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Theriogenology

Comparative Study on the Effect of Three Extenders on Post-thaw Sperm Motility in Arabian Stallions

Mahmoud A. Sultan[1]*, Ahmed H. Zaghloul[1], Emad M. Abd El-Razek[1], Hamed T. Elbaz[1] and Mohamed K. Derbala[2]

(1) Department of Theriogenology, Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Menofia 32897, Egypt.
(2) Animal Reproduction Research Institute, Agriculture Research Centre (ARC), Diagnostic Imaging and Endoscopy Unit, Giza, Egypt.

**Corresponding author:* sultanmahmoud2019@gmail.com *Received: 12/2/2025 Accepted: 27/4/2025*

ABSTRACT

The aim of the present study was to evaluate the effect of three semen extenders on the post-thaw motility of sperm from Arabian stallions. Semen was collected from six Arabian stallions, evaluated for standard semen parameters, diluted with centrifugation extenders in a ratio of 1:1, then cushioned centrifugation 1000 xg for 10 minutes, the sperm pellet was removed using catheter coupled with a syringe and divided into three portions, the first portion was diluted with MX freezing extender, the 2nd portion was diluted with STAR freezing extender while the 3rd portion was diluted with DMF egg yolk based extender, packad in 0.5 ml straws and sealed using glass-balls in manual semen filling and sealing machine at the room temperature , straws were placed on rack, cooled slowly in the refrigerator for 25 minutes in case of DMF egg yolk based extender and for 1 hr. in case of MX and STAR freezing extender, then removed from refrigerator and was put in Styrofoam box contains 4 cm liquid nitrogen -120 ℃ for 13 minutes, evaluated for the motility then plunged in liquid nitrogen at -196 ℃. Results showed that MX and STAR freezing extenders improved the post-thawing motility of sperm but not significantly ($P < 0.05$) like DMF egg yolk based extender. In conclusion, Lyophilized extenders can be used for cryopreservation of stallion's sperm with the advantage of storage and distribution at room temperature.

Keywords: Sperm, Freezing, MX, STAR, Stallion and Post-Thawing Motility

INTRODUCTION

Cryopreservation of stallion 's sperm permits storage of sperm for many years, maintenance of sperm in its viable state and transportation of the insemination doses for long distance especially if the stallion suffers from illness and the cryopreserved sperm can be used after stallion's death (Alvarenga et al., 2016). However, the protocol of cryopreservation causes physical and chemical damage to plasma membrane of sperm which affects its viability and

motility (Barbas and Mascarenhas, 2009). Freezing of stallion's sperm has not reached the efficiency level and positive results that described in other species (Maria et al., 2019). When the semen of stallions exposed to the freezing temperature, a significant rise in ROS levels happen (Castro et al., 2016). Several factors have been detected to be responsible for poor freezing of the sperm of stallions such as the technique of freezing (Metcalf, 2007), the components of the freezing diluent and the cryoprotectant type (Squires et al., 2004). Cryopreservation of stallions semen shows individual variations among stallions in their ability to resist the freezing conditions. The stallions might be classified as good freezers or poor freezers depending on the post-thawing motility characters such as the percent of progressive motile sperm (Hoffmann et al., 2011). Seminal plasma is harmful to the sperm because it affects the post-thawing motility of the sperm of stallions (Love et al., 2005). Presence of the seminal plasma in low percent can improve the quality of stallion sperm and centrifugation using cushion fluid helps in the recovery of more than 90% of the sperm present in the semen (Loomis, 2006). Di-methyl formamide (DMF) cryo-protectant has several advantages as low molecular weight, less toxicity to sperm and more effective in the process of cryopreservation than glycerol because it improves the post-thawing motility and enhances semen freezing ability from poor freezers stallions (Oldenhof et al., 2017). Cryopreservation of stallion's sperm is not popular in Egypt due to low post-thawing motility and low conception rate result from using cryopreserved semen, so the aim of the present study was to detect the ability of stallion's sperm to resist the freezing conditions and study the effect of three different diluents on the post-thawing motility of stallion's sperm.

MATERIAL AND METHODS

This study was assessed and agreed by the Animal Care and Welfare Committee Ethics, University of Sadat City, Egypt.

The freezing extenders

In the current study, three diluents were used and compared between them (Egg yolk based extender provided with DMF as a cryoprotectant and two commercially lyophilized freezing extenders that based on skim milk and also containing egg yolk, produced in Rancho das Americas, Porto feliz, Brazil (MX3 and STAR freezing extenders) which can be stored and distributed at room temperature. The egg yolk based extender consisted of D- glucose, EDTA, tri-sodium citrate dehydrate, sodium bicarbonate, Amikacin sulphate, potassium penicillin G and D-lactose solution (11%w/v). STAR and MX consisted of sugars, amino acids, egg yolk, antibiotics and excipients.

Animals

Six healthy adult arabian stallions aged between 6-12 years old were used in the current study. The study was carried out in a private clinic at Giza governorate, Egypt (from June 2024 to March 2025). The stallions were housed and raised in an individual boxes and fed hay and concentrates and the water was provided adilibitum. The reproductive history showed that these stallions were used for artificial insemination. Stallions were reared in accordance to the experimental designs and andrological examination of these stallions was normal and was

performed by palpation and ultrasonography. Three ejaculates were collected from each stallion.

Semen collection

Semen was collected from stallions using a pre-warmed (45-50 ℃), pressurized and lubricated Missouri type artificial vagina. A disposable gel filter was included to remove the gel fraction of the ejaculate. Semen collection was performed using a dummy mount in the presence of oestrus mare as a teaser. Three ejaculates were collected from each stallion (McKinnon et al., 2011).

Semen evaluation

Immediately following semen collection, the gel filter was removed and the semen was evaluated for different semen characters that include: macroscopic assessment for the ejaculate volume that was detected using graduated collection apparatus (Sieme et al., 2004), detection of the colour by visual examination (Sieme et al., 2004) and the consistency was examined visually (Samper, 2009) and microscopic examination of individual motility of sperm was performed following (Turner, 2005). Detection of sperm cell concentration ($x10^6$/ml) was achieved through using of improved neubauer-haemocytometer (Bailey et al., 2007). Only ejaculate contains at least 60% progressively motile sperm and 100 million sperm cell per ml were used for cryopreservation (Aurich 2020).

Semen cryopreservation and examination

Semen was collected from stallions, then evaluated for different raw semen characters and only stallions with motility not less than 60% and SCC not less than 100 million motile sperm was used for cryopreservation. The semen was diluted with the centrifugation extender in a ratio 1:1, then the cushion fluid (Minitube) was put at the bottom of the diluted tubes to protect the sperm during centrifugation, then centrifuged at 1000 g for 10 min to remove the seminal plasma, then the sperm pellet was removed using catheter coupled with a syringe, then the sperm pellet was divided into three portions, the first portion was diluted with MX freezing extender, the 2nd portion was diluted with STAR freezing extender while the 3rd portion was diluted with egg yolk DMF based extender, then the diluted semen was packed in 0.5 ml straws and sealed using glass-balls using manual semen filling and sealing machine at the room temperature , straws were placed on rack which was designed to keep the straws above the liquid nitrogen by 3-4 cm, cooled slowly in the refrigerator for 25 minutes in case of DMF egg yolk based extender and for 1 hr. in case of MX and STAR freezing extender, then removed from refrigerator and was put in Styrofoam box contains 4 cm liquid nitrogen -120 ℃ for 13 mints, then were placed in liquid nitrogen and evaluated for the motility then plunged in liquid nitrogen tank at -196 ℃ (Alvarenga et al., 2016).

Examination of the post-thawing motility

For examination of the post-thawing motility, at least three straws from the ejaculate (one for each diluent) were placed at 37 ℃ water bath for 30 sec. then evaluated for the motility of sperm.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed

by post-hoc analysis according to the Benjamini-Hochberg procedure. ANOVA assumptions were checked using the Shapiro-Wilk and Levene's tests for normal distribution and homogeneity of variance, respectively. Graphs and statistical analysis were performed in RStudio-2024.4.2.764 (Posit team, 2023) and R programming language v4.4.1 (R Core Team, 2023). $P<0.05$ were considered statistically significant.

RESULTS

The motility of raw semen was significantly higher ($P < 0.05$) than the post-thawing motility in almost all of the stallions in case of DMF egg yolk based extender. The motility of raw semen was significantly higher ($P < 0.05$) than the post-thawing motility in all stallions in case of MX and STAR freezing extenders. The post-thawing motility of DMF egg yolk based extenders was significantly higher ($P < 0.05$) than the post-thawing motility in all stallions in case of MX and STAR diluted semen as in Table and Figure.

Table 1: Effect of three different diluents on the post-thawing motility of stallion's sperm.

Parameter	Raw semen	MX	STAR	DMF egg yolk
Motility (%)	60.8 ± 4.3^{a}	38.6 ± 11^{c}	35.3 ± 10.1^{c}	46.4 ± 12.7^{b}
Stallion 1- motility (%)	65 ± 5^{a}	48.3 ± 2.9^{b}	43.3 ± 5.8^{b}	51.7 ± 2.9^{b}
Stallion 2- motility (%)	61.7 ± 2.9^{a}	45 ± 0^{c}	40 ± 0^{d}	55 ± 5^{b}
Stallion 3- motility (%)	56.7 ± 5.8^{a}	40 ± 5^{bc}	36.7 ± 2.9^{c}	48.3 ± 2.9^{ab}
Stallion 4- motility (%)	63.3 ± 2.9^{a}	40 ± 5^{c}	36.7 ± 2.9^{c}	53.3 ± 5.8^{b}
Stallion 5- motility (%)	58.3 ± 2.9^{a}	41.7 ± 2.9^{c}	40 ± 0^{c}	50 ± 0^{b}
Stallion 6- motility (%)	60 ± 0^{a}	16.7 ± 2.9^{b}	15 ± 5^{b}	20 ± 0^{ab}

N.B: Data are expressed as mean ± standard deviation. Different superscript letters (a, b, c, d) within the same row indicate statistically significant differences ($P < 0.05$).

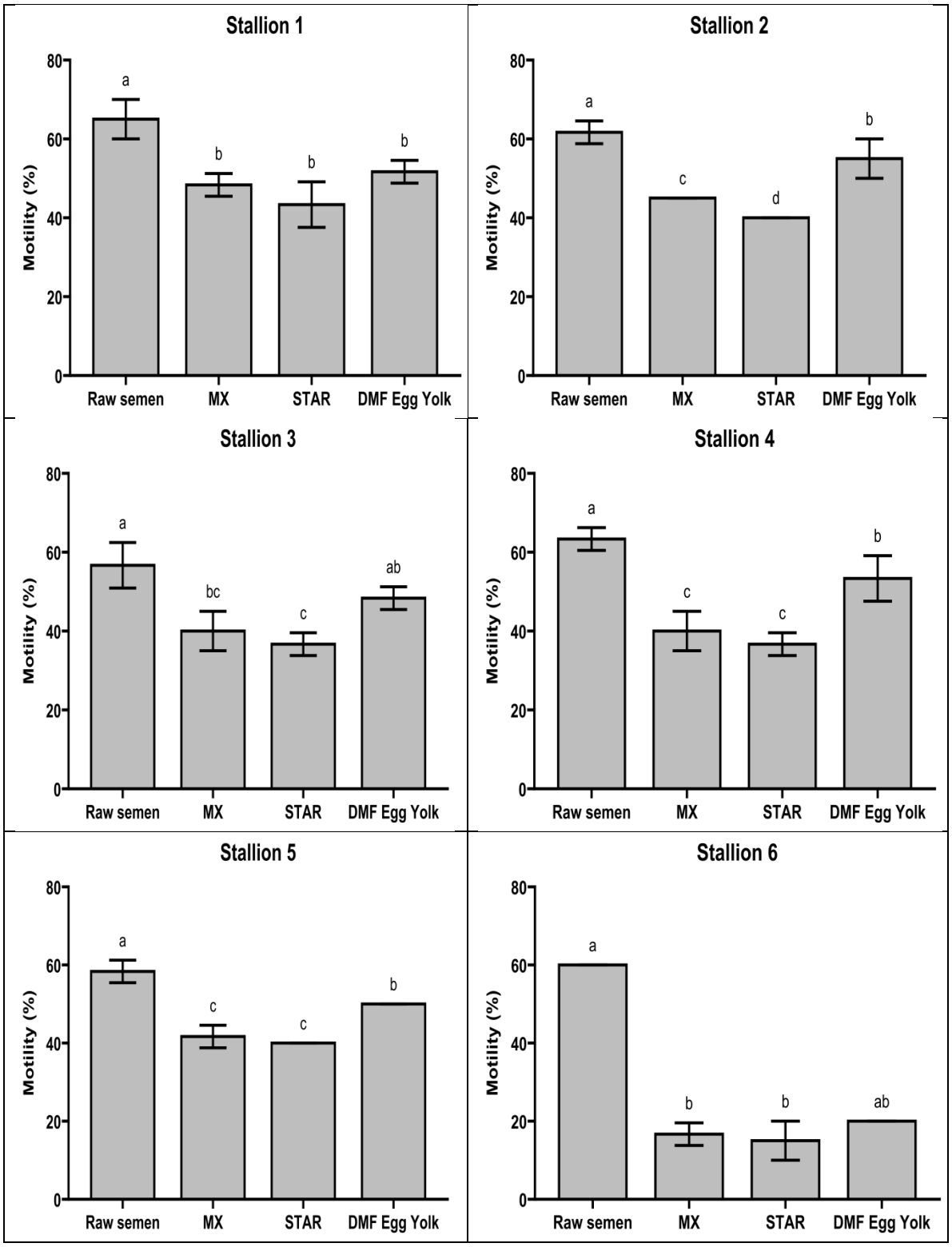

Figure 1: Effect of three different diluents on the post-thawing motility of different stallion's sperm.

DISCUSSION

Cryopreservation of stallion's sperm is an crucial technique in all stallion's artificial insemination centers because it has the ability to keep the sperm alive for long periods and permits distribution of stallions superior genetic merit among different breeds of equine. However, using of frozen semen led to lower fertility among animals because of the lethal damage that caused to the sperm during the process of preservation (Esmail et al., 2023).

Following evaluation of the motility of fresh semen and post-thawing characteristics of sperm, it was appeared that cryopreservation of stallion sperm had a significant effect on the parameters of sperm motility. The motility was extremely decreased after the procedure of cryopreservation. This result was in agreement with all studies performed before as the findings of (Aurich et al., 2020).

In the current study, we used the motility of sperm as the primary character for evaluating the quality of semen of Arabian stallions as similarly used by (Love et al., 2015) in their studies. In our study, only stallions gave post-thawing motility more than 35% motile sperm were used for cryopreservation according to (Aurich et al., 2020). In this study, only one stallion showed post-thawing motility less than 30 %. The stallions which gave post-thawing motility more than 35% were considered good freezers, while stallions gave post-thawing motility less than 35% were considered bad freezers and not used for artificial insemination with cryopreserved semen as in agreement with (Hoffman et al., 2011). The difference among stallions in their ability to resist the freezing conditions may be owed to genetic reasons (Hoffman et al., 2011).

The most important point concerning cryopreservation of stallion's sperm was to choose the best cryo-diluents which not only increase the volume of semen but also preserve the sperm during the dilution time, cooling, freezing and thawing technique (El-sharkawy et al., 2016). Differences in the efficiency of extenders in protecting the stallion's sperm during cryopreservation was based on variation in the constituents of the diluent mainly (Dascanio and Mecue, 2014). Our results showed that cryopreservation of stallion's sperm using egg yolk based extender gave better results than cryopreservation of stallion's sperm using MX freezing extender and STAR extender (skim milk based extender). This result was in a harmony with (Esmail et al., 2023) who proved that egg yolk based extender improved the post-thawing motility of sperm of Arabian stallion than using milk based diluent. However, this result was not in agreement with (Love et al., 2005) who said that the most successful extender used for cryopreservation of equine sperm those which contain milk. Considering that egg yolk based diluent is cheaper and has lower cost than milk extender, this could be used for freezing and cryopreservation of sperm of arabian stallions as an option to lower the expenses and costs of the cryopreservation process of equine sperm.

Cryopreservation of stallion's sperm using MX freezing extender improved the post-thawing motility nearly similar to STAR freezing extender ($P > 0.05$). This result was in agreement with (M´arcio et al., 2024) who said that

cryopreservation of stallion's sperm using lyophilized extenders improved the post-thawing motility and had the advantage of storage and distribution at the room temperature

In the current study the extender that was provided with Di-methyle formamide as a cryoprotectant improved the post-thawing motility of sperm of arabian stallions than diluents which were provided with glycerol as a cryoprotectant. This result was in agreement with (Olaciregui et al., 2014) who told that dimethyl formamide was better than glycerol in improvement of the post-thawing motility. This result was not in agreement with (Moore et al., 2006) as they did not find any significance in the post-thawing motility in diluents containing either glycerol or dimethyle-formamide.

Maintenance of higher motility after thawing of cryopreserved sperm does not mean higher fertility of stallion's sperm, but can be used as a guide for measuring and evaluation of the stored sperm (LeFrapper et al., 2010). So, evaluation of conception in the field is very important to be sure that egg yolk based diluent is the extender of choice for cryopreservation of sperm of Arabian stallions.

Conclusion, the current study revealed that Lyophilized extenders can be used for cryopreservation of stallion's sperm with the advantage of storage and distribution at room temperature, but also egg yolk based extender is the best for improvement of sperm motility after thawing.

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CONFLICTS OF INTEREST:

The authors declare no conflict of interest.

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