Bulletin USAMV-CN, 64/2007 (1-2).

THE INFLUENCE OF DIFFERENT CENTRIFUGATION REGIMES ON DOG SPERMATOZOA

Igna Violeta, Ramona Pană

Facultatea de Medicină Veterinară Timişoara, Calea Aradului Nr.119
E-mail: ignavioleta@gmail.com

Key words: centrifugation, centrifugation regime, semen processing, dog.

Abstract. The present study has the aim of evaluating the centrifugation effects over canine semen. The main objectives are establishment of the four centrifugation regime (650xg/2min., 650xg/5min., 1500xg/2min., 1500xg/5 min.) effects upon spermatozoa by establishing of quantitative and qualitative losses of spermatozoa in centrifugation stage. Centrifugation regime 1500xg for 5 minutes showed the lowest losses of spermatozoa: 4,42%. A short semen centrifugation time (2 min) is correlated to a higher motility percentage. Centrifugation regime 650xg for 2 minutes is the most suitable under the aspect of spermatozoa morphological integrity maintaining. The functional integrity of the spermatozoa membranes is less affected by the short centrifugation time.

INTRODUCTION

Centrifugation immediately after sperm collection is a common method to remove prostatic fluid that is unsuitable for preserving dog semen at 4°C and exerts harmful effects upon the spermatozoa during the freezing process (5). Spermatozoa from mammals showed different sensibility to centrifugation. While the rat, human (3), and mouse (7) spermatozoa have been shown to be very sensitive to mechanical and centrifugal forces, spermatozoa from equine and bovine (2,4) are somewhat less sensitive to centrifugation. This fact indicates that species specificity is very important with respect to spermatozoa injury caused by centrifugation (1). It seem necessary to establish an optimal centrifugation regime for sperm preparation techniques correlated to the species. Moreover, centrifugation increases reactive oxygen species (ROS) formation in semen. High levels of ROS are associated with sperm membrane injury through spontaneous lipid peroxidation, which may alter sperm function (6).

Cryopreservation protocol analysis of dog semen makes evident the presence of numerous variables of every processing stage of the semen. The first unconcordance revealed in the attempt of determining the variation parameters of each work stage, was the stage specifying and succession in a general scheme of semen processing by cryopreservation. In most protocols in semen processing by freezing, centrifugation is included as a first processing step. It was noticed the tendency of it's including as a cryopreservation current step - with the object to remove prostatic fluid, but in a heterogeneous manner, with centrifugation parameters (force/speed and time) very different as values and mode of expression as well (ex. g-force or rpm) and with no conversion possibility, because of the lack of specifying referring to the technical parameters of the equipment used for a certain centrifugation. These aspects create difficulties or the impossibility to compare different research results. The absence of a centrifugation parameters standard in canine species generates a randomly specifying of centrifugation parameters or direct taking-over from other study.

In this way, the present study has the aim of evaluating the centrifugation effects over canine semen. The main objectives are establishment of the centrifugation parameters effect (g force and time) over spermatic cell by establishing of quantitative and qualitative losses of spermatozoa in centrifugation stage.

MATERIALS AND METHODS

Six clinically healthy dogs, aged 2-5 years, were used for semen sample collection.

Semen collection. Semen was collected by digital manipulation of penis in the presence of oestrus bitch. The sperm rich fraction was collected separately, into warmed glass tube.

Semen evaluation. Immediately after collection were assayed: volume, concentration, motility parameters, morphology, and membrane integrity. The concentration of spermatozoa and motility parameters were analyzed by CASA system (IVOS- Hamilton Thorne Biosciences, USA) used *Animal motility* software.

Sperm morphology was evaluated using a light microscope, by counting 200 cells in a smear staining with Spermac. The percentage of total abnormalities for each sample was calculated.

Integrity and functional capacity of spermatozoa membrane were determined trough hypo-osmotic test, by placing 0,1 ml sperm fraction into 1 ml hypo-osmotic medium consist of 75% fructose (150mosmolxl^{-1}) and 25% sodium chloride (150mosmolxl^{-1}) and maintaining to 37°C for 30 min.

Sperm centrifugation. Four centrifugation regime variants were established –Table 1. The 24 sperm samples were centrifuged using Hettich Universal 320 centrifuge.

After centrifugation was removed the supernatant from each sample and was determined it's volume. Next, it was determined the concentration of spermatozoa in supernatant and calculated the losses of sperm cells by supernatant removal. The sediment was diluted and evaluated for the following sperm parameters: motility, morphology and membrane integrity. The same techniques were used as described at sperm evaluation after collection.

Table 1

Regimes of centrifugation and corelation beetwen G force and RPM

Nr. crt.	Regime of centrifugation (G)	Regime of centrifugation (RPM)
1.	650xg / 2 min.	2620 rpm/ 2 min.
2.	650xg / 5 min.	2620 rpm / 5 min.
3.	1500xg / 2 min.	3980 rpm/ 2 min
4.	1500xg / 5 min.	3980 rpm / 5 min

RESULTS AND DISCUSSION

Sperm parameters (concentration, motility, morphology and membrane integrity) of second fraction of the ejaculate, immediately after collection are presented in Table 2.

Table 2

Fresh semen parameters

Nr. crt.	Case	Volume fraction II (ml)	Concentration ($x10^6$/ml)	Number of spermatozoa ($x10^6$)	Total motility (%)	Progresive motility (%)	Morphology (% normal spermatozoa)	Membrane integrity (% swollen sperm)
1	A	1,95	136	265.2	72	53	70	80
2	B	3,1	271,8	842,6	93	66	91	92
3	C	1,3	93,3	121,3	60	36	68	80
4	D	1,3	222,4	289,12	63	37	74	85
5	E	2	454,8	909,6	87	61	84	81
6	F	2,2	293,4	645,5	80	59	77	94
7	$\overline{X}$ ± SD	1,98± 0,67	245,28± 128,44	561,62± 344,68	75,83± 13,17	52,00± 12,71	77,33± 8,76	85,33± 6,25

The quantitative losses after centrifugation (lost sperm by supernatant removal) for the four centrifugation regimes used, are presented in Table 3.

Table 3

Means values of spermatozoa loss by supernatant, dependent upon centrifugation regime (g x force / time)

Case	**Centrifugation force 650 x g**				**Centrifugation force 1500 x g**			
	Lost spermatozoa (%)				**Lost spermatozoa (%)**			
	2 min.		**5 min.**		**2 min.**		**5 min.**	
A	A_1	6,32	A_2	7,25	A_3	7,48	A_4	6,74
B	B_1	14,00	B_2	14,2	B_3	11,3	B_4	8,2
C	C_1	4,58	C_2	2,58	C_3	1,40	C_4	0,62
D	D_1	11,30	D_2	9,20	D_3	2,89	D_4	2,62
E	E_1	2,55	E_2	2,29	E_3	1,67	E_4	1,46
F	F_1	12,14	F_2	9,99	F_3	8,12	F_4	6,87
$\overline{X}$ **± s**	**8,48 ± 4,62**		**7,59 ± 4,59**		**5,48 ± 4,07**		**4,42 ± 3,23**	

The results analysis regarding the effect of different centrifugation regime on the sperm loss, reveal the following aspects:

- The centrifugation force influence the number of spermatozoa loss by supernatant; centrifugation at 1500 x g showed less loss of spermatozoa as compared to centrifugation at 650 x g, in both time variants: 2 min. -fig 1 and 5 min. - fig 2 .

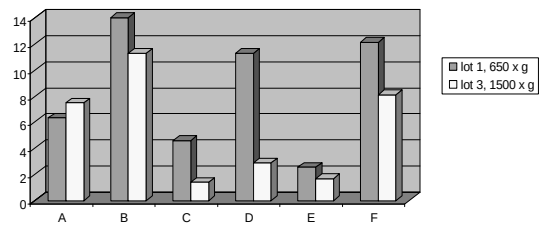

Fig.1. Effect of two centrifugation force (650xg and 1500xg) for 2 minute, on the sperm loss by supernatant

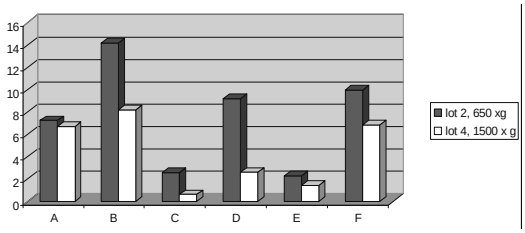

Fig.2. Effect of two centrifugation force (650xg and 1500xg) for 5 minute, on the sperm loss by supernatant

-The time of centrifugation influence, also, the number of sperm cell loss by supernatant; 5 minute to centrifugation showed less numerical loss than 2 minute to centrifugation – fig. 3 and 4.

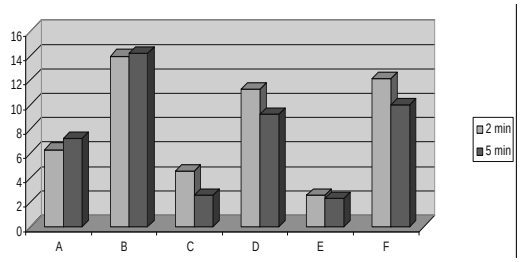

Fig.3. Effect of centrifugation times (2 and 5 min.) to 650xg , on the sperm loss by supernatant

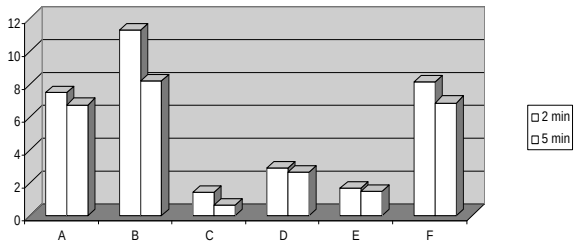

Fig.4. Effect of centrifugation times (2 and 5 min.) to 1500xg, on the sperm loss by supernatant

-Centrifugation regime 1500xg for 5 minutes determined the best recuperative rate of spermatozoa on centrifugation semen.

Total motility values asses by computerized semen analysis after centrifugation, register a decrease of approximately 15%. A short centrifugation time (2 min) is correlated to a higher percent of the total motility –Table 4. There are no significant differences between samples centrifuged with 650g or 1500g, at the same centrifugation time – fig. 5 and 6. We found out just differences between sperm samples from different males, which confirm the implication of individual factor to the stress inducted by various procedures on spermatozoa.

Table 4

Total motility (% of motile spermatozoa) after sperm centrifugation

Case	**Centrifugation force 650 x g**				**Centrifugation force 1500 x g**			
	Total motility (%)				**Total motility (%)**			
	2 min.		**5 min.**		**2 min.**		**5 min.**	
A	A_1	59	A_2	46	A_3	61	A_4	46
B	B_1	90	B_2	89	B_3	87	B_4	84
C	C_1	44	C_2	46	C_3	39	C_4	36
D	D_1	61	D_2	51	D_3	61	D_4	57
E	E_1	68	E_2	65	E_3	72	E_4	71
F	F_1	50	F_2	48	F_3	66	F_4	64
$\overline{X} \pm s$	**62±16,11**		**57,5±17,00**		**64,33±15,74**		**59,66±17,28**	

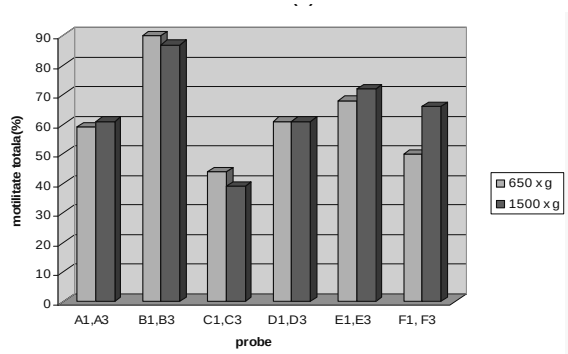

Fig. 5. Motility after centrifugation for 2 min.

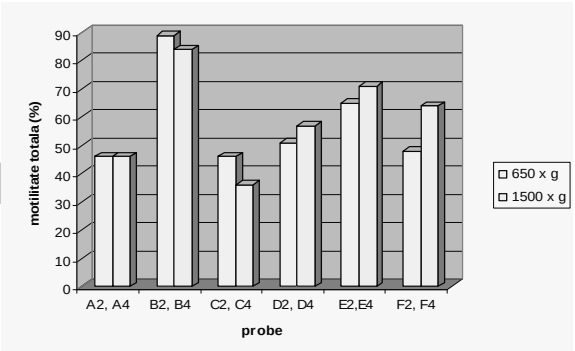

Fig. 6. Motility after centrifugation for 5 min.

The morphological analisys of spermatozoa after centrifugation points out an increase of abnormalities percentage. The main abnormally type detected were: head detached from tail, broken tail and loss acrosome. Using different centrifugation parameters revealed a higher percentage of spermatozoa with a normal morphology in case of 650 g for 2 minute regime:65 % - Table 5. Both variable of centrifugation (force and centrifugation time) have direct

influence over spermatozoa morphological integrity; lower values of time and centrifugation force was associated with minimal sperm damage – fig 7 and 8.

Table 5

Sperm morphology after centrifugation

Case	**Centrifugation force 650 x g**				**Centrifugation force 1500 x g**			
	Morphology (% normal spz)				**Morphology (% normal spz)**			
	2 min.		**5 min.**		**2 min.**		**5 min.**	
A	A_1	60	A_2	57	A_3	52	A_4	50
B	B_1	76	B_2	71	B_3	71	B_4	65
C	C_1	56	C_2	52	C_3	48	C_4	37
D	D_1	66	D_2	56	D_3	56	D_4	49
E	E_1	73	E_2	70	E_3	61	E_4	58
F	F_1	59	F_2	55	F_3	52	F_4	48
$\overline{X} \pm s$	**65±8,09**		**60,16±8,18**		**56,66±8,28**		**51,16±9,53**	

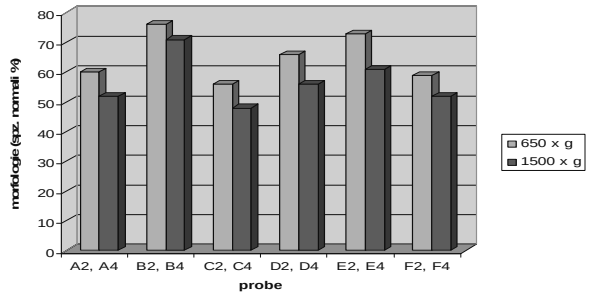

Fig.7.Sperm morphology after centrifugation for 2 min

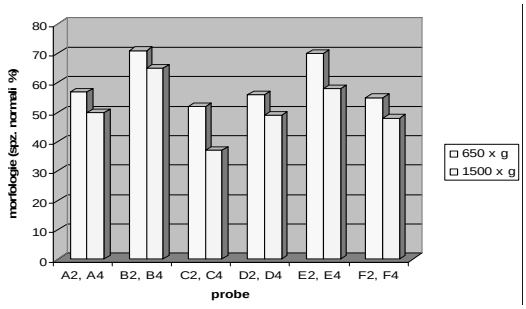

Fig.8.Sperm morphology after centrifugation for 5 min

Functional integrity of spermatozoa membranes is less affected by a short time of centrifugation – table 6. This conclusion is in concordance to the results obtained by Shekarriz and col.(6) after human semen centrifugation at 200 xg and 500 x g for 2 and 10 minute. Their recomandation for a shorter centrifugation period of the semen is based on the observation that the lowest values of ROS were registered in the 2 minute centrifugation case.

Table 6

Membrane integrity (% swollen spermatozoa)

Case	**Centrifugation force 650 x g**				**Centrifugation force 1500 x g**			
	Membrane integrity (% swollen spermatozoa)				Membrane integrity (% swollen spermatozoa)			
	2 min.		**5 min.**		**2 min.**		**5 min.**	
A	A_1	50	A_2	49	A_3	55	A_4	53
B	B_1	60	B_2	65	B_3	67	B_4	61
C	C_1	39	C_2	35	C_3	46	C_4	45
D	D_1	60	D_2	58	D_3	63	D_4	52
E	E_1	51	E_2	52	E_3	68	E_4	60
F	F_1	68	F_2	61	F_3	70	F_4	67
$\overline{X} \pm s$	**54,66±10,15**		**53,33±10,70**		**61,50±9,26**		**56,33±7,84**	

CONCLUSIONS

Centrifugation parameters influence the spermatozoa losses in supernatant. Centrifugation regime 1500xg for 5 minutes showed the lowest losses of spermatozoa: 4,42%. A lower semen centrifugation time (2 min) is correlated to a higher motility percentage. Centrifugation regime 650xg for 2 minutes is the most suitable under the aspect of spermatozoa morphological integrity maintaining. The functional integrity of the spermatozoa membranes is less affected by the short centrifugation time.

The quantitative and qualitative losses registered at the centrifuged semen, correlated to the physiological particularities of canine ejaculation which allow separate collection of the ejaculation fractions, make us question about the inclusion of spermatic fraction centrifugation, as necessary step in dog semen cryopreservation protocols.

BIBLIOGRAPHY

1. CARVAJAL G., C.CUELLO, M. RUIZ, J.M. VAZCUEZ, E.A MARTINES, J. ROCA, 2004, Effects of centrifugation before freezing on boar sperm cryosurviva, J. Androl. 25(3):389-96
2. CROCKETT, E.C., J.K. GRAHAM, J.E.BRUEMER, E.L. SGUIRES, 2001, Effect of cooling on equine spermatozoa before freezing on post-thaw motility: preliminary results, Theriogenology, 55:793–803
3. MAKLER, A., P. JAKOBI, 1981, Effects of shaking and centrifugation on human sperm motility, Arch Androl. 7:21–26
4. PICKETT, B.W., J.J SULIVAN, M.S. BYERS, M.M PACE, E.E. REMMENGA, 1975, Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa, Fertil Steril. 26:167–173
5. RIJSSELARE, T., A. VAN , SOOM , D. MAES, A. DE KRUIF, 2002, Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa, Theriogenology 57(6): 1669-81
6. SHEKARRIZ, M., D.M. DeWIRE, AJ. THOMAS Jr., A. AGARWAL, 1995, A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species, Eur Urol.28 (1):31-35
7. SCHREUDERS, P.D., A.E. JETTON, J.L. BAKER, J.K CRITSER, P. MAZUR, 1996, Mechanical and chill sensitivity of mouse sperm. Cryobiology. 33:676–677.