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## SEMEN COLLECTION, EVALUATION AND CRYOPRESERVATION OF ARABIAN EQUINE STALLIONS

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### ABSTRACT

The purpose of this study was to evaluate the macroscopic and microscopic characteristics of fresh and cryopreserved semen from Arabian stallions. Semen was collected once a week for six weeks from two healthy adult stallions between October and April 2025. Macroscopic evaluation included ejaculate volume, color, and consistency, while microscopic assessment focused on sperm motility, viability, and morphology. Ejaculates were extended using a glucose–sodium citrate extender, diluted at a 1:1 ratio based on sperm concentration, and cryopreserved in 0.25-mL plastic straws. Straws were subsequently thawed at 37°C for 30 seconds for post-thaw evaluation.

Results revealed that fresh ejaculate characteristics varied as follows:  $25.0 \pm 1.63\%$  to  $35.0 \pm 2.9\%$  for sperm motility,  $63.0 \pm 2.9\%$  to  $78.0 \pm 4.6\%$  for viability,  $56.0 \pm 3.1\%$  to  $78.0 \pm 4.2\%$  for plasma membrane integrity, and  $52.0 \pm 1.9\%$  to  $71.0 \pm 3.8\%$  for acrosome integrity. The mean total sperm abnormality ranged from  $8.00 \pm 0.47\%$  to  $22.0 \pm 1.4\%$ , with tail and head abnormalities ranging from  $4.0 \pm 0.07\%$  to  $18.0 \pm 0.96\%$  and  $3.0 \pm 0.06\%$  to  $14.0 \pm 0.78\%$ , respectively. After thawing, highly significant differences were observed, with progressive motility, live sperm, and acrosome integrity reaching  $45.83 \pm 2.01\%$ ,  $68.17 \pm 1.78\%$ , and  $63.17 \pm 1.64\%$ , respectively. The mean total ejaculate volume was 60 mL. Morphometrically, the total length of normal stallion sperm was approximately  $55.0 \pm 0.004 \mu\text{m}$ , comprising a head length of  $5.69 \pm 0.004 \mu\text{m}$ , head breadth of  $4.06 \pm 0.004 \mu\text{m}$ , midpiece length of  $8.17 \pm 0.008 \mu\text{m}$ , and tail length of  $48.0 \pm 0.021 \mu\text{m}$ .

In conclusion, cryopreservation of Arabian stallion semen yields optimal post-thaw quality across all evaluated parameters when utilizing a glucose–sodium citrate extender at a 1:1 dilution rate.

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## INTRODUCTION

Artificial Insemination (AI) is recognized as a modern and effective approach for enhancing genetic traits and improving the reproductive performance of horses. It is success depending on stallion fertility, mare fertility, and overall management practices [21]. Although the first successful pregnancy from frozen semen in mares was recorded in 1975, it was widespread been late until 1980s. Notably. it is estimated that only 30%-40% of stallion produce semen that is suitable for successful cryopreservation. Furthermore, sperm freezability varies notably among breeds, and a post-thaw progressive motility rate of approximately 35% is generally considered acceptable for AI [4]. Currently, no universally effective protocol exists for stallion semen cryopreservation that consistently ensures adequate fertilizing capacity in each ejaculate. This inconsistency is largely due to various factors, including damage to the sperm membrane during freezing and thawing. Additionally, the composition and properties of cryopreservation extender play a crucial role in determining post-thaw sperm quality [3].

Several extenders have demonstrated promising result, including Kenney's extender, cream-gel, skim- based extenders, glycine-egg yolk formulations, sugar-based solutions, and INRA96 [6]. Ejaculation is the natural process through which spermatozoa are expelled during copulation. When a stallion undergoes a prolong period of sexual inactivity, the initial ejaculates often contain a high concentration of deteriorated or aged spermatozoa. Although these ejaculates may present with high sperm count, their viability is usually compromised, necessitating the disposal of the first few collections. This phase is commonly known as the flush period. Additionally, long interval without ejaculation can lead to sperm aggregation within the ampullae of the ductus deferens, potentially causing partial obstruction of the ejaculatory pathway [8]. Total sperm number, viability, and motility evolution during the flush period refers to a period of serial semen collections to clean out the epididymis of older, less viable sperm, improving semen quality.

The approach is particularly beneficial for stallions emerging from periods of sexual inactivity, such as at the onset of the breeding season. During this phase, multiple ejaculate is obtained over consecutive days to optimize the functional quality of the ejaculate and restore reproductive efficiency [12]. The equine spermatozoon is structurally composed of the head, neck, and tail, being the longest component, the total length of a spermatozoon measures approximately 60 $\mu$ m [20]. Before the evaluation gel must be remove for easy to recognize the sperm by 1000x magnification after prerared from unstained sample which fixed in a buffered-formol slaine solution or stained by eosin-nigrosn stain [1, 10]. The number of spermatozoa per 0.25 ml straw ranges from 40- 100 million, depending on the outcome of various laboratory evaluation. Higher sperm concentration per dose are generally correlated with improved fertilization success [16]. However, a method called deep-horn insemination has demonstrated satisfactory pregnancy outcomes while utilizing lower sperm count than conventional uterine body deposition reported conception rate using this approach average between 30% and 55%, though they may vary from 0% up to 70% [18]. The present study was designed to evaluate the stallion semen characterizations of Arabian horses in Iraq before and after freezing process.

## MATERIALS AND METHODS

### Animals

Two healthy Arab horse stallions (Rabia and Assif), aged 19 and 10 years old, were involved in the current study. The stallions belong to AL-Zawraa park horse division, in Baghdad, Iraq and have been used as sires in the stables' regular breeding program. Each stallion was housed in a separate pen under standard conditions of feeding and general management (Table1), and the water was freely available. Animals were exercised daily for at least 1 h. with good histories of fertility were used for the artificial insemination.

Table 1. Daily feeding program of the experimental stallions according to the parks and afforestation department, Al- Zawraa recommendation.

| Ingredient     | Quantity           |
|----------------|--------------------|
| Barley grain   | 1.350 kg / 3 times |
| Glutinous rice | 5.5 kg / 2 times   |
| Fruit          | 1 kg / 1 time      |
| Salt           | 50 g / 3 times     |
| Wheat bran     | 0.125 g / 3 times  |

### Preparation of artificial vaginas for semen collection

The water jacket of the artificial vagina (AV) was filled with water at 45–50 °C, maintaining an internal temperature of 44–48 °C, which aided penile stimulation and facilitated ejaculation. Pressure of the AV was adjusted to provide uniformly good contact around the penis. The penis was full insertion into the AV during the first penile thrust and then maintenance fully inserted position (Photo 1).



**Photo 1. Artificial vaginas for horse stallion semen collection.**

### Semen collection and evaluation

Twelve ejaculates were collected from stallions during the breeding season (October to April) using an Equine Artificial Vagina Kit (Great Farm, Model GF 45, China; temperature 45–50 °C, with appropriate pressure). Collections were performed once a week while the stallions mounted estrous mares. Immediately after collection, sperm-rich ejaculate fraction (fractionated by the kit filters) from each stallion was transferred to the laboratory, placed in a water bath at 37°C and assayed by light microscopy (Olympus CX23) provided with hot stage. Macroscopic examinations of the ejaculates (color, volume, and pH) were performed. The pH was determined using litmus paper (range: 5–8) by applying a single drop of semen and comparing the

resulting color with the reference chart provided in the kit. Microscopic examination (mass activity and sperm individual motility, live sperm percentage and sperm abnormalities) were determined as described by [14]. Those sperm characteristics were determined after semen dilution and cooling to 5°C, after having thawed at 37°C for 30 seconds.

### **Semen dilution and cryopreservation**

All chemical compounds used in this study were purchased from Merck (Merck, Darmstadt, Germany) unless otherwise stated. Glucose-sodium citrate extender (0.15g glucose, 2.6g sodium citrate dehydrate, 0.37g di-sodium EDTA, 0.12g sodium bicarbonate, 0.10g streptomycin and 50000, 100000 unit benzyl penicillin in distilled water to make 100ml as described previously [15]. The ejaculate was diluted at a rate of 1:1(at 37°C) due to the low concentration of semen ejaculate and highly concentration of sperm in straw was required. Briefly, sperm-rich fractions were diluted and filtered by special filter for removing gel.

The diluted semen was cooled to 5°C for 75 min. The cooling was around 0.3°C/min. At 5°C, the diluted semen was packed in 0.25-ml straws (the sperm concentration ranged from 25 to 30 million` sperm/ straw). The straws were placed in racks 4 cm above the liquid nitrogen surface and frozen in foam box to - 120°C within 7 min and stored in liquid nitrogen at - 196°C for at least 48 h before evaluation. Frozen straws were thawed at 37°C for 30 seconds [28].

### **Statistical analysis**

The Statistical Analysis System (SAS) was used to evaluate the effects of different factors on the study parameters. Analysis of variance (ANOVA) was performed using a completely randomized design (CRD), and mean comparisons were conducted using the least significant difference (LSD) test [22]. The statistical model is presented as follows:

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

Where:  $Y_{ij}$ = dependent variable (semen characteristics),  $\mu$ = Overall mean.

$P_j$  = Effect of period( weeks 1-6) and preservation period (cooling and cryopreservation).

$e_{ij}$  = Error term.

## **RESULTS AND DISCUSSION**

Table 2 summarizes the characteristics of stallion semen. Motility ranged from  $25 \pm 1.63$  to  $35 \pm 2.9$ , live sperm from  $63 \pm 2.9$  to  $78 \pm 4.6$ , plasma membrane integrity from  $56 \pm 3.1$  to  $78.0 \pm 4.2$ , and acrosome integrity from  $52.0 \pm 1.9$  to  $71.0 \pm 3.8$ .

The mean total sperm abnormality ranged from  $8.00 \pm 0.47$  to  $22.0 \pm 1.4$ , while tail and head abnormalities ranged from  $4.0 \pm 0.07$  to  $18.0 \pm 0.96$  and  $3.0 \pm 0.06$  to  $14.0 \pm 0.78$ , respectively. The last two weeks, which yielded the best results across all parameters, were considered the green light to begin the cryopreservation step.

Moreover, there was a significant difference in the sperm motility among six week of flushing with highly significant differences for most semen characteristics (Table 2). This results were in agreement with [5, 9] whom summarized that the frequency of semen collection significantly influences quality of all semen parameters by the processed mean ejaculation due to a large number of ageing spermatozoa, dead spermatozoa, agglutinated spermatozoa will be expelled in flush period. On the other hand, inadequate training ejaculation course of the stallion led to a large ejaculate volume and low sperm concentration.

The semen characteristics of Arabian horses are presented in Table 3. Progressive motility, live sperm, and acrosome integrity showed highly significant

differences, whereas no significant differences were observed in sperm cell abnormalities or the plasma membrane integrity.

**Table 2. Effect of collection period (week) on semen characteristics of horse stallions in Iraq (Mean  $\pm$  SE)**

| Collection period (week)                                                                                           | Progressive motility (%) | Live sperm (%)    | Abnormal sperms (%) | Sperm tail abnormalities (%) | Sperm head abnormalities (%) | Plasma membrane integrity (%) | Sperm acrosome integrity (%) |
|--------------------------------------------------------------------------------------------------------------------|--------------------------|-------------------|---------------------|------------------------------|------------------------------|-------------------------------|------------------------------|
| 1                                                                                                                  | 30.0 $\pm$ 2.4 ab        | 76.0 $\pm$ 3.7 a  | 22.0 $\pm$ 1.4 a    | 18.0 $\pm$ 0.96 a            | 14.0 $\pm$ 0.78 a            | 56.0 $\pm$ 3.1 c              | 52.0 $\pm$ 1.9 d             |
| 2                                                                                                                  | 25.0 $\pm$ 1.63 b        | 63.0 $\pm$ 2.9 b  | 18.0 $\pm$ 1.1 ab   | 8.0 $\pm$ 0.57 b             | 10.0 $\pm$ 0.63 ab           | 61.0 $\pm$ 2.3 c              | 58.0 $\pm$ 2.3 cd            |
| 3                                                                                                                  | 30.0 $\pm$ 2.0 ab        | 72.0 $\pm$ 3.1 ab | 12.0 $\pm$ 0.68 bcd | 5.0 $\pm$ 0.22 b             | 7.0 $\pm$ 0.34 bc            | 65.0 $\pm$ 3.1 bc             | 61.0 $\pm$ 2.8 bc            |
| 4                                                                                                                  | 30.0 $\pm$ 2.0 ab        | 70.0 $\pm$ 2.8 ab | 15.0 $\pm$ 0.74 bc  | 7.0 $\pm$ 0.29 b             | 8.0 $\pm$ 0.62 bc            | 66.0 $\pm$ 3.7 bc             | 63.0 $\pm$ 3.5 abc           |
| 5                                                                                                                  | 35.0 $\pm$ 2.9 a         | 78.0 $\pm$ 4.6 a  | 11.0 $\pm$ 0.62 cd  | 4.0 $\pm$ 0.07 b             | 7.0 $\pm$ 0.42 bc            | 72.0 $\pm$ 4.5 ab             | 69.0 $\pm$ 2.9 ab            |
| 6                                                                                                                  | 30.0 $\pm$ 1.7 ab        | 73.0 $\pm$ 2.2 a  | 8.00 $\pm$ 0.47 d   | 5.0 $\pm$ 0.22 b             | 3.0 $\pm$ 0.06 c             | 78.0 $\pm$ 4.2 a              | 71.0 $\pm$ 3.8 a             |
| L.S.D.                                                                                                             | 6.82 *                   | 8.51 **           | 6.511 **            | 5.790 **                     | 5.627 **                     | 9.530 **                      | 8.661 **                     |
| P-value                                                                                                            | 0.0389                   | 0.0001            | 0.0002              | 0.0001                       | 0.0072                       | 0.0001                        | 0.0001                       |
| Means with the different letters in same column differed significantly, * ( $P \leq 0.05$ ), ** ( $P \leq 0.01$ ). |                          |                   |                     |                              |                              |                               |                              |

**Table 3. Effect of cooling and cryopreservation periods on semen characteristics of horse stallions (Mean  $\pm$  SE)**

| Preservation period                                                                         | Progressive motility (%) | Live sperm (%)      | Abnormal sperms (%) | Sperm tail abnormalities (%) | Sperm head abnormalities (%) | Plasma membrane integrity (%) | Sperm acrosome integrity (%) |
|---------------------------------------------------------------------------------------------|--------------------------|---------------------|---------------------|------------------------------|------------------------------|-------------------------------|------------------------------|
| Before cooling (immediately after dilution)                                                 | 60.00 $\pm$ 1.29 a       | 82.00 $\pm$ 1.29 a  | 6.00 $\pm$ 0.58 c   | 3.33 $\pm$ 0.42 c            | 2.67 $\pm$ 0.49              | 73.00 $\pm$ 2.22 a            | 75.33 $\pm$ 2.03 a           |
| Cooling                                                                                     | 52.50 $\pm$ 1.11 b       | 75.00 $\pm$ 1.52 b  | 8.83 $\pm$ 0.70 b   | 5.17 $\pm$ 0.30 b            | 3.67 $\pm$ 0.42              | 69.16 $\pm$ 2.15 a            | 72.17 $\pm$ 1.68 a           |
| Cryopreservation                                                                            | 45.83 $\pm$ 2.01 c       | 68.17 $\pm$ 1.78 c  | 11.67 $\pm$ 1.20 a  | 7.67 $\pm$ 0.88 a            | 4.00 $\pm$ 0.36              | 61.33 $\pm$ 0.80 b            | 63.17 $\pm$ 1.64 b           |
| L.S.D.                                                                                      | 4.586 $P \leq 0.01$      | 4.657 $P \leq 0.01$ | 2.623 $P \leq 0.01$ | 1.783 $P \leq 0.01$          | 1.492 NS                     | 5.559 $P \leq 0.01$           | 5.401 $P \leq 0.01$          |
| P-value                                                                                     | 0.0001                   | 0.0001              | 0.0014              | 0.0004                       | 0.1207                       | 0.0015                        | 0.0007                       |
| Means with the different letters in same column differed significantly. NS: Non-significant |                          |                     |                     |                              |                              |                               |                              |

There was significant differences ( $P \leq 0.01$ ) in the live sperm percentage at cooling and cryopreservation periods giving a good result in all parameter post cryopreservation (Table 3). These results are consistent with those reported by [7], who noted that good motility was achieved after freezing. In that study, only semen samples with a live sperm percentage above 60% prior to cooling were accepted for cryopreservation. This finding also aligns with [24], who reported that the acceptable percentage of live sperm should range between 40% and 60%. The overall proportion of abnormalities was  $11.67 \pm 1.20$ , while head abnormal and tail abnormal was  $4.00 \pm 0.36$  and  $7.67 \pm 0.88\%$  respectively. This result was in agreement with [12] whom reported that the acceptable abnormality percentage was 35% in stallions, with a maximum of 5% and 10% for each primary and secondary abnormality, respectively. This in agreement with [19] who reported that the highest percentage of normal sperm was seen in Diff-Quik (DQ) and Rose Bengal (RB) methods ( $82.55 \pm 2.88$  and  $88.31 \pm 5.19$ ) respectively. (the same result that determined the acceptable normal sperm percentage) decrease the abnormality in semen collections means that management has been improved.

This study observed a significant decrease in both plasma membrane and acrosome integrity; being ranging from  $73.00 \pm 2.22$  and  $75.33 \pm 2.03\%$  to  $61.33 \pm 0.80$  and  $63.17 \pm 1.64\%$ , respectively. The initial membrane integrity was considered good suggesting only minor damage to the plasma membrane. This damage is likely due to the reorganization of membrane proteins and destabilization of plasma membrane, potentially influenced by an elevated concentration of polyunsaturated fatty acids in sperm membranes which interact fatty acids can interact with reactive oxygen species, affecting membrane fluidity and potentially facilitating the entry of calcium ions these findings align with result reported by [11]. In contrast, previous studies have demonstrated an improvement in membrane integrity when alternative antioxidants were incorporated into extender media [13, 29]. The acrosome, a vital sperm structure composed of membranes and proteins, is particularly vulnerable to ROS- induced damage. The result concerning acrosome integrity in this study were deemed acceptable and are consistent with finding from [2], who showed that supplementing extenders with various doses of the antioxidant hydroxytyrosol (ranging from 1.25 to  $10 \mu\text{g/ml}$ ) positively impacted acrosome integrity. Similar beneficial effect has been observed in studies on ram [17].

### **Ejaculate volume**

The ejaculate volume of Arabian stallion fresh semen was  $60.00 \pm 3.34$  ml having a milky white color, gel volume of the ejaculate varies from (10- 20) ml in different semen ejaculate and pH was 7.2.

The ejaculate volume of Arabian horse fresh semen was  $60.00 \pm 3.34$  ml with gel volume of the ejaculate varies from 10 gel to 20 ml in different semen ejaculate. This result was in agreement with [27] who summarized that the differences in total volume and gel-free volume were also found between stallions aged 5–9 years and 10–14 years to stallions aged 20 years and over. Total volume was reduced in the older stallions by 34% to 5–9 year olds and 38% to 10–14 year olds ( $p < 0.0001$ ). This pattern was repeated for gel-free volume with stallions aged over twenty years recording 47% and 59% reduced counts compared to 5–9-year-old and 10–14-year-old stallions, respectively ( $p < 0.004$ )[23].

Ejaculate volume is dependent on species, breed, age, and environment. Environmental factors affecting seminal volume may include feeding, housing, teasing, soundness, method and frequency of semen collection (collection of total ejaculates or seminal fractions), and time of the year (e.g., small volume during the non-breeding season) [26].

### Sperm size measurement

Sperm measurements obtained in this study showed that the total length of normal stallion sperm was approximately  $55 \pm 0.004 \mu\text{m}$ , consisting of a head length of  $5.69 \pm 0.004 \mu\text{m}$ , head breadth of  $4.06 \pm 0.004 \mu\text{m}$ , midpiece length of  $8.17 \pm 0.008 \mu\text{m}$ , and tail length of  $48 \pm 0.021 \mu\text{m}$ , as observed after eosin–nigrosine staining (Figure 1a). Sperm morphometric analysis using an eye-piece micrometer revealed that the mensuration of sperm head length, head maximum breadth, mid-piece length, and tail length were  $5.96 \pm 0.004$ ,  $4.06 \pm 0.004$ ,  $8.17 \pm 0.008$ , and  $48.88 \pm 0.022 \mu\text{m}$ , respectively. This in agreement with [29] who reported that the evaluation of stallion sperm morphology should be performed on fresh unstained samples using a microscope with phase contrast (Figure 1b).

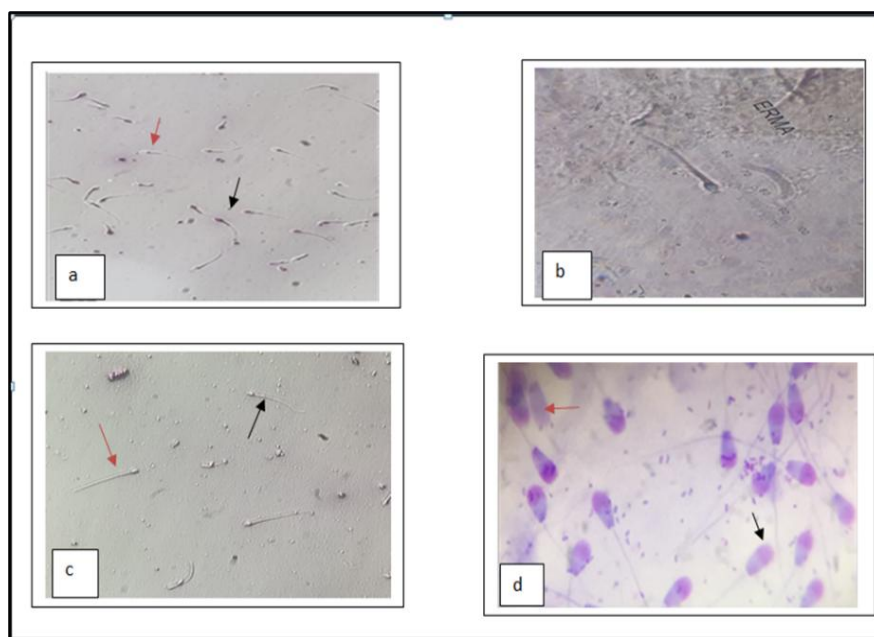


Figure1: Semen evaluation by stain, (a) Eosin and Nigrosine stain, dead sperm (black arrow) and live sperm (red arrow) 40x lens, (b) Measurement of sperm diameter 40x lens, (c) Host test intact (black arrow), defect (red arrow) 40x lens, and (d) Gimesa stain, normal sperm (black stain), abnormal sperm (red arrow) 100x lens.

### CONCLUSION

Glucose-sodium citrate extender has a good quality to improve semen cryopreservation and can be considered as one of the best extenders for the semen cryopreservation in horses.

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## جمع وتقييم وحفظ السائل المنوي بالتجميد لطلائق الخيول العربية

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### الملخص

هدفت الدراسة الحالية تحديد بعض خصائص السائل المنوي لطلائق الخيول العربية. تم جمع السائل المنوي مرة واحدة في الأسبوع من اثنين من طلائق الخيول البالغة للمدة بين شهري تشرين الأول ونيسان 2025. تم تقويم خصائص السائل المنوي لطلائق الخيول عياناً من حيث حجم القذف واللون والقوام، ومجهرياً من حيث الحركة الفردية للنطف، النسبة المئوية للنطف الحية، نسبة التشوهات في النطف، وسلامة الغشاء البلازمي للنطف).

ُجمع السائل المنوي من اثنين من طلائق الخيول العربية لمدة ستة أسابيع باستخدام مهبل اصطناعي خاص بالخيول وخففت بمخفف الكلوكوز-سترات الصوديوم. حُفظت العينات بالتبريد في قصبات بلاستيكية سعة 0.25 مل، باستخدام معدل تخفيف 1:1، بناءً على تركيز النطف. تمت اسالة القصبات عند درجة حرارة 37 درجة مئوية لمدة 30 ثانية.

أظهرت النتائج أن صفات السائل المنوي المحفوظ بالتجميد تراوحت بين  $1.63 \pm 25$  و  $2.9 \pm 35$ ، و  $2.9 \pm 63$  و  $4.6 \pm 78$ ، و  $3.1 \pm 56$  و  $4.2 \pm 78.0$ ، و  $1.9 \pm 52.0$  و  $3.8 \pm 71.0$  % لحركة النطف، وسلامة الغشاء البلازمي والأكروسومي، على التوالي. تراوح متوسط تشوهات النطف بين  $0.47 \pm 8.00$  و  $1.4 \pm 22$ ، و  $0.07 \pm 4.0$  و  $0.96 \pm 18.0$ ، و  $0.06 \pm 3$  و  $0.78 \pm 14$  % لتشوهات ذيل النطف ورأسها، على التوالي.

كانت نسبة الحركة التقدمية للنطف والنطف الحية وسلامة الأكروسوم في المدد التحضيرية ذات فروق معنوية عالية ( $2.01 \pm 45.83$ ،  $1.78 \pm 68.17$  و  $1.64 \pm 63.17$  على التوالي)، بينما انعدمت الفروق المعنوية في الصفات أخرى، مثل تشوهات النطف وسلامة الغشاء البلازمي. بلغ متوسط حجم القذف 60 مل. بلغ الطول الإجمالي للنطف الطبيعية تقريباً  $0.004 \pm 55$  ميكرومتر (طول الرأس:  $0.004 \pm 5.69$  ميكرومتر؛ عرض الرأس:  $0.004 \pm 4.06$ ؛ القطعة الوسطى:  $0.008 \pm 8.17$  ميكرومتر؛ الذيل:  $0.021 \pm 48$  ميكرومتر) في قطر النطف بعد استخدام صبغة الإيوسين-نيكروسين. تم تحقيق أفضل النتائج في حفظ السائل المنوي للخيول العربية بالتجميد باستخدام مخفف سترات الكلوكوز والصوديوم وبمعدل تخفيف 1:1.

الكلمات الدالة: طلائق الخيول العربية، السائل المنوي، الحفظ بالتجميد.

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