Histol Histopathol (1996) 11: 427-430

Histology and Histopathology

Seasonality and freezability vs routine parameters in stallion semen

H. Rodríguez and E. Bustos-Obregón
Department of Cell Biology and Genetics and Department of Experimental Morphology, Faculty of Medicine, University of Chile, Chile

Summary. The fertilizing ability of stallion semen was analyzed using fresh and frozen samples, obtained before (June-July) or during (October-November) the breeding season. Thirty ejaculates obtained from 4 stallions, were used. The analysis comprises routine seminogram; ATP concentration (Comhaire et al., 1983); subjective and objective motility and sperm velocity (Makler, 1980). Freezing was done following the technique of Martin et al. (1979). Sperm velocity, ATP content and objective motility in ejaculates of subjective motility >50% show values of 14.0±0.84 $\mu m\ s^{-1}$; $4.8\pm2.7\times10^{-7}$M and 54.0±7.4%, respectively. For ejaculates with subjective motility <50%, these values are 8.4±2.4; $0.74\pm0.36\times10^{-7}$M and 27.0±0.8%. No significant changes in these characteristics were elicited by freezing, though ATP content dropped to 50% after thawing. These characteristics are highly associated between them ($p<0.05$) and with some conventional parameters of the routine seminogram such as sperm motility, count, total spermatozoal number and morphology. Additionally, sperm chromatin packing was analyzed by decondensing sperm nuclei using a thiol reducing agent. This parameter was not modified by freezing and it may depend on prolonged epididymal sperm storage during the non-breeding season. Epididymal maturation then results in an excessive disulphur bridging in sperm basic proteins, so that hypermaturation with faultly male pronuclear formation can follow.

Key words: Seasonality, Freezability, Seminal parameters, Stallion sperm

Introduction

Prediction of the individual potential fertility based on the analysis of routine semen parameters in the stallion is difficult, although, as is also the case for other species, fertility is best correlated to progressive motility and (to a lesser extent) to morphology of spermatozoa (Eliasson, 1971; van Huffel et al., 1985).

As a consequence, an increasing number of other seminal tests have evolved, such as determination of sperm velocity, ATP content of sperm and sperm chromatin condensation, among many others (van Huffel et al., 1985; Royere et al., 1988; Farlin et al., 1992).

Sperm damage has been reported after storage of stallion semen (and of other mammals) in liquid nitrogen (O'Reilly et al., 1979; Royere et al., 1988). In this respect, individual conditions of the animals as well as the moment of sexual activity seem to be of relevance. Moreover, since the stallion is a seasonal breeder, the quality of the semen varies within the year depending on the sexual regime of the animal (Bustos-Obregon, 1980) though not so markedly as it is for the ram (Rodriguez et al., 1985).

In sexually resting animals, an increased resistance to chromatin decondensation has been found in sperm stored in vitro. It has been attributed to an excessive formation of S-S bonds between nuclear protamines (Calvin and Bedford, 1971), a fact that may interfere with ulterior normal transcription of the paternal genome if such sperm fertilizes an egg (Beil and Graves, 1977; Huret, 1986).

The frozen stallion semen is scarcely used due to its very irregular outcome in terms of pregnancy rates (Tishner, 1979). ATP seminal content has been reported to bear a good correlation with pregnancy rates using frozen human semen (Comhaire et al., 1983) and animal sperm (Glander, 1984).

Therefore, the present work will explore the relationships between seasonality, freezability and routine seminal parameters in the stallion; in addition, ATP seminal content and chromatin condensation will also be assessed as supplementary parameters.

Materials and methods

Four stallions (of heavy race), 3 to 15 years old, weighing from 400 to 600 Kg, in good health and with records of good fertility, were used to obtain at least 4

Offprint requests to: Prof. Eduardo Bustos Obregón, Departamento de Biología Celular y Genética, Universidad de Chile, P.O. Box 70061, Santiago - 7, Chile

semen samples in June-July (resting period) and October-December (breeding period). Semen was collected using a Hannover type of artificial vagina. A total of 30 samples were studied for both periods.

The analysis performed were:

a) Routine seminogram. After filtering fresh ejaculates, evaluation was done of: volume (ml); spermatic count (per ml and total count per ejaculate); % progressive (subjective) motility; % of vitality (eosin unstained spermatozoa 1 hr after ejaculation); of total and % of head sperm anomalies.

b) Objective sperm motility and sperm velocity using the multiple exposure photographic method (Makler, 1980).

c) Chromatin decondensation test, using Na thioglycolate (Lung, 1972, modified by Bustos-Obregón and Leiva, 1983).

d) ATP content of semen, determined by bioluminiscence (Comhaire et al., 1983).

All tests were done for native semen (1 hr after ejaculation) and after thawing for the same seminal samples that were kept frozen for 30 days, using the freezing method described by Martin et al. (1979).

Seminogram values are expressed as routinely used in veterinary practice. Sperm velocity is expressed in $\mu m\ s^{-1}$, and seminal ATP content as $10^{-7}M$ per ml of semen. Statistical analysis was done as described in each Table in Results. (Student's «t» test and correlation «r», with $p<0.05$).

Table 1. Routine seminogram parameters for fresh and frozen stallion ejaculates during and after the breeding season (X±SD).

	BREADING SEASON (n=14)		NON-BREADING SEASON (n=16)	
	Fresh	Frozen	Fresh	Frozen
Motility (%)	42±25	35±16	44±18	38±15
Vitality (%)	49±16	47±15*	52±11	39±8*
Abnormal (total) (%)	32±18		22±16	
Volume (ml)	115±69	188±91		
Count (10^6 spz/ml)	155±132	63±60		
Total count (10^9 spz/ejac)	11±7	12±6		

*: p<0.05.

Table 3. Correlations coefficients for sperm velocity, ATP concentration and objective motility for fresh and frozen stallion ejacultates during and after the breeding season.

	BREADING SEASON (n=14)		NON-BREADING SEASON (n=16)	
	Fresh	Frozen	Fresh	Frozen
Velocity vs objective motility	r=0.89	r=0.92	r=0.80	r=0.94
Velocity vs ATP	r=0.87	r=0.87	r=0.70	r=0.64
ATP vs objecitve motility	r=0.92	r=0.92	r=0.63	r=0.77

All coefficients are significant (p<0.05).

Results

Table 1 shows the routine seminogram parameters for fresh and frozen stallion semen obtained in June-July (non breeding) and October-November (breeding season). Statistical analysis showed no significant differences for any of the parameters evaluated in fresh and frozen semen from both periods, except for sperm vitality which as noticeably decreased upon thawing for frozen samples obtained in the non-breeding season.

In Table 2, sperm velocity ($\mu m\ s^{-1}$), ATP concentration ($10^{-7}M$) and objective motility (%) are shown for fresh and frozen semen obtained during and after the breeding period. Ejaculates were classified as high (subjective) motility (ie >50%) or low (subjective) motility (<50%), following this parameter as predictor of good or low potential fertility, respectively.

Samples classified as of good fertilizing ability did not show significant differences in velocity, subjective motility nor ATP concentration when comparing fresh vs frozen semen. Within ejaculates of low motility there were no significant differences between the same parameters. Significant differences were found for all

Table 2. Sperm velocity, ATP concentration and objective in fresh and frozen stallion semen during and after the breeding season (X±SD). Ejaculates are classified as high or low motility (*, **).

	BREADING SEASON (n=14)		NON-BREADING SEASON (n=16)	
	Fresh	Frozen	Fresh	Frozen
*High motility**				
Velocity (µm/s) (S)	14.0±0.84	14.5±0.60	16.0±1.77	16.0±1.78
ATP (10^{-7} M) (S)	4.8±2.7	2.13±0.82	5.7±3.9	2.52±1.2
Objective motility (%) (S)	54±7.42	54±8.50	54±8.2	50±7.3
*Low motility***				
Velocity (µm/s) (S)	8.44±2.42	8.9±1.7	8.38±2.49	8.31±2.41
ATP (10^{-7} M) (S)	0.716±0.36	0.419±0.25	1.09±0.57	0.66±0.3
Objective motility (%) (S)	27±8.0	21±5.7	22±7.9	21±5.1

*: subjective motility over 50%; **: subjective motility under 50%; S: significantly different * from ** (p<0.05).

Table 4. Percentage of nuclear decondensation of stallion sperm incubated with sodium thioglycolate at different times and from fresh and frozen samples taken during or after the breeding season (X±SD).

TIME OF INCUBATION	6 min	8 min	10 min	12 min	15 min
Breeeding season (n=14)					
Fresh semen	25±6*	30±7	37±9*	42±10*	46±11*
Frozen semen	23±7*	31±8*	36±10*	38±10*	45±11*
Non-breeding season (n=16)					
Fresh semen	14±6*	23±12	24±11*	16±13*	29±13*
Frozen semen	14±4*	20±5*	26±8*	27±7*	34±9*

*: significantly different (between seasons; p<0.05).

sperm parameters measured when high and low motility ejaculates were compared in both seasons, either fresh or frozen.

Correlation coefficients for sperm velocity, ATP concentration and objective motility in fresh and frozen semen from breeding and non-breeding season are shown in Table 3. The three parameters had high correlation coefficients that were highly significant among all of them.

Analysis of sperm chromatin decondensation is shown for fresh and frozen semen of breeding and non breeding seasons in Table 4, where different times of reduction using alkaline thioglycolate were used. Nuclear decondensation increased with time of incubation in thioglycolate. The degree of packing of the chromatin differed among seasons but is not related to the freezing process.

Discussion

Sperm motility is the characteristic of an ejaculate more readily associated to fertility potential both in vivo and in vitro (Makler, 1980; Aitken et al., 1983). Values observed in this paper for stallion sperm motility do not differ from those reported by Gomes (1977) or Setchell (1977). Both authors agree that sperm motility is also a good indicator of sperm viability.

Farlin et al. (1992) reported a detrimental effect of the process of freezing and thawing on stallion sperm. A differential influence of the freezing process between both periods was not found in this work, except for sperm vitality which was lower for thawed sperm, either in the breeding or in the non-breeding season.

Morphology seems not to be altered. Voss et al. (1981) suggest that there may be seasonal fluctuations of morphology which we cannot document. It should be states that abaxial implantation of the flagellum is a normal trait for stallion sperm, though this is an abnormality in other species (Jones, 1975; van der Host, 1975).

Volume of the ejaculate and sperm concentration and total count found in this work were within values reported by other authors (Stabenfelt and Hughes, 1977), and displayed monthly and individual variations (see Table 1).

A good sperm velocity defines a good quality of sperm movement and displacement (van Huffel et al., 1985). These characteristics imply a morpho-functional integrity of the gamete and an adequate epididymal function. Therefore, sperm velocity is a relevant parameter to evaluate the fertilizing ability of an ejaculate (Makler et al., 1984; Budworth et al., 1987).

Sperm velocity depends on the species and the method used. Values found in the literature are highly variable (Tishner, 1979; Makler et al., 1984; van Huffel et al., 1985). Freezing and thawing did not produce significant variations of sperm velocity in our work, though Tishner (1979) found a decrease in velocity after thawing.

ATP content of semen was found to be highly correlated to fertilizing ability in man (Comhaire et al., 1983).

In the present work ATP content in fresh ejaculates was very different among semen of good quality (defined as >50% motility) and poor quality (motility <50%). Therefore, a good correlation exists in the stallion, at least between ATP content and subjective motility. After thawing, ATP content continued to be lower in the low quality ejaculates. However, ATP content does not vary considerably among seasons and is consistently lower than levels found in human or other species, in which artificial insemination using thawed semen has been successful, whereas it is of very low yield in the equine species (Brooks, 1970; Loomus et al., 1983; Royere et al., 1988).

According to Glades (1984), ATP concentration decreases in stallion semen after criopreservation, since this process apparently elicits activation of acrosomic proteases. The sperm cell damage following crio-preservation seems to be due to cell membrane damage (Blach et al., 1988) and loss of ATP and Mg^{+2}.

Ejaculated spermatozoa in eutherian mammals are characterized by a considerable packing of the chromatin, mostly due to disulphur bridges formed between the nuclear basic proteins (Bedford and Calvin, 1974a,b; Huret, 1986; Perreault et al., 1987).

There are suggestions in the literature that additional disulphur bridges may form with time, as happens with spermatozoa kept longer than usual in the epididymis of non breeding stallions (Bustos-Obregón, 1980). However, this was not the case upon storage of frozen sperm for 30 days. Under this condition, the so-called state of hypermaturation was not found, though it has been documented in seasonally resting stallions as well as in the bull (Beil and Graves, 1977) and in other mammals (Calvin and Bedford, 1971). Apparently, additional disulphur bond formation does not proceed in liquid nitrogen-frozen spermatozoa in the stallion, though Royere et al. (1988), suggest that the process of freezing and thawing may result in an increase of nuclear condensation of human spermatozoa.

Since hypermaturation implies the formation of excessive disulphur bonds, it may affect decondensation of the male pronucleus and thus, eventually interfere with further embryonic development (Perreault et al., 1987). Therefore, this parameter (chromatin condensation) must be evaluated if ejaculates obtained in the non-breeding season are to be used in artificial insemination.

Chan and Tredway (1992), have shown a positive correlation between sperm nuclear condensation and fertilizing ability. They stress the relevance of considering nuclear condensation for the analysis of fertilization.

In conclusion, motility and velocity plus ATP determination complement very appropriately classical semen analysis to evaluate the reproductive state of the stallion.

Acknowledgements. Partially supported by FONDECYT 1930/777 (Chile) and Contract Nº CI1*CT92-0022 (European Economic Community).

References

Aitken R., Ross A. and Lees M. (1983). Analysis of sperm function in Kartagener's Syndrome. Fertil. Steril. 40, 696-698.

Beil R. and Graves C. (1977). Nuclear decondensation of mammalian spermatozoa: changes during maturation and in vitro storage. J. Exp. Zool. 202, 235-240.

Bedford J. and Calvin H. (1974a). Changes in S-S cross-linked structures of the sperm tail during epididymal maturation with comparative observations in submammalian species. J. Exp. Zool. 187, 181-200.

Bedford J. and Calvin H. (1974b). The occurrence and possible functional significance of S-S crosslinks in sperm head with particular reference to eutherian mammals. J. Exp. Zool. 188, 137-156.

Blach E., Amann R., Bowen R., Sawyer H. and Herment M. (1988). Use of a monoclonal antibody to evaluate integrity of the plasma membrane of stallion sperm. Gamete Res. 21, 233-241.

Brooks D. (1970). Acid-soluble phosphorus compounds in mammalian semen. J. Biochem. 118, 851-887.

Budworth P., Amann R. and Hammerstedt R. (1987). A microcomputer photographic method for evaluation of motility and velocity of bull sperm. J. Dairy Sci. 70, 1927-1936.

Bustos-Obregón E. (1980). Cytochemical characterization of sperm nuclei during epididymal maturation in mammals. Arch. Biol. Med. Exp. 13, 335-341.

Bustos-Obregon E. and Leiva S. (1983). Chromatin packing in normal and teratozoospermic human ejaculated spermatozoa. Andrologia 15, 468-478.

Calvin H. and Bedford J. (1971). Formation of disulphide bonds in the nucleus and accesory structures of mammalian spermatozoa during maturation in the epididymis. J. Reprod. Fertil. (Suppl) 13, 65-75.

Chan P. and Tredway D. (1992). Association of human sperm nuclear decondensation and in vitro penetration ability. Andrologia 24, 77-81.

Comhaire F., Vermeulen L., Ghedira K., Mas J., Irvine S. and Callipilitis G. (1983). Adenosine triphosphate in human semen: a quantitative estimate of fertilizing potential. Fertil. Steril. 40, 500-504.

Eliason R. (1971). Standards of seminal analysis. Andrologia 3, 49-64.

Farlin M., Jasko D., Graham J. and Squires E. (1992). Assessment of *Pisum sativum* agglutinin in identifying acrosomal damage in stallion spermatozoa. Mol. Reprod. Dev. 32, 23-27.

Glades H. (1984). Sperm metabolism, sperm viability and cryopreservation. Zbl. Gynekol. 106, 892-899.

Glander H. (1984). Sperm metabolism, sperm vitality and cryopreservation. Zentralbl. Gynakol. 106, 892-899.

Gomes W. (1977). Artificial insemination. In: Reproduction in domestic animals. Cole H. and Cupps P. (eds). Academic Press. New York. p 665.

Huret J. (1986). Nuclear chromatin decondensation of human sperm. A review. Arch. Androl. 16, 97-109.

Jones R. (1975). Fertility and infertility in mammals in relation to sperm structure. In: The biology of the male gamete. Duckett J.C. and Racey P.A. (eds). Academic Press. London. p 393.

Loomus P., Amann R., Squires E. and Pickett B. (1983). Fertility of unfrozen and frozen stallion spermatozoa extended in EDTA-Lactose-Egg yolk and packaged in straws. J. Anim. Sci. 56, 687-692.

Lung B. (1972). Ultrastructure and chromatin disagregation of human sperm head with thioglicolate treatment. J. Cell Biol. 52, 179-186.

Makler A. (1980). Use of the elaborated multiple exposure photography (MEP) method in rutine sperm motility analysis and for research purpose. Fetil. Steril. 33, 160-166.

Makler A., Mac Lusky N., Chodos A., Hoseltine F. and de Cherney A. (1984). Rapid microcomputer based analysis of semen characteristics from photographs taken by the MEP mehod. Arch. Androl. 12, 91-95.

Martin J., Klug E. and Gunzel A. (1979). Centrifugation of stallion semen and its storage in large volume straws. J. Reprod. Fertil. (Suppl). 27, 47-51.

O'Reilly P., McComark O. and Mohorny K. (1979). The effects of collection procedure and freezing on concentration of some electrolytes and G.O.T. on stallion semen. Irish Vet. J. 33, 55-59.

Perreault S., Naish S. and Zirquin B. (1987). The timing of hamster sperm nuclear decondensation and male pronucleus formation is related to sperm nuclear disulfide bond content. Biol. Reprod. 36, 239-244.

Rodríguez H., Ohanian C. and Bustos-Obregón E. (1985). Nuclear chromatin decondensation of spermatozoa in vitro: a method for evaluation the fertilizing ability of ovine semen. Int. J. Androl. 8, 147-158.

Royere D., Hamamah S., Nicolle J., Barthlemy C. and Lansac J. (1988). Freezing and thawing alter chromatin stability of ejaculated human: fluorescence acridine orange staining and feulgen-DNA cytophotometric studies. Gamete Res. 21, 51-57.

Setchell B. (1977). Male reproductive organs and semen. In: Reproduction in domestics animals. Cole H. and Cupp P. (eds). Academic Pres. New York. p 665.

Stanbenfelt G. and Hughes I. (1977). Reproduction in horses. In: Reproduction in domestics animals. Cole H. and Cupp P. (eds). Academic Press. New York. p 665.

Tishner M. (1979). Evaluation of deep-frozen semen in stallions. J. Reprod. Fetil. (Suppl). 27, 53-59.

van der Host W. (1975). A study of the morphology of stallion semen during the breeding and non-breeding seasons. J. Reprod. Fertil. (Suppl). 23, 87-89.

van Huffel X., Varner D., Hinrichs K., Garcia M., Sterzemienski P. and Kenney R. (1985). Photomicrographic evaluation of stallion spermatozoal motility characteristics. Am. J. Vet. Res. 46, 1272-1275.

Voss J., Burwash L., Pickett B. and Squire E. (1981). Stallion spermatozoal morphology and its relationship to fertility. J. Am. Vet. Med. Assoc. 178, 287-289.

Accepted December 4, 1995