

Full Length Research Paper

Effect of cooling on sperm motility before and after frozen-thawed stallion semen

R. A. Ledezma-Torres^{1*}, L. Sandoval¹, P. Valladares¹, M. Mellado², F. J. Picón¹, R. Ramírez¹ and F. Sánchez³

¹Universidad Autónoma de Nuevo León, Facultad de Medicina Veterinaria y Zootecnia, México.

²Universidad Autónoma Agraria Antonio Narro, México.

³Universidad Autónoma de Nuevo León, Facultad de Agronomía, México.

Received 15 February, 2012; Accepted 26 February, 2014

The aim of this study was to assess the effect of cooling on sperm motility before and after frozen-thawed stallion semen. Fifteen ejaculates of three stallions were collected with artificial vagina. The progressive motility was determined under microscope immediately after collection, cooling (5°C for 0, 2, 7 or 24 h) before frozen-thawed and cooling (5°C for 0, 2, 7 or 24 h) after the semen was frozen-thawed. Sperm progressive motility (83.1, 78.7, 74.8 or 70.3%, respectively) was significantly different ($P < 0.05$) at different hours of cooling before freezing. Similar pattern was found when semen was subjected to cooling, frozen-thawed and cooling time resulted in a progressive reduction in motility from 39.4 to 26.9%. The motility of semen subjected only to cooling for 24 h before freezing was optimal (70.0%) for artificial insemination. Moreover, semen subjected to cooling for 7 or 24 h before and after frozen-thawed could be used still with some considerations for artificial insemination.

Key words: Stallion, semen, motility, cooling, frozen-thawed

INTRODUCTION

The best semen quality is obtained when the semen is recently collected by artificial vagina. However, for different reasons semen must be preserved during different period of times (For example, advanced age or disability to breed by a stallion, to export and/or sell semen, to inseminate estrus synchronized mares that a single stallion may not inseminate or to research, etc). Preserved semen quality is certainly a factor that impacts the rates of pregnancies in artificial insemination.

Although, a number of studies have been assayed to improve the motility and fertility of stallion semen after

cooling and/or frozen-thawed, no satisfactory results have been obtained (Martin et al., 1979; Palacios and Zarco, 1996; Rugby et al., 2001; Kavak et al., 2002; Warnke et al., 2003; Sieme et al., 2006; Clulow et al., 2008a, b; Deichsel et al., 2008). For the purpose of obtaining satisfactory results, assayed modifications in centrifugations and semen with or without a cushion fluid (Sieme et al., 2006), added substances after cryopreserved spermatozoa (Gradil and Ball, 2000) and removal of seminal plasma (Ramires et al., 2013a; b) had been studied; the incorporation of antioxidant or another

*Corresponding author. E-mail: ledezma210470@hotmail.com.

substance to the diet of stallions (Brinsko et al., 2005; Deichsel et al., 2008). Graham and Mocé (2005) reported that the motility or another laboratory assay does not correlate well with semen fertility. Moreover efforts to keep thawed semen and employ it to freezing process have been made (McCue et al., 2004; Underwood et al., 2009; Underwood et al., 2010a,b,c). In Laboratories where frozen semen is available, this semen is sometimes thawed before examining the mare's ovaries. If follicles are not mature enough, it is required to cool the thawed semen in order to allow a greater development of the dominant follicle. In this case a deep artificial insemination (AI) is practiced using endoscopy.

The importance of this study is that it can be applied in places of sample collection where there is equipment or technical limitation for freezing semen. Therefore, a good option is to transport cooled semen (5°C) and then freeze it in a site that has the required equipment.

MATERIALS AND METHODS

Animals and location

This study was conducted at the Semen Processing Laboratory from the Veterinary Hospital "La Silla" and the Reproduction Department of the Veterinary Medicine Faculty of the Universidad Autónoma de Nuevo León.

Three jumping stallions were selected based on acceptable (>60%) total motility of spermatozoa before treatment. French Saddle or stallion 1; Oldenburg or stallion 2 and Westfalian or stallion 3 with 11, 11 and 15 years old and weighing 600, 524 and 598 kg, respectively. Horses were fed with a commercial diet with free access to water.

Semen collection

Five ejaculates from each of three stallions ($n = 3$, $r = 5$) were collected in September every 7 days by artificial vagina (Missouri, Har-Vet®), non spermicidal lubricant gel was used (H-R of Carter Products®) along with a plastic sump preheated to 37°C and a disposable paper filter (Har-Vet®). The artificial vagina was filled with water at a temperature of 52-54°C. A dummy was used for the collection, plus an ovariectomized mare previously treated with 10 mg of estradiol cypionate IM every 7 days to induce estrous and stimulate the stallion to facilitate semen collection. Before each collection, the penis was washed with warm water (37°C). Ejaculated volume was measured with a graduated cylinder then a semen drop was placed in a photometer microcuvette holder (Sperma CUE, Minitube®) to measure sperm concentration (million/ml).

Diluents preparation

Diluents Kenney E-Z mix (Animal Reproduction System) were prepared to determine sperm motility. After heat up at 37°C, portion A (water diluents) was mixed with portion B (glucose and nonfat milk solids). To avoid agglutination, semen and diluents were mixed in 1:1 proportion. Progressive motility was evaluated using ten microlitres of sperm suspension. This sample was placed on a warm glass slide and observed using phase contrast with an ocular of 10 and objective of 40 (400X).

Cooling technique

The ejaculate was divided into 4 aliquots with a concentration of 25 to 50 million sperm cells/ml. Each aliquot portion was packed in sterile plastic bags and sealed with heat, which were cooled to a temperature of 5°C by an Equitainer II (Hamilton Research, Inc. ®). Aliquots were removed at 0, 2, 7 or 24 h. Hour 0 stayed for an instant during cooling.

Freezing technique

After the cooling process, each aliquot of semen was evaluated for progressive motility. Then, aliquots were centrifuged at 400 g for 15 min. The supernatant was removed and the sperm cells were mixed with the cryopreservation medium (lactose-EDTA: Lactose solution, Glucose-EDTA, Yolk, Glycerol, Equex STM) prepared at the Semen Processing Laboratory. Straws (0.5 ml) were filled and sealed with metal pellets using a manual micropipettes sealer. A freezing cooler was used to freeze semen (Polyfoam Packers Corp®). Micropipettes were exposed in liquid nitrogen vapor for 15 min, immersed and stored in liquid nitrogen at -196°C. For thawed, straws were randomly taken from thermos and submerged in water bath (Lind berg bluem®), for one minute at 37 °C.

Re-cooling and progressive sperm motility

After freezing, straws were randomly thawed in water at 37°C, for one minute. Progressive sperm motility was evaluated at magnification 400X under a phase contrast microscope immediately after collection. The samples were maintained at 37°C and observed at 0, 5, 10 or 15 min after re-cooling (5°C) for 0, 2, 7 or 24 h after frozen-thawed semen (re-cooling hours : post-freezing minutes 0:0, 0:5, 0:10 and 0:15; 2:0, 2:5, 2:10 and 2:15 until 24 h). In all cases semen was diluted with Kenney extender to evaluate motility.

Progressive sperm motility was evaluated after cooling (5°C) for 0, 2, 7 or 24 h before freezing it and re-cooling (5°C) for 0, 2, 7 or 24 h after frozen-thawed semen.

Statistical analysis

Progressive sperm motility was evaluated after cooling (5°C) for 0, 2, 7 or 24 h before freezing it and re-cooling (5°C) for 0, 2, 7 or 24 h after frozen-thawed semen. Progressive sperm motility data were analyzed using the GENMOD procedure of SAS. The basic model included categorical variables defined above as dependent variables. The independent variable was treatment. The PDIF option was used to detect differences between treatments. The PROC MIXED procedure of SAS was used to compare motility before and after frozen-thawed stallion semen.

RESULTS

Semen volume and mean concentration ($n = 3$, $r = 5$) for stallion 1, stallion 2 and stallion 3 were 17.2, 50.2 and 77.0 ml and 427.8, 263.0 and 208.2 million/ml, respectively. The progressive sperm motility at different cooling time (0, 2, 7 or 24 h) before freezing was different ($P < 0.05$). These results showed a difference of 12.8% between hour 0 and 24 (Table 1).

As for progressive sperm motility undergoing cooling, freezing-thawing and cooling, rates were lower but with

Table 1. Progressive sperm motility (%) after different hours of cooling before freezing ($P < 0.05$).

Stallion n	Cooling Time (h)				
	0	2	7	24	
1	15	82.0	78.4	75.0	70.4
2	15	85.4	81.4	78.4	76.0
3	15	82.0	76.4	71.0	64.6
Mean		83.1 ^a	78.7 ^b	74.8 ^b	70.3 ^c

a similar behavior than those who only were cooled before semen freezing. Mean percentages ($n = 5$, $r = 3$) were (re-cooling hours: post-freezing minutes): 39.4 (0:0) and 37.4 (0:15); 39.3 (2:0) and 35.8 (2:15); 37.1 (7:0) and 33.2 (7:15); 33.7 (24:0) and 26.9 (24:15). Figure 1, shows the trends of progressive sperm motility at different hours of cooling before and after frozen-thawed.

DISCUSSION

This is the first study that stallion spermatozoa were cooled, frozen-thawed and again cooled. There are different reasons in which semen must be kept cool or frozen during different period of times. Preserved semen quality is certainly a factor that impacts the rates of pregnancies in artificial insemination (AI). In Laboratories where frozen semen is available, sometimes this semen is thawed before examining the mare's ovaries. If follicles are not mature enough, it is required to cool the thawed semen in order to allow a greater development of the dominant follicle. In this case a deep AI is practiced using endoscopy or deep AI technique.

In this study, progressive sperm motility was different at different cooling times before freezing (from 83.0 until 70.0% for 0 and 24 h, respectively). Brinsko et al. (2000), mentioned that stallion sperm quality is acceptable (for transport and use) after 24 h of cooling at 4-5°C or for up to 40 h after collection (Aurich, 2008). Moreover the results obtained herein were similar to those observed by Sieme et al. (2004), but 17.0% better than Sieme et al. (2006) who obtained 53.1% of progressive spermatozoa motility when the semen was stored at 5°C for 24 h and 30-40% better when stallion spermatozoa was cooled during 24 or 48 h (Dawson et al., 2000). There are a number of studies to improve the motility and fertility of stallion semen after cooling and/or frozen-thawed, however, no satisfactory results have been obtained (Martin et al., 1979; Palacios and Zarco, 1996; Rugby et al., 2001; Warnke et al., 2003; Sieme et al., 2006; Deichsel et al., 2008; Elhordoy et al., 2008).

To date, there are not studies regarding the use of cooled, frozen-thawed-cooled spermatozoa in domestic animals. However, there is a study in sheep where it was used for first time; the frozen-thawed, sex sorted and refrozen-thawed semen procedure with successful births

of lambs (Graaf et al., 2007). In addition, there are studies where the double frozen-thawed sperm procedure in stallion was used (McCue et al., 2004) and bull for artificial insemination (Saragusty et al., 2009; Underwood et al., 2009; 2010a; 2010b) or in vitro fertilization and production of embryos (Underwood et al., 2010c). Besides, birth of calves has been done after using the IVF and ET procedure with frozen-thawed, sex-sorted and refrozen-thawed sperm (Puglisi et al., 2006).

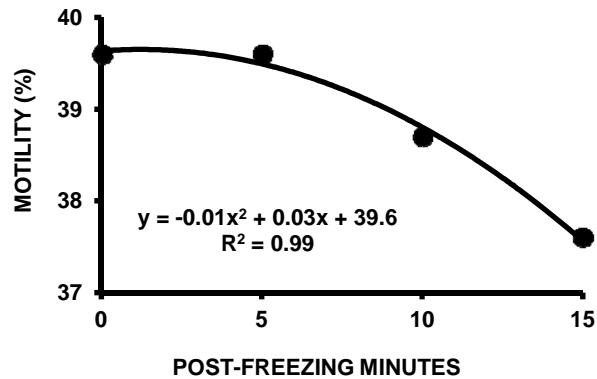
McCue et al. (2004) found that the motility of stallion spermatozoa with refreezing (double freezing) was about $45.7 \pm 10.4\%$, which was similar to that observed in our study. Besides, the same or lower results were found by Underwood et al. (2009) and Saragusty et al. (2009) who determined the in vitro progressive motility of frozen-thawed dairy bull sperm with or without sex-sorting and refreezing and thawing (0, 2 and 4 h post-thaw at 37°C; around 30.0, 10.0 and 1.0%, respectively). Moreover, when sperm of bull was frozen-thawed, sex-sorted and incubated at 15°C during 30 h the motility was around 70.0 until 12.0% (Underwood et al., 2009).

In the present study semen was made to undergo cooling, frozen-thawed and cooling procedure, the result revealed that by increasing the cooling time after being frozen-thawed reduced progressively the motility from 39.4 until 26.9%. These results were better than those reported by Warnke et al. (2003), who found after freezing and thawing stallion semen that the mean motile sperm was between 11.3 and 15.6% or 43.3 to 11.0%, when stallion spermatozoa was cold storage during 24-48 h (Dawson et al., 2000; Clulow et al., 2008b; Deichsel et al., 2008). Kavak et al. (2002) found 43.4% of spermatozoa motility when the semen was frozen-thawed. It is important to mention the large variations in semen quality from stallions and efficiency to withstand the process of freezing and thawing (Graham, 1996). However, if motility or another laboratory assay do not correlate well with semen fertility (Graham and Mocé, 2005), successful pregnancy rates using semen with 30.0% motility have been obtained (Volkman and Van Zyl, 1987).

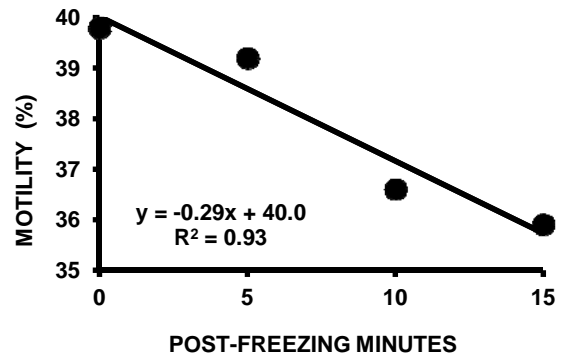
This study can be applied in places of semen collection where there is equipment or technical limitation for freezing semen. Therefore, a good option is to transport cooled semen (5 °C) and then freeze the semen in a site that has the required equipment.

Conclusion

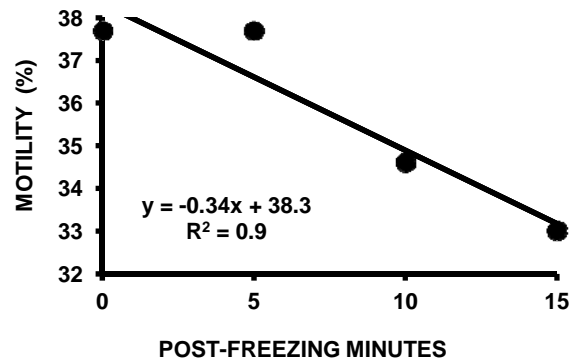
In conclusion, this study shows that stallion semen subjected only to cooling for 24 h before freezing-thawing is optimal for artificial insemination. Moreover, cooled semen before freezing-thawing and subsequent cooling or re-cooling for 7 or 24 h could be considered for AI with some limitations for example by knowing the time of ovulation and deep semen deposition in the uterine horn by deep insemination technique or endoscopy. These results indicate a good option for laboratories with limited



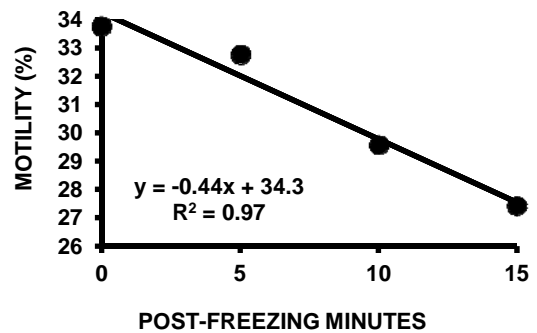
A



B



C



D

Figure 1. Sperm motility (%) in stallions ($n = 3$; $r = 5$) in function of time of cooling after frozen-thawed. Semen cooled for 0 h after thawed (A), Semen cooled for 2 h after thawed (B), Semen cooled for 7 h after thawed (C), Semen cooled for 24 h after thawed (D).

equipment or technical limitation for freezing semen. Therefore, a good option is to transport the cooled semen (5°C) and then freeze it in a laboratory with the required equipment to freeze stallion semen.

ACKNOWLEDGEMENTS

The authors wish to thank the Equestrian Club “La Silla” (Monterrey, N.L.) and to the PROMEP/103.5/04/125, PROMEP/103.5/05/3365 and the Programa Integral de Fortalecimiento Institucional (PIFI) of Mexican Government.

REFERENCES

- Aurich C (2008). Recent advances in cooled-semen technology. *Anim. Reprod. Sci.* 107:268-275.
- Brinsko SP, Crockett EC, Squires EL (2000). Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. *Theriogenol.* 54(1):129-136.
- Brinsko SP, Varner DD, Love CC, Blanchard TL, Day BC, Wilson ME (2005). Effect of feeding a DHA-enriched nutraceutical on the quality of fresh, cooled and frozen stallion semen. *Theriogenol.* 63:1519-1527.
- Clulow JR, Buss H, Sieme H, Rodger JA, Cawdell-Smith AJ, Evans G, Rath D (2008a). Field fertility of sex-sorted and non-sorted frozen-thawed stallion spermatozoa. *Anim. Reprod. Sci.* 108:287-297.
- Clulow JR, Mansfield LJ, Morris LHA, Evans G, Maxwell WMC (2008b). A comparison between freezing methods for the cryopreservation of stallion spermatozoa. *Anim. Reprod. Sci.* 108:297-308.
- Dawson GR, Webb GW, Pruitt JA, Loughin TM, Arns MJ (2000). Effect of different processing techniques on motility and acrosomal integrity of cold-stored stallion spermatozoa. *J. Equine Vet. Sci.* 20(3):191-194.
- Deichsel K, Palm F, Koblichke P, Budik S, Aurich C (2008). Effect of a dietary antioxidant supplementation on semen quality in pony stallions. *Theriogenol.* 69:940-945.
- Elhordoy DM, Cazales N, Costa G, Estévez J (2008). Effect of dietary

- supplementation with DHA on the quality of fresh, cooled and frozen stallion semen. *Anim. Reprod. Sci.* 107:319.
- Graaf SP, Evans G, Maxwell WMC, Cran DG, O'Brien JK (2007). Birth of offspring of pre-determined sex after artificial insemination of frozen-thawed, sex-sorted and re-frozen-thawed ram spermatozoa. *Theriogenol.* 67:391-398.
- Gradil CM, Ball BA (2000). The use of pentoxifylline to improve motility of cryopreserved equine spermatozoa. *Theriogenol.* 54(7):1041-1047.
- Graham JK (1996). Cryopreservation of stallion spermatozoa. *Vet Clin North Am Equine Pract.* 12(1):131-147.
- Graham JK, Mocé E (2005). Fertility evaluation of frozen/thawed semen. *Theriogenol.* 64:492-504.
- Kavak A, Johannisson A, Lundeheim N, Rodriguez-Martinez H, Aidnik M, Einarsson S (2002). Evaluation of cryopreserved stallion semen from Tori and Estonian breeds using CASA and flow cytometry. *Anim. Reprod. Sci.* 76: 205-216.
- Martin JC, Klug E, Gunzel AR (1979). Centrifugation of stallion semen and its storage in large volume straws. *J. Reprod. Fertil. Suppl.* 27:47-51.
- McCue PM, Moore AI, Bruemmer JE (2004). Refreezing stallion spermatozoa for assisted reproduction. *Reprod. Fertil. Dev.* 16(2):176-177.
- Palacios AA, Zarco QL (1996). Efecto de la sustitución de yema de huevo por albúmina sérica bovina, suero equino o suero bovino en el diluyente de congelación sobre la viabilidad posdescongelación del espermatozoide equino. *Vet Méx.* 27(3):221-227.
- Puglisi R, Vanni R, Galli A, Balduzzi D, Parati k, Bongiono G, Crotti G, Duchi R, Galli C, Lazzari G, Aleandri R (2006). In vitro fertilization with frozen-thawed bovin sperm sexed by flow cytometry and validated for accuracy by real-time PCR. *Reproduction.* 132:519-526.
- Ramires Neto C, Monteiro GA, Soares RF, Pedrazzi C, Dell'aqua Jr JA, Papa FO, Alvarenga MA (2013a). Effect of Removing Seminal Plasma Using a Sperm Filter on the Viability of Refrigerated Stallion Semen. *J. Equine Vet. Sci.* 20(1):40-43.
- Ramires Neto C, Monteiro GA, Soares RF, Pedrazzi C, Dell'aqua Jr JA, Papa FO, Castro-Chaves MM, Alvarenga MA (2013b). New seminal plasma removal method for freezing stallion semen. *Theriogenology.* 79(7):1120-1123.
- Rugby SL, Brinsko SP, Cochran M, Blanchard TL, Love CC, Varner DD (2001). Advances in cooled semen technologies: seminal plasma and semen extender. *Anim. Reprod. Sci.*, 3, 68(3-4):171-180.
- Saragusty J, Gacitua H, Zeron Y, Rozenboim I, Arav A (2009). Double freezing of bovine semen. *Anim. Reprod. Sci.* 115:10-17.
- Sieme H, Katila T, Klug E (2004). Effect of semen collection practices on sperm characteristics before and after storage and on fertility of stallions. *Theriogenol.* 61:769-784.
- Sieme H, Knop K, Rath D (2006). Effects of cushioned centrifugation on sperm quality in stallion semen stored cooled at 5 °C for 24 h, and stored cooled for 2 or 24 h and then frozen. *Anim. Reprod. Sci.* 94:99-103.
- Underwood SL, Bathgate R, Maxwell WMC, Evans G (2009). In vitro characteristics of frozen-thawed, sex-sorted bull sperm after refreezing or incubation at 15 or 37°C. *Theriogenology.* 72:1001-1008.
- Underwood SL, Bathgate R, Ebsworth M, Maxwell WMC, Evans G (2010a). Pregnancy loss in heifers after artificial insemination with frozen-thawed, sex-sorted, re-frozen-thawed dairy bull sperm. *Anim. Reprod. Sci.* 118:7-12.
- Underwood SL, Bathgate R, Maxwell WMC, Evans G (2010b). Birth of offspring after artificial insemination of heifers with frozen-thawed, sex-sorted, re-frozen-thawed bull sperm. *Anim. Reprod. Sci.* 118:171-175.
- Underwood SL, Bathgate R, Pereira DC, Castro A (2010c). Embryo production after in vitro fertilization with frozen-thawed, sex-sorted, re-frozen-thawed bull sperm. *Theriogenol.* 73:97-102.
- Volkman DH, Van Zyl D (1987). Fertility of stallion semen frozen in 0.5 ml straws. *J Reprod. Fertil. Suppl.* 35: 143-148.
- Warnke C, Tuchscherer A, Alm H, Kanitz W, Blottner S, Torner H (2003). Characterisation of movement pattern and velocities of stallion spermatozoa depending on donor, season and cryopreservation. *Acta Vet Hung.* 51(3):395-408.