production and the antioxidant protection. However, imbalance occurs due to a reduction in the antioxidant capacity and/or to increase ROS production which leads to oxidative stress (Alahmar, 2019). The seminal plasma has an important role in supporting sperm and protecting the male gametes from ROS damaging effects (Szczykutowicz *et al.*, 2019). Methionine and Lysine are an essential part of seminal plasma and sperm. They have an impact on sperm metabolism, sperm motility, and male gamete quality and performance (Kwasek *et al.*, 2014). This makes the effects of amino acid supplementation on male gamete quality and performance fascinating. Nizza *et al.*, (2000) observed that Methionine supplementation dramatically increased libido and the motility of rabbit sperm. Additionally, Ezzat *et al.* (2019) showed that male laying hens fed with zinc methionine had significantly less dead and total aberrant sperms than the control group. Our results were agreed with those of El-Sharawy *et al.* (2012), who found that soaking zinc

Methionine had a significant impact on the semen traits of crossbred Rams. This outcome is inconsistent with research of Shamiah *et al.* (2017), who observed that selenomethionine supplementation (0.30mg. kg diet⁻¹) significantly increased the testosterone level of Cockerels. Male broiler testosterone concentration did not significantly change as a result of the Methionine addition in the feed (Zhai *et al.*, 2016). This rise in testosterone levels might be due to the methionine supplementation that increased growth and live body weight. Testosterone hormone and body weight have a positive correlation, an improvement in growth and live body weight may also increase testosterone levels (Ashton *et al.*, 1995). The type of amino acids, including Methionine and Lysine, were responsible for inducing spermatogenesis and sexual behavior (Nizza *et al.*, 2000). Additionally, adding more Lysine to the food dramatically increased the ejaculate volume (Huang *et al.*, 2020), there is link between dietary intakes of specific amino acids and spermatogenesis, for example, adding certain amino acids, such as Lysine, to goat semen during incubation improves the pH and metabolic activity of the sperm as well as the production of ATP (nmol.10⁻⁸ spermatozoa), which is necessary for sperm motility (Zoca *et al.*, 2022). Although it has been shown that Lysine can shield cells from the damaging effects of excessive reactive oxygen species (ROS), it has also been shown that Lysine and their metabolites can encourage oxidative processes by taking part in metal ion-mediated events that produce ROS and free radicals (Ritagliati *et al.*, 2018; Collin, 2019). This startling finding highlights amino acid's crucial role in male gamete function. Another important finding in the current study was the

apparent improvement in testicular tissue represented by well-developed seminiferous tubules and the increase in the numbers of Leydig cells and Sertoli cells in the two treated groups with methionine and lysine and their combination. Sertoli cells regulated the secretion and synthesis of testosterone from Leydig cell, they are working together to regulate the spermatogenesis. The Sertoli cells increment provides a lot of growth factors for spermatogenesis process, which is important for spermatogenic cells differentiation, development and maintaining spermatogenesis (Cupp & Skinner, 2001). Also in this current study there is more pronounced effect as shown in the histological sections reviewed in the results of the study. All these histological studies were combined with macroscopic and microscopic examinations of semen samples for the treatments of the experiment.

Conclusion

It was concluded from this study that protected methionine and lysine and their combination have a beneficial effect on semen and testicular performance.

Acknowledgments

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Ethics

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Conflicts of Interest

The authors declare no conflicts of interest.

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تأثير المثيونين واللايسين المغلف ومزيجهما على خصوبة ذكور الاغنام العواسي

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المستخلص: اجريت هذه الدراسة لتحديد تأثير المثيونين واللايسين المغلف على الاداء التناسلي لذكور الاغنام العواسية، استخدم في هذه الدراسة عشرون من الذكور العواسي تتراوح اعمارهم بين 10-12 شهراً ووزنها 32 ± 0.5 كغم بدأت الدراسة من شهر أيار الى شهر اب 2022، تم توزيع الحيوانات عشوائياً على اربع مجاميع متساوية ، مجموعة السيطرة تم تغذيتها على عليقة قياسية بدون أي ضافة، المجموعة الثانية تم تغذيتها على المثيونين المغلف بجرعة (1.5 غم / راس/يوم)، المجموعة الثالثة تم تغذيتها على اللايسين المغلف بجرعة (2.5 غم / راس / يوم) ،المجموعة الرابعة تم تغذيتها على المثيونين واللايسين المغلف بنفس الجرع المستخدمة بالمجموعة الثالثة والرابعة بالإضافة للعليقة القياسية ، اظهرت النتائج تحسن معنوي في خصائص الحيامن في المجموعة الرابعة مقارنة مع مجموعة السيطرة وباقي المجاميع، ايضا اظهرت المجموعة الرابعة ارتفاع مستوى هرمون التستوستيرون مقارنة مع باقي المجاميع، كذلك اظهرت المجموعة الرابعة تطور في عملية تكون النطف في النبيبات المنوية وخلايا سيرتولي وخلايا ليديج مقارنة مع المجموعة الاولى ،واظهرت المجموعة الرابعة قراءة نسيجية متطوره لنسيج الخصية خاصة في خلايا سيرتولي مقارنة مع المجموعة الاولى والثانية والثالثة. نستنتج من هذه الدراسة بان المعاملة بالمثيونين واللايسين المغلف احدثت تحسن في السائل المنوي ومستوى التستوستيرون وخصائص الجهاز التناسلي الذكري لذكور الاغنام العواسي.

الكلمات المفتاحية: اللايسين المغلف، المثيونين المغلف، السائل المنوي، ذكور الاغنام، التستوستيرون