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Effect of Cryopreservation on Arabian Stallion Semen After Adding INRA96 and DMF Based Extenders

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Abstract

Cryopreservation is very important technique in AI centers of stallions, it preserves sperms for long period and spread the superior genetic merits between different animal's breeds. However, using of cryopreserved sperms lead to decreasing the fertility between animals, due to lethal damage of sperms during preservation process; so, this study aimed to use the two different freezing media with decreasing post thawing sperm damage. A total of 54 ejaculates were collected from nine pure fertile Egyptian Stallions (6 ejaculates per stallion), individually housed at a Veterinary Clinic in Giza Government. Semen sample was collected by Missouri AV on a regular basis (two collections / week) during the 2021 breeding season in presence of teaser mare. The collected ejaculates were sent to the laboratory immediately for evaluation by CASA (total concentration, progressive motility, static motility, and sperm abnormalities). Ejaculates were filtrated for removal gel fraction; filtrated ejaculates were diluted by EDTA-glucose media for centrifugation and the resulting sperm pellets were split into 2 equal aliquots and then extended in freezing media. INRA96 Milk-based Extender with glycerol and Egg yolk- based Extender with DMF (dimethylformamide) were used in this study as freezing extenders. Diluted semen was packaged into 0.5 ml straw then cooling at 4 ◦C and freezing by vapor of liquid nitrogen and after that preserved by freezing in liquid nitrogen container at -196º C. After keeping the frozen straws in liquid nitrogen for one week, at least 2 straws were taken for thawing and evaluating post thawing-freezing motility. Finally thawed-frozen semen was inseminated inside fertile mares for calculation the conception rate after one month. Post thawing motility were evaluated in extended semen by two different extenders. The obtained results showed a change in the motility by decreasing in INRA-diluted semen compared to DMF-diluted semen. Conception rate was recorded after insemination and showed a high significant in DMF-diluted semen than INRA diluted semen. We concluded that the frozen semen with DMF based diluent did not decrease the motility of sperms after thawing and achieved high conception rate when compared with INRA based glycerol diluent.

KEYWORDS
Stallion, Cryopreservation, INRA, DMF, CASA, Semen.

INTRODUCTION

The horse industry has been incorporating modern biotechnologies that allow for the reduction of production costs and the selection of breeding animals with better genetic value because productivity is essential for good economic outcomes (Varner *et al.*, 2000). By the time, the male gamete had shown to be the most efficient tool for enhancing animal breeding activities. Unlike cattle, semen cryopreservation in horses progresses slowly, yet equine AI employment has been increasing despite these obstacles (Kirk *et al.*, 2005). Although sperm motility results from frozen/thawed semen vary from 30 to 50% on average, semen cryopreservation is still a relatively new technique in equine reproduction (Zúccari, 1998).

The equine industry is increasingly using frozen stallion spermatozoa for artificial insemination. Since it allows for the long-term storage and transportation of semen (regardless of the location and availability of a stallion), also it allows for the preservation of semen from genetically superior animals and the creation of a successful breeding programme, as well as the control or eradication of venereal and other infectious diseases, this technology is crucial for equine breeding. Breeders can more readily inseminate a mare at the ideal breeding time thanks to the availability of frozen sperm rather than having to rely on the availability of stallion sperm (Squires, 2013). Moreover, banking gametes with cryopreservation is a secure option that promotes biodiversity preservation and endangered species protection. Sperm can be preserved by being kept in liquid nitrogen at a temperature of -196 degrees Celsius. This prolongs the life of the cells (Moore *et al.*, 2005).

Regrettably, there is inter-individual heterogeneity in the cryo-survival of their semen, and the fertilizing power of cryopreserved stallion spermatozoa is typically regarded as lower than that of some other domestic species, particularly that of dairy cow. This decrease in fertility is probably caused by the fact that stallions are chosen for their pedigree, performance history, and

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conformation traits rather than their fertility accomplishment (Hussain *et al*., 2011).

The cryopreservation technology is still in a suboptimal level of development, and cause extensive chemical and physical cryo-damages (lethal or sub-lethal) to the structural integrity, biochemistry, and biophysics of the spermatozoa (Sieme *et al*., 2008). Phase transitions in the plasmalemma, oxidative stress, apoptotic-like changes, capacitation-like changes, mechanical stress on cell membranes due to osmotic stress, temperature changes during the process of freezing and thawing, and sperm death or, if surviving, reduced fertilizing capacity have all been characterized as contributing to cryo-injury (Ortega *et al*., 2009; Mara *et al*., 2013). Many laboratories have experimented over time in an effort to enhance present cryopreservation procedures and comprehend the physiology of the spermatozoa subjected to freezing and thawing (Vidament, 2005; Oldenhof *et al*., 2010; Salazar *et al*., 2011), and direct comparisons among extenders or cryoprotectants have been performed (Squires *et al*., 2004); The evaluation of modifications in membrane lipid structure or minor sperm damage has not yet taken place. The long-term preservation and global distribution of valuable genetic resources are two benefits of cryopreserving stallion spermatozoa. It also reduces the danger of disease transmission associated with transportation and spontaneous mating. The successful outcome depends on a number of elements, including the best choice of cryo-protectant, freezing protocols, holding temperatures, and thawing procedures. Cryopreservation and the following thawing/warming are very precise processes. Because every stage of this process has the potential to reduce fertility, careful supervision is essential (Alvarenga *et al*., 2005; Vidament, 2005). The "preferential exclusion" theory, the "water replacement" theory, and the "water entrapment" theory are the three basic hypotheses that explain how cryoprotectants work. According to the "preferential exclusion" theory, biomolecules interact with water more frequently than with additional co-solutes. This indicates that water molecules are drawn to the membrane's biomolecules rather than interacting with the cryoprotectants, keeping the cells from becoming dehydrated (Crowe *et al*., 1992). The stabilizing effects of additional compatible solutes on proteins at supra-zero temperatures and the stabilizing effects during freezing are explained by Sieme *et al*. (2016). So, the goal of this study was to cryopreserve stallion semen using two different extenders, compare them, and select the optimal extender with the least amount of harmful damage to sperms. In this investigation, the conception rate in mares who were inseminated with this cryopreserved semen was also calculated.

MATERIALS AND METHODS

Semen collection and evaluation

This study was used a total of 54 ejaculates, six ejaculates per stallion, from nine pure, fertile Egyptian stallions that were kept separately at a veterinary clinic in Giza Government. The stallions were given a balanced diet and unlimited access to water. During the breeding season of 2021, a regular semen sample collection regime (two collections per week) was carried out; the teaser mare was present during the collection process. Missouri style artificial vagina was used for semen collection from these stallions, it was be lubricated by non-spermicidal gel, and pre-warmed to 45–50 degree, and fixed from inner by filter to separate the gel fraction. The collected ejaculate was immediately transported to the laboratory (Giza, Egypt) for evaluation (macroscopic, motility, sperm concentration and abnormalities) by computer assisted semen analyzer (CASA), (IMV Company, France).

Only ejaculates with sperm that was >65% motile, morphologically normal, and had >70% viable sperm were used in this study. At 37°C, a 1:1 ratio of centrifugated extender (EDTA-glucose medium, Germany Company) was added to the filtered ejaculate. At room temperature, 50 (ml) centrifuge tubes containing the expanded semen were centrifuged at 600g for 10 minutes. The sperm pellets were separated into two equal aliquots after the supernatant was removed, and they were extended once more in freezing medium.

Freezing extender

This study used two extenders and compared between them (INRA96 Milk-based Extender with glycerol) and Egg yolk- based Extender with DMF (dimethylformamide). The INRA96 extender consisted of a HGLL solution containing Hank's salts, glucose and lactose, buffered by Hepes (4.76 $g{\cdot}L^{-1}$ Hepes buffer) and supplemented with a purified fraction of caseins (native phosphocaseinate). The pH is adjusted to 7.1. INRA96 also contained 50000 UI penicillin, 50 mg gentamicin, and 0.25 mg amphotericin B per liter. INRA96 is distributed in a ready to-use liquid form by IMV-Technologies (L'Aigle, France). Egg yolk- based Extender with DMF that was consisted of D-Glucose – Trisodium citrate dihydrate-disodiumethylene diamine tetra acetic acid (EDTA), sodium bicarbonate, potassium penicillin G, Amikacin sulfate, and D-lactose solution (11%w/v). those extenders are sterilized by the manufacturer and stored at 4◦C before use.

Semen Dilution

It was performed, by mixing the semen with an appropriate centrifugation medium to minimize the cellular damage. A centrifugation force of 500 g force for 10 minutes was used for obtaining the semen pellet. The cushion fluid was used in the bottom of 50 ml conical tubes. The pellet was put back into suspension then divided into 2 aliquots, one aliquot was diluted with egg yolk based DMF based extender and the other aliquot was diluted with milk-based glycerol extender (INRA 96) (Brinsco and Varner, 1992).

Packaging of semen

The 0.5-mL straws were used which contain considerably fewer sperms (50 to 100 million), so more than one straw may have to be used to provide an adequate insemination dose (often six to eight straws) (Diego *et al*., 2013).

Cooling and freezing

Sperms were cooled from 37° C to 4°C in a refrigerator cabinet at 4°C for 30 minutes or 60 minutes depending on the cryoprotectant employed after being diluted with freezing extenders. Following equilibration, the straws were frozen by immerged them for ten minutes in vapors that were 4 cm above liquid nitrogen (-120°C). They were then submerged in a container of liquid nitrogen (-196°C) (Kavak *et al*., 2003).

Evaluation of frozen- thawed semen

At least two straws were picked up from the liquid nitrogen container by plastic forceps and then immersed in thermometer at 37°C for one minute. The frozen-thawed semen was evaluated by CASA as total and progressive motility.

Insemination of the female and calculation the conception rate

In this study, 74 mares were inseminated with DMF diluted semen and 72 mares were inseminated with INRA diluted semen. Mares that inseminated were monitored daily using ultrasonography during estrus and hCG was used to induce ovulation (1500 IU). Every six hours, an ultrasound evaluation is required. Once ovulation is observed, insemination is carried out. After 15 days of insemination, inseminated mares underwent ultrasound examination to determine whether they were pregnant and to determine the rate of conception.

Statistical analysis

Prior to statistical analysis, all percentage was normalized with an arcsine transformation. Data of semen quality were subjected to one-way ANOVA test to estimate the effect of animal age (7-8, 9-10, and > 11 years). The effect of animal age, extender type (DMF and INRA 96), and their interaction on conception rate, raw motility, post thawing motility and motility change was tested by two-way ANOVA test (2x3 factorial design). Data of conception rate were also subjected to chi square test of independence to determine the effect of age and extender on frequencies of pregnant and non-pregnant animals. The P-value ≤0.05 was used to indicate a statistical significance. Multiple comparisons among mean values of intervals of ages (7-8, 9-10, and > 11 years) and / or extender type (DMF and INRA96) were conducted according to Tukey HSD test (P ≤ 0.05). SPSS software version 24.0 was used to conduct the statistic tests.

RESULTS

Semen evaluation after collection

This study was employed on 9 Arabian pure stallions with different ages ranged from 7 to 11 years. Immediately after semen collection from those stallions, all ejaculates were sent to the laboratory for evaluation of sperm parameters (total motility, progressive motility, slow motility, sperm concentration and sperm abnormalities) by CASA, then comparing between different ejaculates according to animal age as in Table 1. A data in Table 1. showed semen sample analysis in stallions used in this study that was depended on the age; stallions with 7 to 8 years gave good semen sample with high sperms concentration reached to 306.3±26.14 million sperms, normal motility above 80% and low sperm abnormalities in compared with the others stallion. Sperm abnormalities were recorded within high percent in stallions aged 9-10 years, especially bent tail abnormalities reached to 10.87±4.293%, but recorded with low percent in stallions aged 7-8 years and >11 years as in Table 2.

Evaluation of thawed-frozen semen

After adding two different extenders on semen samples for

Table. 1. Sperm parameters of fresh ejaculates from the different ages' stallions, which analyzed by CASA.

Sperm parameters	7-8 years (N:3)		9-10 years (N:3)		>11 years (N:3)		P-value
	Conc	(%)	Conc	(%)	Conc	(%)	
Total Conc.	306.3±26.14	100%	310.4±30.58	100%	272.6±37.53	100%	$0.664^{N.S}$
Static Conc.	74.83±9.273	24.45±2.508	102.0±9.663	35.55±6.461	90.99±24.56	32.63±7.952	$0.549^{N.S}$
Progressive Conc.	88.15±14.58	28.24±3.598	66.33±18.15	19.67±3.623	63.76±15.49	23.51±4.071	$0.550^{N.S}$
Motile Conc.	231.4±20.63	75.55±2.508	208.4±37.35	64.45±6.461	181.6±33.33	67.37±7.952	$0.577^{N.S}$
Slow Conc.	55.90±2.389	18.68±1.472	59.05±7.944	19.02±2.169	50.14±7.877	18.34±1.462	$0.652^{N.S}$
Normal fraction	270.3±32.44	87.25±4.093	246.0±42.93	76.94±8.135	227.8±35.71	83.89±4.608	$0.745^{N.S}$

Data are presented as Mean±SE; Conc.: Concentration

Table. 2. Semen analysis by CASA from different age stallions especially normal and abnormal sperm parameters.

Sperm parameters	7-8 years (N:3)		9-10 years (N:3)		>11 years (N:3)		P-value
	Conc	(%)	Conc	(%)	Conc	(%)	
Normal Fraction Conc.	270.3±32.44	87.25±4.093	246.0±42.93	76.94±8.135	227.8±35.71	83.89±4.6%	$0.664^{N.S}$
Bent Tail Conc.	16.30±3.536	5.820±1.611	29.85±10.35	10.87±4.293	16.82±4.666	6.133±1.477	$0.549^{N.S}$
Coiled Tail Conc.	3.254±1.004	1.120±0.373	2.538±0.728	0.883±0.303	2.260±0.546	0.898±0.227	$0.550^{N.S}$
Distal Droplet Conc.	6.090±3.213	2.206±1.342	13.40±5.948	4.887±2.345	8.527±3.796	2.935±1.230	$0.577^{N.S}$
Proximal Droplet Conc.	7.720±3.182	2.726±1.334	15.92±4.449	5.518±1.717	13.20±5.385	4.613±1.710	$0.652^{N.S}$
DMR (Distal midpiece reflex)	2.630±0.845	0.882±0.278	2.722±0.476	0.900±0.174	4.028±1.399	1.522±0.415	$0.745^{N.S}$

Data are presented as Mean±SE; Conc.: Concentration

Table 3. Changes in motility of sperms from different aged stallions after freezing and thawing.

Age	Extender	Raw motility after adding extender	Post thawing motility	Motility change
7-8 years	DMF	61.82±2.053	55.91±2.002	5.909±0.610
7-8 years	INRA 96	63.64±1.664	51.36±1.664	12.27±0.787
9-10 years	DMF	62.50±2.329	56.79±2.321	5.714±0.485
9-10 years	INRA 96	61.79±2.003	51.79±1.933	10.00±0.741
> 11 years	DMF	64.29±2.766	58.57±2.608	5.714±0.714
> 11 years	INRA 96	64.29±2.766	51.43±1.429	12.86±1.487
P-value		$0.830^{N.S}$	$0.850^{N.S}$	0.16

dilution and freezing, those frozen straws still kept in liquid nitrogen container for one week. At least 2 straws were taken from each ejaculate for thawing and reevaluating by CASA as in Table 3. Motility change was recorded after thawing of frozen semen which extended by two different extenders and we noticed decreasing in sperm motility after adding INRA96. The change was reached into 12.27% and 12.86 % in 7-8 years and >11years old stallions, respectively as in Fig. 1.

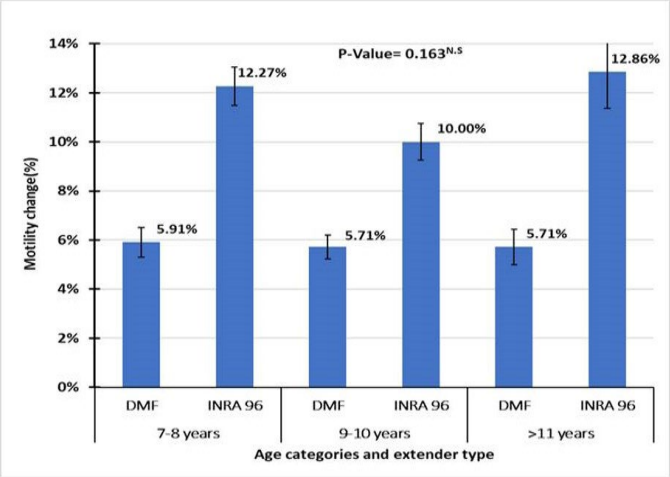

Fig. 1. Graphic of the percent of the Motility changes immediately post thawing

Calculation of conception rate

After insemination the fertile mares with thawed-frozen semen, conception rate was calculated as in Table 4. The conception rate was measured highly significant in semen sample diluted with DMF extender than INRA 96 diluted semen. When compared the effect of the different ages of stallions on conception rate in inseminated mares, was recorded that the high conception rate appeared in 9-10 years old stallion as in Table 5.

DISCUSSION

One of the most important jobs of sperm's cryopreservation is choosing cryo-diluents that are not only does it increase sperm volume, but also, it protects sperms upon dilution, chilling, freezing and thawing (Monteiro *et al*., 2011). Variation of effectiveness of diluents in maintaining motility and functional sperm integrity, cryopreservation may be due to their difference's component (Dascanio and mecue, 2014). In general, the extenders that are used to freeze sperm are made of ingredients that stabilize pH, remove harmful byproducts of sperm metabolism, provide energy, protect against temperature shock, preserve electrolytic and osmotic equilibrium, and suppress bacterial development (Jorg *et al*., 2020). The quantity of semen doses, together with the costs associated with gathering and freezing one ejaculate, are two criteria that impact how economically efficient semen cryopreservation is. The proportion of frozen-thawed ejaculates deemed appropriate for use in commercial AI programs is also a factor (Metcalf, 2007). A frozen semen program's main objective is to work out on how to use semen as effectively as possible without lowering pregnancy rates (Watson, 2000).

Cryoprotectants must be present in the extenders to prevent the development of intracellular and extracellular ice crystals. In a comparison of the sperm motility characteristics assessed by CASA, the sperm motility in extended DMF semen did not significantly alter, but it did decrease after extending with INRA96 and achieved a 12% change by lowering motility. Cryopreservation causes acute necrotic changes to spermatozoa mainly because of osmotic imbalance (Morris, 2005; Ortega *et al*., 2008); Yet, following thawing, a sizable fraction of the spermatozoa that survive have sublethal damage and are forced to undergo "apoptosis-like" cell death, which reduces their longevity (Medeiros, 2003; Graham, 2000). So; the present study used egg yolk based DMF diluent to decrease sublethal damages and preserve the fertility capacity of sperms. This result accord with Medeiros *et al*. (2002) who reported that sperm motility and morphology improved by adding glycerol based DMF freezing diluent and also pregnancy rate was elevated to 40%. Also agreed with the study of Hammerstedt and graham (1992) who noticed that when compared to MF or DMF, the proportion of motile spermatozoa after thawing was significantly lower for semen frozen in skim milk egg yolk extender containing either acetamide or methyl acetamide. The same result was reported in the study of Glazar *et al*. (2009) who demonstrated that post thaw motility (total and progressive) was significantly improved when sperm was frozen in the presence of 3% DMF compared with glycerol (20 stallions; 1 ejaculated/stallion). Adding of glycerol to INRA based diluent occurred lowering in total and progressive motility, this is contributed into toxic effect of glycerol. Protein denaturation, altered actin interactions, and the induction of protein-free membrane blisters were some of the symptoms of glycerol toxicity. Additionally, according to report of Moffet *et al*. (2003) cytoplasmic changes, altered tubulin polymerization, altered microtubule association, and osmotic stress also appeared after glycerol addition. Another study by Vidament (2005) showed that stallion spermatozoa are substantially more permeable to lower molecular cryoprotectants (CPAs) such dimethylformamide (DMFA) than to glycerol, resulting in reduced osmotic stress than the latter. In order to assess the fertility of thawed-frozen semen, the conception rate was measured using either an INRA- or DMF-based diluent. In our results, DMF based diluent did not affect the viability and fertilizing capacity of sperms after insemination in the female and achieved high pregnancy rate in compared with INRA diluent. This was agreed with the experiment of Anger *et al*. (2003) who reported that pregnancy rate was zero % when frozen semen with glycerol used compared with frozen semen with DMF diluent. In contrast, another study by Jorg *et al*. (2020), they reported that there was no difference in the rates of conception in mares that were inseminated with frozen sperm in either DMF or glycerol-containing semen. The sperm membrane's permeability to the cryoprotectant is a key component in determining the degree of damage to frozen semen. Glycerol appears to be more harmful to sperm than DMF and penetrates the sperm membrane more slowly (Moffet *et al*.,

Table. 4. Percent of conception rate after insemination mares by one month with DMF and INRA 96 diluted semen.

Conception rate	DMF	INRA 96	χ^2	df	*P*-Value
Non pregnant mares	18 (31.03%)	27 (42.19%)	1.63	1	$0.202^{N.S}$
Pregnant mares	40 (68.97%)	37 (57.81%)			

Table. 5. Calculation of conception rate after inseminated with thawed-frozen semen from different age stallions.

Conception rate	7-8 years	9-10years	>11 years	χ^2	df	*P*-Value
Non pregnant	15 (50.00%)	11 (23.40%)	19 (42.22%)	6.44	2	0.040*
Pregnant	15 (50.00%)	36 (76.60%)	26 (57.78%)			

2003).

CONCLUSION

It was concluded that the frozen semen with DMF based diluent did not decrease the motility of sperms after thawing and achieved high conception rate when compared with INRA based glycerol diluent.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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