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Effect of dimethyl-formamide and glycerol cryoprotectants on frozen stallion spermatozoa

Aya M. Fadl[1]; El-Badry, D.A.[2]; Abou-Ahmed, M.M.[1] and Ghallab, A.M.[1]
[1]Theriogenology Dept., Faculty of veterinary medicine, cairo university
[2]Artificial insemination and embryo transfer Dept., Anim. Reprod . Res. Instit.

Abstract

This study aimed to compare the efficiency of dimethyl-formamide (DMF), glycerol and their combination on freezability of Arabian stallion spermatozoa. Fourty semen samples were collected from four Arabian stallions, diluted with INRA-82 supplemented with 5% glycerol, 5% DMF or 2.5% glycerol+2.5% DMF and subjected to cryopreservation. The results revealed the superiority of DMF vs. glycerol in terms of post-thaw motility (56.25 ± 0.89 vs. 46.50 ± 1.14%, respectively), viability index (167.75 ± 3.49 vs. 135.50 ± 4.49, respectively), survival rate (74.58 ± 1.03 vs. 61.59 ± 0.38%, respectively) and sperm acrosome integrity (78.83 ± 0.74 vs. 75.95 ± 0.74%, respectively). In conclusion, The protocols of Arabian stallion sperm cryopreservation could be enhanced using DMF as a cryoprotectant (CPA) used with INRA-82 extender.

Key words: Arabian stallion- glycerol- dimethyl-formamide- cryoprotectant - freezing

Introduction

Cryopreservation of stallion sperm is an active field of research, since it allows for long-term sperm storage, transportation and insemination of mares at the optimal breeding time instead of relying on the availability of short-lived and cool-transported semen **(Choi et al., 2002).** A large part of the stallion population, however, remains unqualified for semen freezing programs because of unsatisfactory post-thaw sperm quality and fertility rates **(Loomis and Graham, 2008)**.The factors potentially affecting the success of cryopreservation assessed in the different studies were the composition of the freezing medium, the cryoprotectant and its concentration, the freezing conditions and cooling and warming temperatures, as well as individual animal variation **(Mocé and Vicente, 2009)**. Among the various factors, the choice of cryoprotectant (CPA) is certainly one of the most important for an effective freezing protocol for equine semen. The formation of intracellular ice crystals during the cryopreservation process causes cell destruction and this can be avoided by dehydrating the cells using a permeable CPA in the freezing solution **(Fuller et al., 2004)**. Although the cryoprotectant properties of glycerol were described 70 years ago by **Polge et al. (1949)**, many studies showed that glycerol was toxic to spermatozoa during the cryopreservation process **(Kundu et al., 2000)**. This toxicity is partly due to osmotic stress, because glycerol permeates the cell membrane more slowly than other CPAs **(Gilmore et al., 1995)**. Therefore, use of glycerol as a CPA could be a factor involved in poor post-thaw motility and fertility rates in frozen stallion spermatozoa **(Alvarenga et al., 2005)**. During the last decades, the search for alternative CPAs has been approached with the aim of improving equine sperm freezing procedures. Among the available CPAs that contain amide groups, dimethyl-formamide (DMF) has shown promising cryoprotective effects on different breeds of equine spermatozoa **(Vidament et al., 2002; Squires et al., 2004; Alvarenga et al., 2005; Betancur et al., 2012; Gibb et al., 2013; Mellisho et al., 2013; Alvarez et al., 2014)**, but to the best of our knowledge this CPA has not yet been tested in Arabian stallion sperm freezing . So, this study was designed to compare the cryoprotective efficiency of DMF, glycerol and its combination for cryopreservation of Arabian stallion semen .

Materials and Methods

Preparation of extender:

Modified INRA-82 **(El-Badry et al., 2014)** extender was prepared as followed : 25 g\L glucose monohydrate, 1.5 g\L lactose monohydrate, 1.5 g\L raffinose pentahydrate, 0.4 g\L potassium citrate monohydrate, 0.3 g\L sodium citrate dihydrate, 4.76 g HEPES, pH 7.0, 500 mg\L gentamycin, 0.035% sodium dodecyl sulphate (SDS) and 0.15% skim milk. Aliquots of INRA-82 extender were supplemented with 5% glycerol (G) , 5% dimethyl-formamide (DMF) or 2.5% glycerol + 2.5% DMF (G + DMF).

Animals and semen collection:

On a regular basis (one ejaculate / week), ten ejaculates per stallion were obtained from four Arabian stallions , aged 11-12 years, and individually housed at Al-Zahraa horse stud, Cairo, Egypt.The experiment was carried out during the period from August to October 2015. The animals were fed hay, tibn and concentrate

pellets supplemented with minerals. Water was provided adlibitum. At the time of collection, early in the morning, a mare in estrus was used as a mount animal. Semen was collected using a lubricated and pre-warmed (45 to 50 °C) Missouri-model artificial vagina with an inline filter to separate the gel fraction

Processing of semen:

Immediately following collection, the gel-free portion of the ejaculate was evaluated for volume and progressive motility. Also, Concentration of spermatozoa was determined using the hemocytometer. Only ejaculates with at least 60% progressively motile sperm and 250 x 10^6 sperm cell/ml were used for freezing. The semen was extended 1:1 (semen:extender) in INRA-82 extender that had been warmed to 38°C. The diluted samples were placed into 15-mL tubes and centrifuged for 10 minutes at 400 xg. **(Cochran et al., 1984).** At least 95% of the supernatant was removed **(Loomis and Graham , 2008)** and the remaining pellet was diluted with modified INRA-82 (containing 15% egg yolk in addition to 5% glycerol, 5% DMF or 2.5% glycerol + 2.5% DMF) to a final sperm concentration of 100x 10^6 motile sperm/ml. Each aliquot was cooled slowly to 5 °C over one hour, under aerobic conditions, and then incubated at 5 °C for 30 min **(Crockett et al., 2001).** The extended semen was drawn into 0.5-ml straws (Minitube, Germany), sealed by polyvinyl powder and placed 4 cm above liquid nitrogen in the vapor phase in foam box for 10 min before being plunged into the liquid phase **(Cristanelli et al., 1985).** The straws were then stored in goblets and kept immersed in liquid nitrogen container (LN). The cryopreserved samples were stored in LN for a minimum of one week until examination. For thawing, two straws per treatment were warmed in a water bath at 38 °C for 30 sec.

Evaluation of frozen-thawed semen:

Spermatozoa motility was examined and recorded using a pre-warmed stage of phase contrast microscope (200X) just after thawing, 1, 2 and 3 h post-thawing. The post-thawing viability indices were calculated according to **Milovanov (1962).** The percentage of HOS-positive cells and acrosome integrity in each sample were determined according to **Nie and Wenzel (2001)** and **Chan et al. (1999),** respectively.

Statistical analysis:

One way analysis of variance and Duncan's multiple range tests (using **SPSS program version 22.0**) were done for the obtained data after transformation of percentages to their corresponding arcsin values **(Snedecor and Cochran, 1989).** $P < 0.05$ was considered as statistically significant.

Results

As shown in table 1, the post-thaw motility and viability index of DMF group (56.25% and 167.75, respectively) were significantly ($P < 0.05$) higher than those of glycerol (46.50% and 135.50, respectively) and DMF + glycerol (48.88% and 143.44, respectively) groups. Also, the pos-thaw survival rate was higher ($P < 0.05$) in DMF group (74.58%), than those in DMF + glycerol group (64.78%) and in glycerol only (61.59%). Regarding the sperm membrane integrity, there were no statistically significant differences between the DMF and glycerol groups (46.48 and 45.88%, respectively). The percentage of spermatozoa with intact acrosomes was higher ($P < 0.05$) in DMF group (78.83%) than in glycerol only (75.95%) and DMF + glycerol groups (70.83%).

Table 1: Effect of different cryoprotectants on post-thaw total motility, membranes and acrosomal integrities of frozen-thawed Arabian stallion spermatozoa (Means ± SEM).

Semen parameters (n=40)		Type of cryoprotectant		
		Glycerol	DMF	Glycerol + DMF
Pre-freezing sperm motility (%)		75.50 ± 0.49		
Post-thaw sperm motility (%) at:	0 h	46.50 ± 1.14^b	56.25 ± 0.89^a	48.88 ± 0.94^b
	1 h	43.50 ± 1.27^b	53.38 ± 0.90^a	45.88 ± 1.21^b
	2 h	37.25 ± 1.37^b	46.25 ± 1.14^a	39.88 ± 1.25^b
	3 h	31.50 ± 1.47^b	40.00 ± 1.33^a	33.25 ± 1.40^b
Viability index		135.50 ± 4.49^b	167.75 ± 3.49^a	143.44 ± 4.13^b
Survival rate (%)		61.59 ± 1.38^c	74.58 ± 1.03^a	64.78 ± 1.12^b
Sperm membrane integrity (HOS%)		45.88 ± 0.39^a	46.48 ± 0.30^a	43.71 ± 0.39^b
Intact acrosomes (%)		75.95 ± 0.74^b	78.83 ± 0.74^a	70.83±0.50^c

Within rows, means with different alphabetical superscripts are significantly different at least at $P < 0.05$ n=Number of samples

Discussion:

The composition of the extender and the inclusion of suitable CPAs are important factors in successful sperm cryopreservation **(Vidament et al., 2000)** .All CPAs have different chemical structures and therefore may react differently with the sperm cells of different species **(Blanco et al., 2000).** It has been suggested that the ideal CPA should have a low molecular weight, great water solubility and minimal toxicity **(Alvarenga et al., 2005).** DMF has a lower molecular weight (73.09) compared with glycerol (92.05) and these CPA may induce less osmotic damage **(Alvarenga et al., 2005; Alvarez et al., 2014).**

In the current study, the decline in sperm parameters observed when using glycerol as a CPA was consistent with the previous findings of several authors that the use of glycerol as a CPA when freezing equine semen could be a factor involved in the poor post-thaw motility and fertility rates (reviewed by **Fahy et al. 1990**). Glycerol toxicity is partly due to osmotic stress, because glycerol permeates the cell membrane more slowly than other CPAs **(Gilmore et al., 1995).** Moreover, glycerol toxicity may result in protein denaturation, alteration of actin interactions and induction of protein-free membrane blisters, resulting in a detrimental effect on the fertility of fresh cooled and frozen–thawed equine semen **(Pace and Sullivan, 1975; Demick et al., 1976).** Furthermore, higher concentrations of glycerol had been shown to be deleterious to DNA of Arabian stallion sperm **(El-Badry et al., 2014)** and could lead to cell death **(Swelum et al., 2011).**

In our study, when DMF was used as an alternative cryoprotectant to glycerol, all semen parameters evaluated were improved (table , 1). In equine, DMF superior capacity as CPA is consistent with previous works **(Vidament et al., 2000; Gomes et al., 2002; Medeiros et al., 2002; Squires et al., 2004; Alvarenga et al., 2005; Alvarez et al., 2014)** Moreover, many authors determined its beneficial CPA effect for those stallions whose semen freeze poorly when glycerol is used ('bad freezer' stallions) **(Gomes et al. 2002; Medeiros et al. 2002; Squires et al. 2004; Alvarenga et al. 2005; Carmo et al. 2005; Mesa and Henao, 2012).** In contrast, glycerol was found to be more effective as CPA than DMF in bovine **(Gonzalez, 2004)**, ovine **(Moustacas et al., 2011; Jerez et al., 2016)** and canine semen **(Lopes et al., 2009).**

Based on our data, the combination of DMF and glycerol as CPA in INRA medium reduced the post-thaw quality of Arabian stallion sperm as compared to DMF alone in terms of post-thaw motility, viability, survival rate as well as membrane and acrosome integrities. In contrary, the CPA combination of DMF and glycerol enhanced equine epididymal **(Alvarez et al., 2014)** and ejaculated spermatozoa quality after cryopreservation **(Squires et al., 2004; Alvarez et al., 2014; Wu et al., 2015).** This discrepancy of results may be attributed to the findings that CPA had different effect on different diluents **(Ariantie et al., 2013),** to different equine breeds used or owing to individual variations within the same breed, as phospholipid levels in seminal plasma of 'good freezer' Arabian horses were found to be higher than 'poor freezers'**(El-Badry and Gabr, 2013).** Since it was suggested that differences among animals in the quantity and type of phospholipids could interfere with stability of the sperm membrane during cryopreservation **(Hammersted et al., 1990).**

In conclusion, the protocols of Arabian stallion sperm cryopreservation could be enhanced using 5% DMF as a cryoprotectant agent with INRA-82 extender. Subsequent fertility tests were necessary to verify if beneficial effects of DMF on sperm post-thaw characteristics are manifested in a higher conception rate.

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الملخص العربي

تهدف هذه الدراسة لمقارنة تاثير كلا من الجليسرول و الداي ميثيل فورماميد كلا علي حدي وكذلك مع اضافتهم سويا وذلك علي قدرة حيامن الخيول العربية علي التجمد. تم تجميع اربعين عينة سائل منوي من اربعة خيول عربية اصيلة وتم تخفيفهم باستخدام مخفف الانرا-82 مع اضافة كلا من الجليسرول 5 % او الداي ميثيل فورماميد 5% او الاثنان سويا كلا بنسبة 2.5 % ثم اخداعها لعملية التجميد. النتائج اوضحت تفوق الداي ميثيل فورماميد 5% علي الجليسرول 5% وذلك في حركة الحيامن الامامية بعد التجميد وكانت نسبتهم كذلك (56.25% في مقابل46.50% , علي الترتيب), معامل الحيوية المطلق (167.75 في مقابل 135.55 علي الترتيب), معامل البقاء (74.58% في مقابل 61.56%, علي الترتيب) و سلامةقلنسواتللحيامن (78.83% في مقابل 75.95%, علي الترتيب). وعلي ذلك يمكن ان نستخلص من النتائج السابقة انه يمكن تحسين طرق تجميد حيامن الخيول العربية الاصيلة باضافة الداي ميثيل فورماميد كمادة محافظة من التجميد مع مخفف الانرا-82.