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Effect of different extenders and seminal plasma on the susceptibility of equine spermatozoa to lipid peroxidation after single-layer centrifugation, through Androcoll-E.

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ABSTRACT

The susceptibility of ejaculated stallion spermatozoa to lipid peroxidation (LPO) was studied in ejaculates (n=4) from each of three stallions before and after a single layer centrifugation (SLC) through Androcoll™-E. Each ejaculate was split, and aliquots extended with one of three different commercial extenders: INRA 96, KENNEY's or Equipro extenders and stored for 24 hours at 5°C (i). From the extended samples, an aliquot was kept as a control and the other was subjected to SLC through Androcoll-E™. The selected spermatozoa were re-suspended in the appropriate extenders, without (ii) or with (iii) addition of 50% (v/v) pooled homologous seminal plasma (SP) for 24 hours at 5°C. Using FeSO₄ as pro-oxidant, the susceptibility for LPO was flow-cytometrically assessed using the probe Bodipy^{581/591}-C₁₁. Sperm motility, monitored with a Qualisperm™ motility analyser, varied among treatments and stallions and was best among the SLC-selected spermatozoa. The

response to induced-LPO was very low and variable (from $1.1\pm 0.87\%$ to $3.8\pm 0.93\%$) among stallions, treatments and concentrations of FeSO_4 . Removal of SP by the SLC procedure did not significantly increase the susceptibility of stallion spermatozoa to induced LPO.

INTRODUCTION

Artificial insemination (AI) using extended, cooled semen is becoming common in equine breeding worldwide since most stud books have removed restrictions to the use of this reproductive technology. There is, however, large variation in pregnancy rates. Some of this variability can be attributed to differences in the ability of the ejaculate from different stallions to survive handling, such as cooling, storage or transport (*Squires et al, 1998*). Research results have suggested that the decrease in quality of equine spermatozoa after storage of extended ejaculates at $+5^\circ\text{C}$ or after freezing and thawing may be due to oxidative damage from an enhanced generation of reactive oxygen species (ROS) by cooling (*Kankofer et al, 2005; Ball BA, 2008; Ortega-Ferrusola et al, 2008*). In spermatozoa, as in other cells, ROS are metabolites produced mainly by mitochondria, in aerobic conditions (*Peña et al, 2009*). Low ROS-levels are necessary to maintain normal cell function (*Agarwal et al, 2003*). *Aitken et al (1994)* demonstrated that low levels of ROS enhanced the ability of human spermatozoa to bind to the zona pellucida, as well as stimulating sperm capacitation, hyperactivation, acrosome reaction and oocyte fusion. However, when there is an excess in the generation of ROS, due to a Fenton (H_2O_2) and a Haber-Weiss (O^{2-}) reactions, sperm quality and function worsen, possibly compromising fertility. In a report in humans, around 25-40% of infertile men had high ROS-levels in semen (*Padron et al, 1997*). Both equine and human spermatozoa are seemingly very vulnerable to oxidative insults due to the high content of

polyunsaturated fatty acids in their plasmalemma (*Macías-García et al, 2010*) and the low concentration of scavenging enzymes in the sperm cytoplasm (*Aitken and Fisher, 1994; Alvarez and Storey 1992; Almeida and Ball, 2005*). When there is an increase of ROS production and the antioxidant system (intra- or extra-cytoplasmatic) is unable to neutralize them, intrinsic pathways similar to apoptosis are triggered. As already mentioned, the seminal plasma (SP) appears to be the major modulator of the effects of ROS. In human semen, SP-proteins could avert deleterious ROS effects during short-term sperm storage at 5°C (*Rossi et al, 2001*). Studies with stallion spermatozoa are somewhat contradictory, probably owing to the routine of removing or decreasing the amount of SP maintained in contact with the processed spermatozoa. A study by *Moore et al (2005)* concluded that the production of ROS not only increases with cooling, but also causes LPO and produces sperm damage, when semen has been cooled or undergone freezing in the absence of SP. These deleterious effects of SP vary largely between stallions, handling procedures, extenders and the relative amounts of SP present (*Rigby et al 2001; Ball 2008; Love et al 2005, Kareskoski and Katila 2008; Kareskoski et al, 2010*). Therefore, further studies are needed, to determine which roles SP and/or extenders could play in preventing the induction of LPO in stallion semen.

Handling of stallion semen has recently included the use of sperm selection from sub-standard ejaculates, in order to reach acceptable pregnancy rates after AI. Among the methods available, the use of SLC (single-layer centrifugation, *Morrell & Rodriguez-Martinez 2009*) through a species-specific colloid has been proven as particularly suitable in laboratory studies. The SLC-selected spermatozoa do not show deterioration in sperm viability or chromatin integrity with storage, unlike their unselected counterparts (*Johannisson et al, 2009; Morrell et al, 2009*). Since SLC allows normal, viable spermatozoa to be separated from dead and dying ones in the ejaculate, it should remove them from these potential sources of ROS. However, as SLC also separates the selected spermatozoa

from the SP, thereby removing them from its potential modulating effects, it would either facilitate damage by ROS or prevent SP-induced damage, depending on which studies are being considered.

This study aimed, therefore, to test the degree of susceptibility shown by ejaculated, extended stallion spermatozoa to LPO, after a single layer centrifugation (SLC) in Androcoll-E. Moreover, we attempted to determine eventual protective effects of three different commercial extenders, supplemented or not with a high proportion of homologous seminal plasma (SP)(50% v/v).

MATERIAL AND METHODS

SEMEN COLLECTION AND PROCESSING

Four ejaculates per stallion were obtained from three stallions. One of them (number 1) was housed at the Division of Reproduction, SLU, Uppsala and the other two stallions (number 2 and 3) were individually housed at Gurresta Sire and Semen Station, Knivsta, Sweden. The stallions were housed and managed according to institutional and European regulations, including ethical permission from the local committee for experimentation with animals, Uppsala, regarding stallion number 1. Semen was collected twice weekly from each stallion during September-October, i.e. outside the normal breeding season in Sweden. Ejaculates were collected using an artificial vagina (Colorado model, Minitüb, Germany), lubricated and pre-warmed at 45-50°C. The gel portion was separated with an in-line filter. Immediately after collection, semen was transported to the laboratory for evaluation. Sperm concentration was determined with a Sperma-Cue (Minitüb, Germany). The ejaculate was split and aliquots extended with one of three different commercial extenders: 1) INRA 96 (IMV, L'Aigle, France), 2) KENNEY's extender, made at SLU (*Kenney et al, 1975*), 3) EquiPro (Minitüb, Tiefenbach,

Germany). From the extended samples, three aliquots of 1 ml each were subjected to different treatment: 1-Control; 2-Centrifuged by SLC through Androcoll-E™; 3-Centrifuged by SLC through Androcoll-E™ and adding 50% seminal plasma.

After centrifugation through Androcoll-E™, the pellets (the selected spermatozoa) were re-suspended in the appropriate extenders, with and without addition of 50% (v/v) pooled homologous SP. All samples were kept 24 hours at 5°C. The final sperm concentration of every sample was evaluated with a Nucleocounter SP-100 (ChemoMetec Technology, Allerød, Denmark), before and after centrifugation (*Morrell et al, 2010*), and adjusted by adding the appropriate semen extender so that every aliquot was at the same sperm concentration (50×10^6 /mL spermatozoa). After 24h of storage, all samples were incubated during 30 minutes with ferrous sulphate (FeSO₄) as pro-oxidant, in different concentrations (0-10-30-50-80 μM FeSO₄).

SINGLE-LAYER COLLOID CENTRIFUGATION (SLC)

The colloid for SLC consisted of glycidoxypopyl-trimethoxysilane-coated silica particles, in a species-specific, buffered salt solution (Androcoll-E™; SLU, Sweden). The colloid was prepared in a single batch and stored at 4°C. Briefly, 4mL of Androcoll-E™ (patent applied for) were pipetted into a centrifuge tube and an aliquot (1.5mL) of extended semen containing up to 100×10^6 spermatozoa/mL was pipetted on top. After centrifugation at $300 \times g$ for 20min, the supernatant and most of the colloid was discarded and the sperm pellet was transferred to a clean centrifuge tube containing 1mL of the same extender (INRA, Kenney or Equipro) (*Morrell et al., 2009*).

INRA			KENNY			EQUIPRO		
Control	SLC	SLC+50%SP	Control	SLC	SLC+50%SP	Control	SLC	SLC+50%SP

SPERM MOTILITY

Sperm motility was monitored using a QualiSperm™ motility analyser (Biophos, Switzerland) (Morrell *et al*, 2009). A sample of each aliquot extended in INRA 96 (a-control, b- after SLC) was evaluated after adding ferrous sulphate (FeSO₄) at one of 5 different concentrations (0, 10, 30, 50, 80 μM). Sperm motility was analyzed 10 and 60 minutes after incubation at 37°C to simulate physiological conditions.

EVALUATION OF LPO

The degree of LPO was measured using the fluorescent probe BODIPY^{581/591}-C11 (Molecular Probes, Leiden The Netherlands) and a LSR flow cytometer (Becton Dickinson, San José, CA, USA), equipped with standard optics. Following 30 minutes of incubation with the pro-oxidant (0-10-30-50-80 μM FeSO₄), an aliquot of approx 5x10⁶/ml spermatozoa was loaded with the probe at a final concentration of 2 μM. Spermatozoa were then incubated further at 37°C each for an additional 30 minutes, and then washed by centrifugation to remove the unbound probe. The samples were then analyzed with FC, and the forward scatter (FSC), side scatter (SSC), green fluorescence (FL1) and red fluorescence (FL3) were collected from each cell. The percentage of cells showing increased green fluorescence (percentage of peroxidation) was quantified.

STATISTICAL ANALYSIS

The data were first examined using the Kolmogorov-Smirnov test to determine their distribution. In view of the non-Gaussian distribution of most of the data gathered, multivariate analysis of variance was performed, and, when significant differences were found, the nonparametric Mann-Whitney U-test was used to directly compare pairs of values. All analyses were performed using SPSS ver.15.0 for Windows (SPSS Inc., Chicago IL). Significance was set at $P \leq 0.05$.

RESULTS

MOTILITY OF SLC-SELECTED SPERMATOZOA

SLC-selected spermatozoa showed higher progressive and total motility, especially in stallions which presented poor sperm motility i.e. stallion 1 (Table 1 and 2). Significant differences were found among stallions ($p < 0.001$). However, there were no differences (ns) between either the different concentrations of FeSO_4 or treatment (SLC vs control) in stallions with good motion parameters at any time considered. Only in stallion 1 (which presented the worst result in TM and PM) was the effect of SLC statistically significant ($p < 0.001$). Stallions 2 and 3 (which showed better results in these parameters) maintained similar results at various times and treatments (Tables 1 and 2). The increase of induced LPO by different concentration of FeSO_4 did not decrease the motility parameters either before or after SLC.

LIPID PEROXIDATION AND SLC

The basal levels of LPO of the sperm membrane were low in all control samples before and after centrifugation through Androcoll-E (Table 3). The SLC-selected spermatozoa (SLC, table 4) showed a tendency for a lower susceptibility to suffer LPO, independently of the extender used, although these results were not significant.

Table 1: Changes in total motility (TM) after 24h of storage at 5°C, before and after SLC, and incubated with 4 different concentrations of Ferrous sulphate (10, 30, 50, and 80 mM). Measurements done at time 0 (T0) and after 60 minutes (T60) of incubation at 37°C.

TOTAL MOTILITY	STALLION 1*		STALLION 2*c		STALLION 3*d	
	T0	T60	T0	T60	T0	T60
INRA	41.9 ± 11.09a	46.3±16.68a	55.8±14.89	44.7±13.55	82.4±2.19	80.3±5.88
I+10 mM FeSO ₄	49.8±14.3a	39.7±7.87a	56.5±25.66	55.3±24.95	79.3±4.88	65.6±11.82
I+30 mM FeSO ₄	44.7±3.97a	52.8±8.28a	55.5±9.92	50.0±13.78	72.8±10.63	66.9±13.28
I+50 mM FeSO ₄	53.2±7.4a	47.6±15.16a	44.2±17.64	56.6±20.16	78.0±5.01	75.0±13.55
I+80 mM FeSO ₄	50.4±7.72a	33.1±7.69a	38.1±13.16	47.8±9.64	77.8±4.41	67.9±11.36
I+COLLOID	83.2±11.62b	82.9±7.96b	67.1±10.11	54.2±3.21	85.1±4.51	80.2±3.32
I+C+10 mM FeSO ₄	82.7±5.5b	67.5±7.99b	51.7±17.58	44.1±12.03	90.5±2.81	83.9±3.82
I+C+30 mM FeSO ₄	73.1±7.01b	77.4±7.89b	52.9±7.49	59.6±13.75	86.5±5.66	81.9±3.88
I+C+50 mM FeSO ₄	70.5±13.04b	73.2±13.82b	50.6±25.8	51.5±10.54	82.7±8.45	81.2±9.81
I+C+80 mM FeSO ₄	76.1±5.83b	74.3±6.44b	54.0±20.76	39.6±4.69	87.3±8.74	77.0±13.52

- *Stallions 1, 2 and 3 presented statistically different variables among them p<0.001

- For stallion 1, there were significant differences observed between centrifugation in SLC and within SLC treatment a,b<0.001

- ^c: For stallion 2, there were no significant differences observed between SLC treatment and time (0 and 60 minute incubation).

- ^d: For stallion 3, there were no significant differences observed between SLC treatment and time.

Table 2: Changes in progressive motility after 24h of storage at 5°C, before and after SLC, and incubated with 4 different concentrations of Ferrous sulphate (10, 30, 50, and 80 mM). Measurements done at time 0 (T0) and after 60 minutes (T60) of incubation at 37°C

VELOCITY	STALLION 1*		STALLION 2*c		STALLION 3*d	
	T0	T60	T0	T60	T0	T60
INRA	8.9±2.39	9.9±3.25	14.9±7.59	16.2±7.26	39.1±11.44	30.1±16.84
I+10 mM FeSO ₄	11.8±3.10	10.8±4.10	16.2±8.09	14.9±8.24	36.5±17.33	27.0±7.23
I+30 mM FeSO ₄	8.9±4.12	7.6±1.77	16.3±14.35	16.2±9.07	29.5±18.24	26.2±6.30
I+50 mM FeSO ₄	10.0±3.84	7.4±1.80	11.7±6.85	13.9±6.92	27.1±2.37	24.5±5.30
I+80 mM FeSO ₄	11.2±5.91	7.6±3.08	14.0±8.71	14.0±5.98	31.2±5.76	20.5±10.15
I+COLLOID	38.1±5.16	28.1±2.76	22.2±5.78	14.7±5.69	26.9±9.60	20.0±10.83
I+C+10 mM FeSO ₄	42.3±7.53	29.6±6.72	25.3±10.62	14.7±6.57	22.4±9.5	20.2±11.15
I+C+30 mM FeSO ₄	35.5±4.06	31.3±9.90	20.3±9.57	11.5±4.07	24.6±11.08	19.13±11.31
I+C+50 mM FeSO ₄	34.4±6.45	24.4±6.64	20.0±9.57	9.9±1.99	25.5±12.72	17.9±8.09
I+C+80 mM FeSO ₄	39.6±8.63	75.8±10.77	18.6±12.09	14.1±6.10	30.2±7.78	17.6±11.65

- *Stallions 1, 2 and 3 had significantly different variables, p<0.001

- In the stallion 1 significant differences were observed between centrifugation in SLC and within SLC treatment a,b<0.001

- ^c: In stallion 2 any significant difference was observed between treatment in SLC and time (0 and 60 minute to incubated) in the same stallion.

- ^d: In stallion 3 any significant difference was observed between treatment in SLC and time in the same stallion

Table 3: Basal levels of LPO (%) before and after SLC in three commercial extenders without stimulation with ferrous sulphate.
(P < 0,05)

INRA					
Stallion 1		Stallion 2		Stallion 3	
Non-SLC	SLC	Non-SLC	SLC	Non-SLC	SLC
0.2±0.17	0.7±1.30	0.3±0.3	0.1±0.03	0.5±0.83	0.0±0.02

Equipro					
Stallion 1		Stallion 2		Stallion 3	
Non-SLC	SLC	Non-SLC	SLC	Non-SLC	SLC
0.1±0.06	0.3±0.23	0.2±0.11	0.2±0.08	0.1±0.13	0.1±0.05

Kenney					
Stallion 1		Stallion 2		Stallion 3	
Non-SLC	SLC	Non-SLC	SLC	Non-SLC	SLC
0.3±0.40	0.1±0.05	0.1±0.02	0.0±0.02	0.3±0.40	0.0±0.02

Table 4: Changes in the susceptibility of the plasma membrane to LPO in three different stallions with three different treatment and commercial extenders

LIPID PEROXIDATION	STALLION 1			STALLION 2			STALLION 3		
	Control	SLC	SLC+50%SP	Control	SLC	SLC+50%SP	Control	SLC	SLC+50%SP
INRA Control	0.2±0.17	0.7±1.30	0.0±0.05	0.3±0.3	0.1±0.03	0.0±0.03	0.5±0.83	0.0±0.02	0.0±0.0
I+80mM FeSO ₄	81.6±30.37	76.2±12.24	92.4±4.22	49.2±17.94	32.9±18.6	45.3±8.78	57.7±16.64	15.4±2.00	75.8±21.93
Kenny Control	0.3±0.40	0.1±0.05	0.1±0.09	0.1±0.02	0.2±0.03	0.1±0.03	0.3±0.40	0.0±0.02	0.0±0.03
80 mM FeSO ₄	87.2±6.81	81.5±8.25	93.7±2.52	53.7±22.02	23.7±6.37	33.2±6.13	60.9±22.7	81.6±25.04	92.4±6.41
EquiPro Control	0.1±0.06	0.3±0.23	0.1±0.07	0.2±0.11	0.2±0.08	0.1±0.052	0.1±0.13	0.1±0.05	0.1±0.03
80 mM FeSO ₄	81.2±16.98	72.7±21.66	91.7±6.56	55.7±4.86	22.3±11.01	37.0±15.55	73.7±17.15	46.8±14.78	91.0±5.79

EFFECT OF THE EXTENDER AND THE SEMINAL PLASMA ON LPO. RELATION WITH MOTILITY.

There were not statistically significant differences in LPO between the stallions 2 and 3 ($p>0.05$). LPO levels were not significantly affected by either extender type or by the addition of 50% (v/v) of pooled homologous SP after stimulating with ferrous sulphate (FeSO_4) (Figure 1). Apparently, the percentage of LPO of sperm membrane was not directly related with impaired equine sperm motility (Figure 2).

DISCUSSION

There is documented stallion-to-stallion variation regarding fertility when using extended, cooled semen for AI. Overall, storage of semen at 5°C is associated with a decrease in motility and fertility rate over time that may be due to oxidative stress (*Kankofer et al, 2005; Ball BA, 2008; Ortega-Ferrusola et al, 2008*). In mammalian spermatozoa, as in other cells, ROS are metabolites produced mainly by mitochondria, in aerobic conditions (*Peña et al, 2009*).

The plasma membrane of the stallion spermatozoon has a high content in polyunsaturated fatty acids (*Aurich C et al, 2005, Macías García et al, 2010*), which makes them vulnerable to oxidative stress. Semen processing, such as centrifugation, extension, cooling or storage has documented effects on the sperm membrane. Several reports have suggested that the centrifugation used to remove the SP appears to be deleterious *per se* (mechanically) to the sperm plasma membrane, and could induce ROS, that finally cause the LPO of the sperm membrane (*Parinaud et al, 1997*). *Aitken and Clarkon (1988)* demonstrated that centrifugal pelleting of unselected human sperm populations caused the production of ROS within the pellet, that induced irreversible damage to spermatozoa and the impairment of their *in vitro* fertilizing ability. Researchers have

Figure 1: LPO of sperm membrane (%) using BODIPY-C11 and the protective effect against LPO of sperm membrane by means of three different extenders (INRA, EQUIPRO and KENNEY) and three different treatment, using FeSO₄ as pro-oxidant.

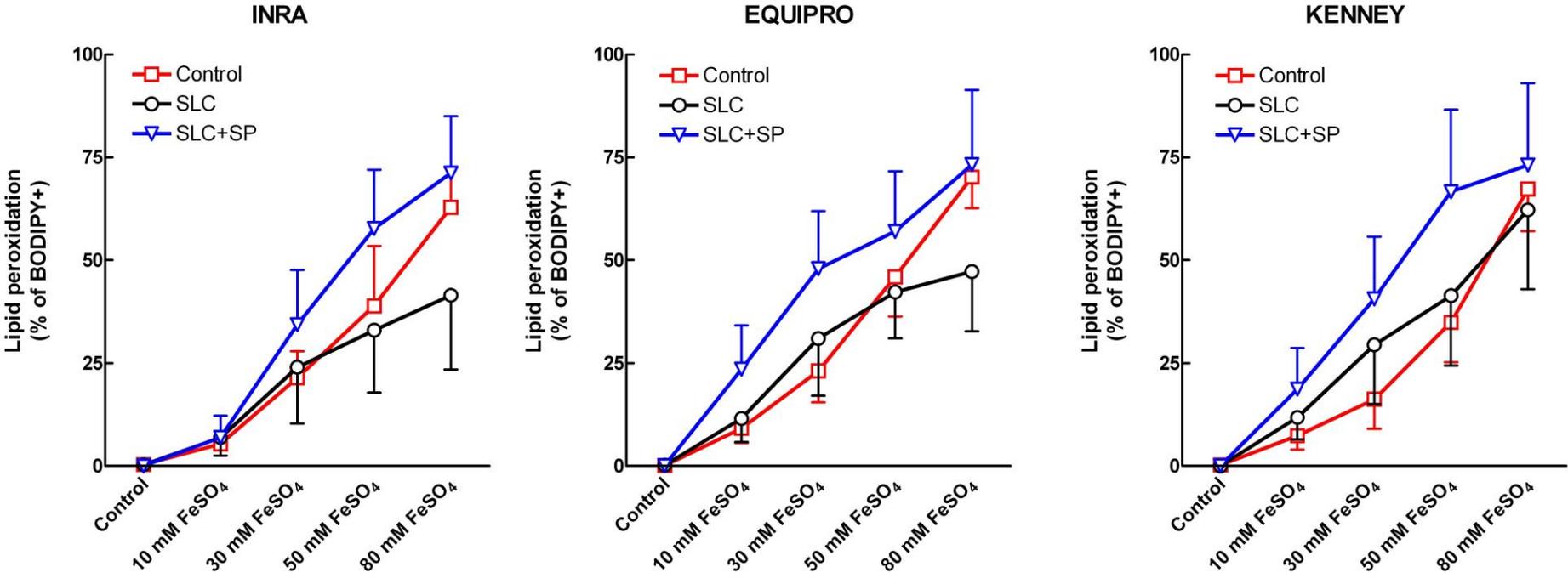
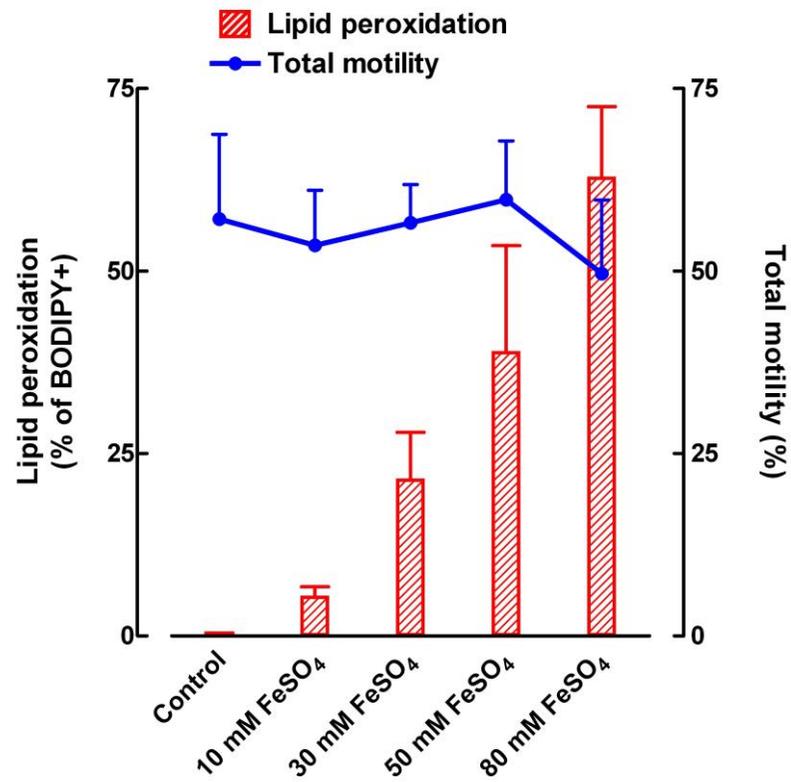


Figure 2: Correlation among total motility (%) and LPO (%) of equine sperm after stimulate with different concentration of FeSO_4 like pro-oxidant. The increase of induced LPO by FeSO_4 did not decrease the motility parameters either before or after SLC.



concluded that the time of centrifugation is more critical than the g-force for inducing sperm damage (*Shekarriz et al 1995; Carvajal et al, 2004*). Consequently, in the present study, we wanted to investigate if the centrifugation during 20 minutes at 300 g in Androcoll-E™ would increase ROS production and furthermore, injure the plasma membrane.

We decided to monitor the peroxidative damage through BODIPY^{581/591}-C11. This probe is incorporated into cells, producing a green fluorescence when it is excited at 488 nm if there is peroxidative damage caused by ROS or red fluorescence if there is no peroxidative damage. In recently research in human sperm (*Aitken et al, 2007*) it was demonstrated that the levels of lipid peroxidation detected with this probe were significantly correlated with the ROS production by the spermatozoa ($P < 0,001$). In this study, the basal level of LPO was quite low in all samples, confirming previous findings from our laboratory (*Ortega-Ferrusola et al 2009*). In the latter report, we observed that the equine sperm membrane is very resistant to LPO even after freezing and thawing. We suggested that the LPO appears to be overestimated as a major factor involved in cryodamage of stallion spermatozoa. In the present study, there was basically no effect, either before or after SLC-centrifugation, indicating that SLC in equine-specific Androcoll- E™ does not cause LPO of the plasma membrane of stallion spermatozoa.

The high levels of lipid peroxidation after stimulation with different concentration of ferrous sulphate were not associated with a significant impairment of sperm motility, in contrast to other research (*Jones et al, 1979; Alvarez and Storey 1989; Aitken et al, 1993*). SLC-selected spermatozoa improved progressive and total motility in the stallion 1, which presented poor sperm motility (Table 1); and kept the same motion parameters in stallion with good motility initially. These findings were similar to previous results in our laboratory (*Macías-García et al, 2008*). There were no differences (ns) between the different concentrations of ferrous sulphate (FeSO_4). The reasons for these differences to other studies, might be

related to different experimental conditions, intensities of the oxidative insult and stallion variability in the susceptibility to LPO

Our second aim was to check the protective effect of SP and extenders against peroxidative damage, after processing in Androcoll-E™. The use of this colloid removes all SP. The presence of SP in cooled extended stallion semen is very controversial. Seminal plasma has a powerful antioxidant system, like superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase activity, which prevent the potential toxic effect of ROS (Rossi *et al*, 2001, Ortega-Ferrusola *et al*, 2009). Nevertheless under *in vivo* conditions, spermatozoa are in contact with SP only during a brief time. Hence, it is suggested that the main functions of SP are the transport and the capacitation of spermatozoa within the female genital tract (Calvete *et al*, 1994). Research has demonstrated that the presence of SP for long time, in cooled storage or previous to freezing can be harmful to sperm quality (Love *et al*, 2005, Moore *et al*, 2005). On the other hand, Jasko *et al* published in 1992, that the total removal of SP after centrifugation decreased the motility of stored semen, thus suggesting that low concentrations of SP are necessary to preserve sperm motion. In our study, we wanted to check if the addition of high percentage of pooled homologous SP would be able to prevent such a possible injury of oxidative stress. Restoring SP to 50% (v/v) of the initial contented, did not significantly affect the levels of LPO, not even after ferrous sulphate (FeSO₄) induction, as a pro-oxidant. Only a slight, although not significant, increase of LPO of the sperm membrane could be observed. A report in rabbits (Alvarez *et al*, 1982) noticed that high sodium or potassium media could induce spontaneous LPO of the membrane, and even lead to chromatin fragmentation. Stallion SP has a high sodium concentration compared to other species. Therefore, sperm exposure to these high concentrations in SP during storage could initiate LPO, at least theoretically. As well, it was demonstrated that the centrifugation in this species-specific colloid formulation Androcoll- E™, selected a

subpopulation of stallion spermatozoa depicting best morphology, motility and integrity of DNA and prolonging their viability (*Johannisson et al, 2009*), and remove abnormal spermatozoa with excess residual cytoplasm (i.e. immature) from the sperm suspension, thus decreasing the sources of ROS (*Morrell JM et al 2009*). This could support the above hypothesis, because the results after simple SLC were better than SLC+50% of SP. In addition, *Rigby et al* demonstrated in 2001, that the pregnancy rate in the absence of SP was very similar to extended semen with SP. There is evidence that the SP of different fractions exert different modulatory effects on spermatozoa (*Rodriguez-Martinez et al 2009*) but it would be necessary to know which are the relevant components of SP and what are their functions in order to confirm this hypothesis.

In relation to the effect of extenders, *Kankofer et al (2005)*, suggested that the addition of extender to semen increases the antioxidative activity of stallion SP and that this combination could improve its protective action against ROS. We did not find significant differences among extenders in relation to any protective effect against LPO, nor any interaction between seminal plasma and extenders. These findings with these commercial extender are opposite to a previous study (*Rigby et al 2001*) using other extenders, where interactions between them (*Kenney and KMT*) and SP were observed.

CONCLUSION:

The levels of LPO are quite low in equine spermatozoa during semen storage. Removal of SP by the SLC does not significantly increase the susceptibility of stallion spermatozoa to induced LPO. Further studies are needed to unravel the mechanisms by which SP and/or extender prevent the induction of LPO in equine semen.

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