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# EFFECT OF ADDING L-ARGININE TO TRIS-YOLK EXTENDER OF SUB-FERTILE EJACULATES IN RAMS

By

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## ABSTRACT

Forty ejaculates of poor motility (50 to 55%) were collected from 8 rams and extended with Tris-yolk extender (1:4) supplemented with varying amounts of L-arginine (0, 2, 4 and 6 mM) in order to study the effect of L-arginine supplementation on sub-fertile ram ejaculates. Computer-assisted semen analysis was used to assess semen samples that were incubated at 5ºC for 4 hours. The findings indicated that, in comparison to the control semen extender, the addition of 6 mM L-arginine increased ($P<0.05$) the percentages of total and rapidly progressing sperm motility while decreasing the percentages of non-motile and static sperm. In comparison to L-arginine free extender, the addition of L-arginine (4 and 6 mM) increased the percentages of vitality and normalcy and decreased the percentages of aberrant neck and tail. No significant ($P>0.05$) change was seen in the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in semen extender upon addition of all L-arginine doses. After four hours of cold-temperature incubation, the ejaculate of a sub-fertile ram can have improved sperm motility, velocity, and vitality by supplementing the Tris-yolk extender with L-arginine (4 or 6 mM).

**Keywords:**

Arginine. Rams. CASA. Poor Semen, Subfertility.

## INTRODUCTION

L-arginine has been used as a necessary amino acid to enhance the efficiency of reproduction **(Mateo *et al.*, 2007).** Previous research has demonstrated a connection between arginine deficit and sperm cell motility reduction as well as loss of spermatogenesis **(Jungling and Bunge, 1976).** According to **Altınısık(2016),** arginine is a fundamental part of the nucleoprotein of spermatozoa of different species,and as several nucleoproteins are needed for spermatogenesis's mitotic and meiotic processes, L-arginine is required to keep spermatogenesis going. Due to its

ability to scavenge superoxide anions ($O_2$) and hydrogen peroxide ($H_2O_2$), L-arginine possesses antioxidant characteristics **(Vergnani *et al.*, 2000).** Through the synthesis of nitric oxide (NO), arginine inhibits the peroxidation of the membrane phospholipid bilayer, safeguarding the spermatozoa's structural integrity **(Susilowati *et al.*, 2019).** Because it protects the sperm plasma membrane from lipid peroxidation and improves cell metabolism, arginine is important for sperm motility, capacitation, and acrosome reaction **(Silva *et al.*, 2014).** Sperm motility and viability were enhanced when arginine was added to semen diluents containing low-motile sperm **(*et al.*, 2012).** According to **Özer Kaya *et al.* (2018),** adding 0.5 mM arginine in vitro to ram semen in sheep may be beneficial, while adding 10 mM may degrade the quality of the spermatozoa when stored for an extended period. Ram sperm may become toxic if L-arginine is added to an extender for an extended period at high doses (4 and 5 mM) **(El-Shahat *et al.*, 2016).** After adding 4 mM L-arginine, the goat spermatozoa's viability and motility were the highest, while the percentage of necrosis, apoptosis, and malondialdehyde (MDA) were the lowest **(Susilowati *et al.*,2019).** When goat semen was added to a skim milk extender and refrigerated for five days, it maintained its quality. According to **Susilowati *et al.* (2019),** the addition of 4 mM L-arginine was the most effective treatment for preserving the viability, motility, and intact plasma membrane of goat spermatozoa, as well as lowering the level of malondialdehyde (MDA),the percentage of necrosis, and the rate of apoptosis. The amount of L-arginine and the species have an impact on the outcomes of arginine supplementation. Computer-assisted semen analysis (CASA) technologies offer a viable substitute for traditional techniques in visualizing sperm motility or concentration. With CASA systems, individual sperm can be identified and assessed. Through precise and reproducible measurements, CASA also enables the simultaneous computation of sperm cell viability, motility class, morphological forms, anomalies percentages, and kinetic parameters in a relatively short amount of time **(Amann and Waberski, 2014).**

Rams occasionally generate ejaculates that are of poor quality, particularly in terms of sperm motility. As a result, inferior ejaculates are never processed or used for artificial insemination. There is a theory that arginine can enhance the caliber of subpar ram ejaculates. Thus, the goal of the current investigation was to determine how adding various concentrations of arginine (0, 2, 4, and 6 mM) to Tris-yolk extender affected the quality of the sperm in the ejaculates of subfertility rams.

## MATERIAL AND METHODS

**Animals:**

Eight Rahmani rams, aged between two and three years old, were utilized to collect samples of semen. Clinical testing revealed that these rams were free of all general and reproductive disorders and in good overall health. These rams' reproductive clinical examination produced normal results. But occasionally, these rams would ejaculate with subfertile semen that had abnormalities (>20%), vitality (<60%), and motility (50 -55%). Every ram was kept in a yard owned by the Animal Production Research Institute, Egypt's Mehllet Moussa Experimental Research Station. For the duration of the collecting period, all rams were housed under identical management, feeding, and watering circumstances. The Animal Production Research Institute, Ministry of Agriculture, Egypt, authorized the Guidelines for the Care and Use of Animals, which were followed in all trials (Code number: 331429).

**Extender preparation and experimental design:**

According to ***et al.*** **(2011),** 0.25 M Tris, 0.08 M citric acid, and 0.05 M fructose were dissolved in 100 mL of bi-distilled water to create the tris-yolk extender. Next, 6% egg yolk was added. **El-Shahat *et al.* (2016)** generated five experimental extenders in total by adding L-arginine 99% to the Tris-yolk extender for biochemistry [(S)-2-Amino-5-guanidinopentanoic acid, Loba Chemie Pvt. Ltd., 107, Mumbai, India] at a level of 0 (Control), 2, 4, and 6 mM, respectively. For five weeks (n = 40 ejaculates), semen was taken weekly from eight rams using an artificial vagina. Following semen collection, the ejaculates were brought right away to the lab, where they were assessed for sperm motility individually using a phase-contrast light microscope that was attached to a specialized camera.The ejaculates were then placed in a water bath at 37°C. Three to five ejaculates from various rams that were subfertile and had poor initial motility (estimated to be between forty and fifty percent) were combined and diluted at a ratio of one to four using various kinds of experimental extenders. Following a 2-hour incubation period at 5°C, the diluted semen was gradually chilled to reach 5°C. Specimens of each type of extender were then assessed by CASA. There were eight replications of the entire experiment.

**Evaluation of semen by computer assisted analysis:**

Following a 4-hour incubation period with varying concentrations of L-arginine, semen samples were evaluated using CASA (SPERMOLAB®, Cairo, Egypt). Before the analysis, a pre-warmed dual chamber disposable Leja slide containing about 5 L of semen diluted with

various experimental extenders was left to settle on the mini-thermal heating stage (38°C). For every specimen, at least 200 spermatozoa from two distinct drops of the sample were examined. After manually removing the quantity of items that were mistakenly recognized as spermatozoa, each sample underwent a final examination.

The percentages of total sperm motility (%), progressive sperm motility (%), non-progressive sperm motility (%), non-motile sperm (%), class A (rapid progressive motility), class B (Slow progressive motility), class C (non-progressive motility), and class D (Static) were among the data from CASA that were used to analyze the sperm motility parameters in the semen of rams. Additionally, CASA automatically calculated and reported the percentage of viable sperm, normal sperm, abnormal heads, abnormal necks, and abnormal tails.

**Biochemical evaluation:**

Semen samples were incubated with varying doses of L-arginine for 4 hours. To separate the spermatozoa from the extender, they were centrifuged at 1000× g for 10 minutes. After collecting the supernatant, it was centrifuged at 3000×g for 15 minutes at 4°C. It was then frozen at -20°C until it was evaluated. Using a spectrophotometer (Spectro UV-VIS Auto UV-2602; Labomed, Los Angeles, USA) and commercial kits (Biodiagnostic, Egypt), the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured as per the manufacturer's instructions.

**Statistical analysis:**

The obtained data were statistically analyzed by the SAS (2002), using one-way ANOVA. Succeeded by Duncan's new multiple range test (DMRT) was set at ($P<0.05$), which was used to compare differences among individual means according to Duncan (1955). Using arcsine transformation for percent data.

## RESULTS AND DISCUSSION

Poor ejaculates are never processed and used for artificial insemination since they were produced in subfertile rams with low quality, particularly in sperm motility. The current study added L-arginine to Tris-extender at three different concentrations (2, 4, and 6 mM/mL) to improve the quality of sperm in the semen of these rams following dilution and four hours of storage at $5^{o}$C. The current study chose a four-hour incubation period because this is the typical amount of time utilized to equilibrate semen with cryoprotectant before freezing.

In this investigation, the majority of semen parameters, including motility class, vitality, and normal and aberrant sperm, were checked and evaluated using the CASA approach.

Sperm motility is still widely regarded as one of the most significant indicators of semen quality, according to Amann and **Waberski (2014)**, who used new methods (CASA) that provide more precise and trustworthy data.

The percentages of total sperm motility (78.8), progressive motility (51.2), non-progressive motility (27.6), and non-motile sperm (31.5) decreased when L-Arginine was added to Tris-extender at a level of 6 mM (P<0.05) as opposed to levels of 2 (73.3, 42.8,30.5 and 39.2) or 4 mM L-Arginine (72.6, 41.1,31.5 and 37.8) or control extender (65.8, 37.4, 28.4 and 42.3) (Table 1)

**Table (1):** Effect of L-arginine supplementation in extender on motility kinetic parameters of rams spermatozoa incubated at 5$^{o}$C for 6 h.

| Sperm parameters | Semen diluted with L-arginine (mM) | | | | P value |
|---|---|---|---|---|---|
| | 0 (Control) | 2 | 4 | 6 | |
| Total sperm motility, % | 65.8$^{c}$±2.52 | 73.3$^{b}$±1.66 | 72.6$^{b}$±3.51 | 78.8$^{a}$±1.23 | <.0001 |
| Progressive motility, % | 37.4$^{d}$±3.45 | 42.8$^{b}$±2.01 | 41.1$^{c}$±2.54 | 51.2 $^{a}$±2.21 | <.0001 |
| Non-Progressive motility, % | 28.4c±1.18 | 30.5$^{b}$±1.12 | 31.5$^{a}$±1.21 | 27.6$^{d}$±2.01 | <.0001 |
| Non-motile sperm, % | 42.4$^{a}$±2.01 | 39.2$^{b}$±1.14 | 37.8$^{c}$±2.01 | 31.5$^{d}$±1.11 | <.0001 |

**Means in the same raw with different superscripts (a, b) differ significantly (P<0.05).**

According to **Schulze *et al.* (2013),** sperm motility (%) is evaluated in terms of the functional test, which measures the sperm's motor skills and energy level because a sizable portion of the energy generated by mammalian sperm is used to sustain motility. Goat spermatozoa's sperm motility was reported to be sustained by L- arginine addition by **Susilowati *et al.* (2019),** which is consistent with the current results of motility metrics.

When compared to the levels of 2 mM (18.4) or control extender (15.9), the rapid progressive motility (Class A) reached 21.8 and 24.2 when the amount of L-arginine was increased to 4 or 6 mM (P<0.05). (Table 2). Given the similar tendency on class B (Slow progressive motility), the elevated L-arginine was 19.8, 24.1, 26.7, and 27.7 for the control extender, 2, 4, and 6 mM L-arginine. The equivalent values for D (Static) were 33.9, 30.9, 28.7, and 29.6, whereas the values for C (non-progressive motility) were 20.3, 24.6, 27.3, and 28.7 (Table 2).

**Table (2):** Effect of L-arginine supplementation in extender on sperm motility categories (%) of ram's spermatozoa incubated at 5°C for 4 h.

| Sperm parameters | Semen diluted with L-arginine (mM) | | | | P value |
|---|---|---|---|---|---|
| | 0 (control) | 2 | 4 | 6 | |
| Class A | 15.9d±1.51 | 18.4c±1.12 | 21.8b±0.56 | 24.02a±1.11 | <.0001 |
| Class B | 19.8b±0.28 | 24.1c±1.85 | 26.7b±2.01 | 27.7a±0.12 | <.0001 |
| Class C | 20.3d±1.21 | 24.6c±2.02 | 27.3b±1.96 | 28.7a±1.21 | <.0001 |
| Class D | 33.9a±1.41 | 30.9b±1.54 | 28.7d±2.21 | 29.6c±0.52 | <.0001 |

**Means in the same raw with different superscripts (a, b, c) differ significantly (P<0.05).**

Sperm cell motility was enhanced by L-arginine administration, particularly in conditions of high oxidative stress **(Alizadeh *et al.*, 2016).** According to **Yildiz *et al.* (1998),** L-arginine improved sperm motility in pigs by acting as a scavenger and antioxidant against free radicals such as ROS and RNS. The study found that utilizing concentrations of L-arginine (6 mM) increased the motility of low-motility ram semen. This effect may be attributed to the L-arginine's ability to enhance sperm metabolism and sperm metabolic activity, which in turn stimulates the rate of glycolysis and increases the synthesis of ATP, both of which are necessary for sperm motility **(Patel *et al.*, 1998).** When comparing the results of the L-Arginine supplementation of Tris extender with other L-arginine treatments at 2 mM (31.1, 18.4 and 24.3) or 4 mM (33.8, 20.4 and 24.1), with control (28.5, 17.7 and 22.5), there was a significant (P<0.05) improvement in the involving curve linear velocity, straight linear velocity, and average path velocity (36.7, 22.4 and 24.1, respectively) (Table 3).

**Table (3):** Effect of L-arginine supplementation in extender on sperm velocity (μm/s) of ram's spermatozoa incubated at 5°C for 4 h.

| Sperm parameters | Semen diluted with L-arginine (mM) | | | | P value |
|---|---|---|---|---|---|
| | 0 (control) | 2 | 4 | 6 | |
| Involving curve linear velocity | $28.5^{d}$±1.63 | $31.1^{c}$±0.22 | $33.8^{b}$±2.66 | $36.7^{a}$±0.82 | <.0001 |
| Straight linear velocity | $17.7^{d}$±2.21 | $18.4^{c}$±1.25 | $20.3^{b}$±1.52 | $22.4^{a}$±1.14 | <.0001 |
| Average path velocity | $22.5^{b}$±1.21 | $24.3^{a}$±1.63 | $23.5^{a}$±2.41 | $24.1^{a}$±1.54 | <.0001 |

**Means in the same raw with different superscripts (a, b) differ significantly (P<0.05).**

Table (4) demonstrates that, when compared to free extender (Control), semen diluted with L-arginine at levels of 4 and 6 mM demonstrated a significant (P<0.05) improvement in semen quality by increasing the percentages of vitality (80.5 and 88.2) and normality sperm (45.7 and

52.3) and decreasing the percentages of abnormal neck and tail. It is noteworthy that aberrant head and tail percentages rose significantly (P<0.05) at all L-arginine doses (Table 4).

Accordingly, it was shown that in rams' semen kept for four hours at five degrees Celsius, L-arginine at a level of six milligrams had the greatest influence on sperm parameters (vitality and normalcy) as markers of fertility. According to **Susilowati *et al.* (2019),** the addition of L-arginine preserved the vitality of goat spermatozoa, which is consistent with the reported results. Additionally, several writers discovered that treating sperm cells with L-arginine improved their survival, normalcy, morphology, mitochondrial membrane potential, and DNA integrity **(Abbasi *et al.*, 2011).**

**Table (4):** Effect of L-arginine supplementation in extender on vitality and normality of semen rams (%) incubated at 5$^{o}$C for 4 h.

| Sperm parameters | Semen diluted with L-arginine (mM) | | | | P value |
|---|---|---|---|---|---|
| | 0 (control) | 2 | 4 | 6 | |
| Sperm vitality (%) | $66.1^{d}\pm8.41$ | $76.5^{c}\pm2.10$ | $80.5^{b}\pm2.70$ | $88.2^{a}\pm0.82$ | <.0001 |
| Normal sperm (%) | $36.5^{d}\pm2.40$ | $41.6^{c}\pm1.96$ | $45.7^{b}\pm1.96$ | $52.3^{a}\pm1.14$ | <.0001 |
| Abnormal head (%) | $13.5^{c}\pm1.56$ | $20.6^{a}\pm2.45$ | $17.5^{b}\pm2.63$ | $17.9^{b}\pm1.54$ | <.0001 |
| Abnormal neck (%) | $8.23^{d}\pm3.52$ | $11.2^{c}\pm2.05$ | $19.1^{a}\pm1.96$ | $15.9^{b}\pm1.63$ | <.0001 |
| Abnormal tail (%) | $6.1^{a}\pm1.42$ | $3.3^{b}\pm0.65$ | $2.2^{b}\pm1.20$ | $2.1^{b}\pm0.11$ | <.0001 |

**Means in the same raw with different superscripts (a, b) differ significantly (P<0.05).**

Malondialdehyde (MDA) is a lipid peroxidation marker, and **Susilowati *et al*. (2019)** found that adding L-arginine reduced the level of MDA in goat semen. Due to its antioxidant properties, L-arginine may help spermatozoa resist lipid peroxidation by promoting the synthesis of nitric oxide, which inhibits lipid peroxidation by deactivating free radicals. Nitric oxide's ability to scavenge gives it a significant intracellular and extracellular action against oxidative stress **(Hogg and Kalyanaraman, 1999).** This action may protect sperm cells' bimolecular components from oxidative damage, prolonging sperm vitality and reducing abnormalities in sperm. A family of isoenzymes known as the NO syntheses produces NO from L-arginine **(Moncada and Higgs, 1993).** As reported in (Table 5), there was no significant difference (P>0.05) between the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes in the extender when L-arginine concentrations were varied to 2 mM (7.85 and 7.75), 4 mM (8.20 and 6.88), and 6 mM (7.99 and 8.13) for AST and ALT, respectively, and control (7.88 and 7.40) for ALT and AST, respectively.

**Table (5):** Effect of L-arginine supplementation in extender on AST and ALT enzymes activity in semen incubated 5ºC for 4 h.

| Blood parameters | Semen diluted with L-arginine (mM) | | | | P value |
|---|---|---|---|---|---|
| | 0 (control) | 2 | 4 | 6 | |
| AST (U/ml) | 7.88$^{a}$±2.05 | 7.85$^{a}$±1.89 | 8.20$^{a}$±2.14 | 7.99$^{a}$±1.96 | 0.9223 |
| ALT (U/ml) | 7.47$^{ab}$±1.21 | 7.70$^{ab}$±2.63 | 6.87$^{b}$±1.63 | 8.22$^{a}$±1.78 | 0.1848 |

Sperm viability may benefit from increased AST and ALT enzyme activity. In order to evaluate the quality of the sperm, demonstrate metabolism, shield the sperm from oxidative stress damage during chilling conservation, and ultimately increase the sperm's vitality, enzyme activity in semen medium was employed as an indication **(Dhami and Kodagali, 1990).** The study's negligible AST and ALT activity observations may point to the preservation of sperm membrane integrity in semen that has been diluted to varying degrees by L-arginine. Ram sperm quality indices may be improved by intramuscular treatment of L-arginine, according to an in vivo study by **Özer Kaya *et al*. (2018).** By inhibiting the peroxidation of the membrane phospholipid bilayer via the generation of NO, L-arginine treatment preserves the spermatozoa's structural and functional integrity **(Susilowati *et al.*, 2019).** The sperm membranes' fluidity increased in response to rising superoxide radical levels **(Purohit *et al.*, 1998).** According to reports, membrane fluidity is crucial for sperm maturation in rams **(Wolf and Voglmayr, 1984).** As a result, membrane fluidity is preserved through regulated ROS peroxidation of the membrane phospholipids **(Jain *et al.*, 1993),** which in turn increases sperm motility. According to the previously mentioned findings, L-arginine significantly contributes to the improvement and maintenance of sperm function over the hour-long equilibration period at 4-5°C by enhancing sperm metabolism and reducing plasma membrane lipid peroxidation **(Silva *et al.*, 2014).** Various L-arginine concentrations had different outcomes for the quality of the sperm, despite non-significant variations in the enzyme activity (AST and ALT levels). This could be explained by a likely improvement in a few undetermined markers, such as NO, MDA, glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD). In conclusion, after a 4-hour cooled incubation period-which serves as an equilibration period-the addition of L-arginine to Tris-yolk extender at a concentration of 4 or 6 mM can enhance sperm motility, velocity, and vitality and reduce abnormalities of ram semen.

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# تأثير أضافة أرجينين الى مخفف الترس لسائل منوى الكباش المنخفض الحيوي

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[2] قسم الانتاج الحيوانى والداجنى والسمكى ، كلية الزراعة ، جامعة دمياط

## ملخص عربى

-أرجينين على سائل منوى الكباش منخفض الحيوية ، تم جمع 40 قذفة ضعيفة الحركة (50 إلى L لدراسة تأثير اضافة -أرجينين (0، 2، 4 و 6 55L٪) من 8 كباش وتم استخدام مخفف الترس بنسبة (1: 4) وباستخدام تراكيز مختلفة من (مليمول). تم تقييم عينات السائل المنوي المحفوظة على 5 درجات مئوية/4 ساعات عن طريق تحليل السائل المنوي في حركة P<0.05-أرجينين أدى إلى زيادة معنوية (L بمساعدة الكمبيوتر. أظهرت النتائج أن إضافة 6 مليمول الحيوانات المنوية الكلية ونسب الحركة التقدمية السريعة، في حين انخفضت نسبة الحيوانات المنوية غير المتحركة -أرجينين (4 و 6 مليمول) إلى تحسين L والحيوانات الساكنة مقارنة بالسائل المنوي فى المجموعة الضابطة. أدت إضافة -أرجينين. لم L نسب الحيوية وتقليل النسب غير الطبيعية للشواذ فى الرقبة والذيل للحيوانات المنوية مقارنة بعدم استخدام ( في السائل ALT) و (AST) على نشاط انزيم (P>0.05-أرجينين أي تأثير معنوي (L يكن لإضافة جميع مستويات -أرجينين (4 أو 6 مليمول) حيث حسن حركة الحيوانات L المنوي. انتهت الدراسة انه يمكن اضافة صفار تريس مع المنوية وسرعتها وحيويتها لسائل منوى الكباش منخفض الحيوية عند التحضين لمدة 4 ساعات في درجة حرارة باردة.