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The quality of chilled stallion semen in relation to its Freezability potential.

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ABSTRACT

The present study aimed to compare the quality of chilled stallion semen depending on its frozen-thawed quality. Semen was collected once weekly for 3 consecutive weeks from 10 fertile Arabian stallions belonging to Al-Zahraa stud, Cairo, Egypt, using a stallion artificial vagina. Semen was extended with INRA-96 and centrifuged for 10 minutes at 400 ×g. The sperm pellets were re-suspended to a final sperm concentration of 100×10^6 motile sperm/ml with INRA-96 either with 5% glycerol and 15% egg yolk (frozen semen) or without (chilled semen). After cooling to 5 °C, the chilled semen was maintained at 5 °C for 72 hours. The frozen semen was packed in 0.5 ml straws, equilibrated at 5 °C for 30 min, and placed 4 cm above the liquid nitrogen level. The chilled and frozen-thawed semen samples were evaluated for progressive motility, plasma membrane integrity (Hypoosmotic swelling test), acrosome integrity (Giemsa staining), mitochondrial activity (MTT assay), and DNA integrity (Comet assay). Depending on the results of frozen-thawed semen, stallions were classified as good and poor freezers. The results of chilled semen were compared between the good and poor freezer stallions. The results revealed that the chilled and frozen semen of good freezer stallions showed higher values of all semen parameters than that of poor freezer stallions ($P< 0.01$). In conclusion, the present study revealed that semen from good freezer stallions have higher post-storage semen quality either after chilling or freezing compared to that of poor freezer stallions.

1. INTRODUCTION

Artificial insemination is common in equine clinical practice and most breed associations (Conboy, 2011; Morrell et al., 2014). The fertilizing capacity of stallion semen is a pivotal aspect of equine reproduction, influencing the success rates of breeding programs and pregnancy rates (Rota et al., 2004; Kopec et al., 2022). Achieving high fertilization rates depends on a comprehensive understanding of several factors affecting semen quality, including collection, evaluation, and preservation techniques (Vidament et al., 2012; Kopec et al., 2022). High-quality semen, with good motility, viability, and morphology, is essential for successful fertilization and the propagation of desired genetic traits (Kopec et al., 2022). Stallion semen can be extended and used in fresh insemination, cooled and stored at 5 °C for 48 hours before insemination, or frozen in liquid nitrogen (Samper, 2009; Sanchez et al., 2009).

Stallion semen cryopreservation has many advantages including the preservation of genetic material for future use, the ability to breed mares in the absence of stallion, and the shipping of frozen semen all over the world (Loomis, 2001; Barbacini, 2011). In the meantime, stallion semen cryopreservation has many disadvantages including the declined post-thawing semen quality and conception rate compared to fresh or cooled-stored semen due to the adverse effect of the freezing-thawing process that causes cellular damage, reducing sperm viability and motility (Prell et al., 2020; Kopec et al., 2022). In addition, specialized equipment and training are required for storing, handling, and evaluating frozen semen (Loomis and Squires, 2005). So Good freezer stallions maintain high post-thawing semen quality depending on the intrinsic properties of the sperm cell membrane and its ability to withstand the stresses of freezing and thawing (Vidament, 2005). The poor freezers experience a marked decline in semen quality after thawing due to various factors, including the composition of the seminal plasma, sperm cell membrane integrity, and reactive oxygen species (ROS) (Ball, 2008).

The differences in seminal plasma composition between good and poor freezer stallions affect sperm cryotolerance. (`Mráčková, M., Zavadilová, M., & Sedlinská, M. , 2015) Seminal plasma with higher levels of antioxidants protects sperm cells from oxidative damage during cryopreservation (Graham, 1996). In addition, the presence of specific proteins in the seminal plasma stabilizes the sperm cell membrane, reducing ice crystal formation, and improving post-thaw sperm quality (Bailey et al., 2000).

The chilling of stallion semen maintains its viability for up to 48 hours, providing practical flexibility for transportation and use in breeding programs (Pickett and Amann, 1993). Additionally, the cooling process minimizes cellular damage

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and preserves sperm membrane integrity by reducing osmotic and mechanical stress. This results in higher sperm motility and viability compared to frozen semen (Aurich, 2012). On the other hand, the cooled semen storage time of up to 48 hours is a limited period that restricts its use to short-term applications (Aurich, 2012). In addition, stallions vary in their response to semen cooling, with some showing a marked decline in semen quality after cooling (Loomis and Graham, 2008). Therefore, this study aimed to compare the quality of chilled stallion semen depending on its frozen-thawed quality.

2. MATERIAL AND METHODS

All procedures and experiments were carried out following the regulations approved by the Ethical Approval Committee of the Faculty of Veterinary Medicine at Benha University, Egypt, under approval number (BUFVTM04-10-24).

2.1. Semen collection

Ten healthy Arabian stallions of proven fertility aged 5-21 years, belonging to Al-Zahraa stud, Cairo, Egypt, were used in this study. Semen was collected from each stallion once weekly for 3 consecutive weeks (n= 30 ejaculates). Semen collection was conducted in the early morning using a lubricated pre-warmed (42-45°C) Colorado model artificial vagina with an inline filter to separate the gel fraction, and an estrus mare as a mounted animal.

2.2. Semen processing

Semen was evaluated for volume, progressive motility, and concentration. Only ejaculates with at least 60% progressively motile sperm and 250 × 10^6 sperm cells/ml were used for processing. Semen was extended 1:1 (semen: extender) using an INRA-96 (IMV, France) extender that had been warmed to 38 °C. The diluted samples were divided into two equal portions in 15-mL Falcon conical centrifuge tubes and centrifuged for 10 minutes at 400 ×g (Cochran et al., 1984). After centrifugation, the supernatant was removed, and pellets were re-suspended to a final sperm concentration of 100 × 10^6 motile sperm/ml with INRA-96 either supplemented with 5% glycerol and 15% egg yolk (frozen semen) or without (chilled semen).

The semen slowly cooled to 5 °C within one hour under aerobic conditions. After cooling, the chilled semen portion was kept at 5 °C for 72 hours. The semen portion for cryopreservation was filled into 0.5-ml straws (Minitube, Germany) equilibrated at 5ºC for 30 min (Crockett et al., 2001), then underwent manual freezing by placing the straws at 4 cm above liquid nitrogen in the vapor phase in a Styrofoam box for 10 min before being plunged into the liquid nitrogen at -196 °C till the analysis (Cristanelli et al., 1985). For thawing, 4 straws per replicate from each stallion were thawed in a water bath at 38 °C for 30 sec.

2.3. Evaluation of semen quality

2.3.1. Assessment of sperm motility

According to Aurich et al. (1997), sperm motility was examined using a warmed-stage phase contrast microscope (Advanced Automated Research Microscope System, Nikon Eclipse E200, phase contrast at 40 and 100 magnifications). For chilled semen, the sperm motility was examined at four different times: directly after cooling (0 hr.), 24, 48, and 72 hours after storage at 5ºC.

For the frozen-thawed semen, the sperm motility was examined at four different times: directly after thawing (time zero) and after 1, 2, and 3 hours of incubation at 37 °C. The post-thawing viability indices were calculated according to Milovanov (1962) to be equal to half of the post-thaw motility in addition to the summation of recorded motility at 1st, 2nd, and 3rd hour post-thawing.

2.3.2. Assessment of membrane integrity

According to Nie and Wenzel (2001), the hypoosmotic swelling test (HOST) was used to assess the sperm membrane integrity. Briefly, A 100 µl aliquot of each semen sample was mixed in 1.0 ml of a pre-warmed 100 mOsm sucrose solution (1.712 g sucrose dissolved in 50 ml of sterile de-ionized water). The mixture was incubated at 37 °C for 1 hour. Following incubation, semen slides were prepared and examined under phase contrast microscopy (400×). About 200 spermatozoa were counted and the percentage of cells with swollen/curled tails (intact plasma membranes) was recorded as HOS positive.

2.3.3. Assessment of acrosomal integrity

Acrosomal integrity was assessed using the Giemsa staining technique according to de Oliveira et al. (2013). A 10 µl of thawed semen was smeared, air-dried, and fixed in a neutral red solution for 4 minutes. The fixed smears were washed with distilled water, air dried, and immersed in Giemsa stain solution (7.5%) for 4 hours. two hundred spermatozoa were counted under a phase contrast microscope (400×) and classified for the percentage of cells with intact Dark blue or purple and damaged/lost acrosome (unstained).

2.3.4. Assessment of sperm mitochondrial activity

The sperm mitochondrial activity was assessed using the MTT assay (Mosmann, 1983)The EZcount™ MTT Cell Assay Kit (Code: CCK003, HiMedia Laboratories) was employed following the manufacturer's protocol. Briefly, A 100 µl semen sample was mixed with 10 µl of MTT stock solution (5 mg MTT/ml of PBS) in wells of a 96-well microplate. The rates of MTT reduction were determined by using an ELISA reader at a wavelength of 550 nm (D.A. El Badry, et al,2008).

2.3.5. Assessment of sperm cell DNA integrity:

The sperm DNA integrity was assessed using the alkaline Comet assay (Hughes et al., 1996) as follows; full frosted glass slides were covered with 100 µl of 0.5% normal melting point agarose (Sigma-Aldrich, St. Louis, MO, USA).) and allowed to solidify. Frozen-thawed semen was diluted to a final concentration of 1×10^5 sperm cells in 50 µl PBS (7.2 pH) and mixed with 50 µl of 1.2% low melting point agarose and used to form the second layer. The slides were placed in lysis buffer for 1 h (2.5 M NaCl, 100 mM Na EDTA, 10 mM Tris, 1% Triton X at a pH of 10) after which, incubated at 37 °C in 100 µl/ml of proteinase K in lysis buffer overnight. After draining the proteinase K solution from the slides, they were placed in a horizontal electrophoresis unit filled with freshly prepared alkaline electrophoresis solution containing 300 mM NaOH and 1 mM EDTA for 20 min to allow the DNA to denature. Electrophoresis was performed at room temperature at 25 V (0.714 V/cm) and 300 mA, obtained by adjusting the buffer level, for 10 min. The slides were then washed with a neutralizing solution of 0.4 M Tris at pH 7 to remove alkali and detergents. After neutralization, the slides were each stained with 50 µl of 20 µg/ml ethidium bromide and mounted with a coverslip. A total of 200 sperm cells were examined under a fluorescent microscope (400×). The intensity of the stain in the comet tail region is presumed to be related to the DNA content. DNA damage was estimated

from measurements of the DNA percent in the tail, tail length, and tail moment, using an image analysis system (Comet-Score program) was developed by TriTek Corp, which is based in the United States.

2.4. Statistical analysis

The data were analyzed and presented as Mean ± SEM using SPSS software (IBM® SPSS® Statistics Version 25). One-way ANOVA with Duncan's multiple-range test was used to define statistical differences between groups and to measure significance. The statistical significance was set at P≤ 0.05.

3. RESULTS

Depending on the results of post-thawing sperm motility, stallions were classified as good (motility ≥ 35%) and poor (motility < 35%) freezers. Hughes, C.M., McKelvey-Martin, V.J., & Lewis, S.E.M. (1996). The results of chilled semen were compared between the good and poor freezer stallions (Five stalions in each group).

3.1. The frozen-thawed sperm motility

The total sperm motility of frozen-thawed semen differed significantly (P ≤ 0.01) between the groups of stallions after the different incubation periods at 37 °C with the highest value being recorded with the semen of good freezer stallions (Table 1).

3.2. The frozen-thawed sperm viability, membrane integrity, and acrosomal intactness.

As shown in Table (2), a significant (P≤ 0.01) increase in the viability index, membrane integrity, and acrosome intactness has been recorded in the frozen-thawed semen of good freezer stallions compared to the poor freezers.

3.3. The frozen-thawed sperm mitochondrial activity and DNA integrity

A significant (P< 0.01) increase has been recorded in the percentage of mitochondrial activity, non-fragmented DNA, and DNA of the comet head with a marked decrease in the percentage of DNA in the comet tail, tail length as well as the tail moment in the frozen-thawed semen of good freezer stallions compared to the poor freezers (Table 3).

Table 1 Total motility of frozen-thawed Arabian stallion spermatozoa

Animals	Good freezers	Poor freezers
Zero hour	50.00±3.47^{a}	24.44±2.48^{b}
1st hour	39.53±3.22^{a}	0.56±0.56^{b}
2nd hour	30.00±2.50^{a}	0.00 ± 0.00^{b}
3rd hour	24.00± 2.50^{a}	0.00±0.00^{b}

Values with different superscripts within the same row differed significantly at P <0.01

Table 2 Viability index, plasma membrane integrity, and acrosomal intactness of frozen-thawed Arabian stallion spermatozoa.

Animals	Good freezers	Poor freezers
Viability Index (%)	118.33 ±9.47^{a}	4.86±0.27^{b}
Plasma Membrane integrity (%)	46.33±1.33^{a}	32.00±2.74^{b}
Acrosome Intactness (%)	43.20±1.85^{a}	19.05 ± 1.33^{b}

Values with different superscripts within the same row differed significantly at P <0.01

Table 3 Mitochondrial activity and DNA integrities of frozen-thawed Arabian stallion spermatozoa.

Animals	Good freezers	Poor freezers
MTT-RR (%)	^{a}59.43 ± 2.92	33.76±1.64^{b}
Sperm with non-fragmented DNA (%)	94.78±0.83^{a}	86.10±1.23^{b}
DNA in the head of comet (%)	96.39±0.82^{a}	92.13 ± 0.96^{b}
DNA in the tail of comet (%)	3.61±0.82^{b}	8.51±0.79^{a}
Tail length (pixel)	6.91±0.30b	15.08±1.06^{a}
Olive tail moment	0.43±0.03b	0.98±0.10^{a}

Values with different superscripts within the same column differed significantly at P <0.01

3.4. The cooled-stored sperm motility, plasma membrane integrity, and acrosomal intactness

As shown in Table (4), a significant (P≤ 0.01) difference was found between good and poor freezer stallion semen in the percentage of total sperm motility, plasma membrane integrity, and acrosomal intactness after the different storage periods at 5 °C.

Table 4 Total motility, plasma membrane integrity, and acrosomal intactness of cooled-stored Arabian stallion spermatozoa after different storage periods (n=30)

Parameters	Storage time (hrs)	Good freezers	Poor freezers
Total sperm motility (%)	0	71.83 ± 2.08^{a}	45.56 ± 4.12^{b}
	24	65.00±1.87^{a}	27.78±8.30^{b}
	48	58.67±1.90^{a}	22.22±8.54^{b}
	72	50.33±1.67^{a}	17.78±7.37^{b}
Plasma membrane integrity (%)	0	71.00±1.50^{a}	42.63±6.19^{b}
	24	66.28±1.36^{a}	29.50±8.71^{b}
	48	58.57±1.34^{a}	22.71±8.70^{b}
	72	50.55±1.33^{a}	18.38±7.22^{b}
Acrosomal intactness (%)	0	71.02±0.86^{a}	42.90±7.05^{b}
	24	65.63±1.04^{a}	31.94±7.87^{b}
	48	59.57±1.21^{a}	23.83±8.17^{b}
	72	54.03±1.34^{a}	20.48±7.55^{b}

Values with different superscripts within the same row differed significantly at P< 0.01

3.4. The cooled-stored sperm mitochondrial activity and DNA integrities

The sperm mitochondrial activity and DNA integrities of cooled-stored Arabian stallion spermatozoa are presented in Table (5). A significant (P ≤ 0.01) difference has been found between good and poor freezer stallions in the percentage of sperm mitochondrial activity and DNA integrity.

Table 5 Mitochondrial activity and DNA integrities of cooled-stored Arabian stallion spermatozoa immediately after thawing.

Parameters	Good freezers	Poor freezers
MTT-RR (%)	79.56±1.60^{a}	56.11±5.10^{b}
Sperm with non-fragmented DNA (%)	97.48±0.36^{a}	90.87±1.48^{b}
DNA in the head of comet (%)	98.12±0.34^{a}	92.69±1.30^{b}
DNA in tail of comet (%)	1.88±0.34^{a}	7.31±1.30^{b}
Tail length (pixel)	4.56±0.15^{a}	6.61±0.49^{b}
Olive tail moment	0.25±0.01^{a}	0.46±0.06^{b}

Values with different superscripts within the same row differed significantly at P <0.01

4. DISCUSSION

The sperm's in vitro survival and fertilizing ability are affected by the handling procedures and storage periods (Heiskanen, 1994). The present study provides valuable insights into the semen quality characters influencing the quality of stallion semen and its fertilizing capacity, focusing on the comparison between good and poor freezer stallions of chilled and frozen-thawed semen. It has been stated that the success of fertilization and the desired genetic traits propagation depends on the fertility parameters of sperm including good motility, viability, and morphology (Kopec et al., 2022). Stallions differ in their ability to sustain their fertility during the storage of spermatozoa (Katila, 1997) The present results revealed the prominent differences in the different semen parameters between the good and poor freezer stallions which are coordinated with those reported by Rota et al. (2004), Vidament (2005), Ball (2008), Vidament et al. (2012), and El-Badry et al. (2014 & 2016)

The difference between the good and poor freezer stallions in their response to cryopreservation effects has been attributed to the intrinsic properties of the sperm cell membrane and its ability to withstand the stresses of freezing and thawing (Vidament, 2005), and the levels of antioxidants and specific proteins in the seminal plasma which protects sperm cells from oxidative damage and stabilizes the sperm cell membrane during cryopreservation (Graham, 1996; Bailey et al., 2000). In addition, the hormonal content of seminal plasma may have a role as good

freezer stallions were found to have higher cortisol, estradiol, insulin IGF-1, T3, and T4 (El-Badry et al., 2016). For equine breeding, chilled semen offers practical versatility, maintaining viability for up to 48 hours, and facilitating transportation and use in breeding programs (Pickett and Amann, 1993). Despite these advantages, the limited storage time of cooled semen presents a significant drawback, restricting its use to short-term applications (Aurich, 2012). Additionally, individual variability in response to cooling can impact semen quality, with some stallions showing a marked decline after cooling (Loomis & Graham, 2008). Ensuring optimal storage conditions and handling practices is critical to preserve semen quality and achieve successful breeding outcomes. The present study confirms the clear difference between the two types of stallions (good and poor freezers) in the semen characteristics parameters of chilled semen. In addition, it confirms that the cooling process reduces cellular damage and maintains sperm membrane integrity, resulting in higher motility and viability compared to frozen semen (Aurich, 2012). This finding is consistent with previous research highlighting the benefits of cooled semen (Kopec et al., 2022).

5. CONCLUSIONS

In conclusion, this study presented a clear difference between the stallions in their response to semen cryopreservation (Good and poor freezer stallions). Moreover, it reveals that good freezer stallions have good quality chilled semen compared to poor freezer stallions.

CONFLICT OF INTEREST

The authors announce that they have no Conflict of interest.

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