



Lyophilized seminal plasma can improve stallion semen freezability

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Received: 23 August 2019; Accepted: 20 September 2019

ABSTRACT

The aim of this study was to evaluate the effect of lyophilized seminal plasma (LSP) on stallion semen freezability. Seminal plasma from 30 stallions was lyophilized to obtain a pool of LSP. Fifteen ejaculates from five stallions were supplemented before freezing with 0 mg/mL (Control), 1.44 mg/mL (LSP1), 5.04 mg/mL (LSP2) or 8.68 mg/mL (LSP3) of LSP. Total antioxidant capacity (TAC) of LSP was assessed using Oxygen Radical Absorbance Capacity (ORAC) assay. Post-thaw motility and kinetics, sperm viability, normal morphology and membrane integrity were evaluated. Completely randomized mixed models were fitted for data analyses. The results were analyzed based on freezability of semen samples. TAC for LSP pool was 13679.4 ± 911.6 $\mu\text{mol Trolox 100/g}$ (ORAC units). Semen supplementation with LSP1 and LSP2 showed a positive effect on post-thaw total motility and membrane integrity. Supplementation with LSP3 showed a decrease in post-thaw total and progressive motility, straight line velocity and sperm viability. For poor freezability semen samples, supplementation with LSP1 and LSP2, showed higher post-thaw total motility and membrane integrity than good freezability semen samples. In conclusion, supplementation with LSP can improve the post-thaw seminal quality of stallion semen with poor freezability.

Key words: Equine, Oxidative stress, Semen freezing, Seminal plasma, Sperm

Reactive oxygen species (ROS) are produced by all metabolizing cells; however, stallion spermatozoa have a high ROS production compared with other species (Johannisson *et al.* 2018). Additionally, there is a differential production of ROS between spermatozoa from fresh and frozen-thawed semen that could be of interest to implement better strategies to enhance the fertilizing potential of raw and / or cryopreserved stallion sperm (Treulen *et al.* 2019). The most important form of defense against ROS available to spermatozoa is the antioxidants present in the seminal plasma (SP) (Fazeli and Salimi 2016). SP contains many chemical components with antioxidant properties, enzymatic and non-enzymatic (Bromfield 2014), which together determine its total antioxidant capacity (TAC) (Mehrotra *et al.* 2013). For this reason, removal of SP before freezing causes loss of antioxidants present in semen, exposing spermatozoa to excessive ROS damage (Oliveira *et al.* 2014). Adding SP has been found to minimize or repair the cryoinjuries on sperm (Al-Essawe *et al.* 2018), which has been related to the contribution of the seminal plasma to the TAC of the cryopreserved semen (Barranco *et al.* 2015, Restrepo *et al.* 2015). The aim of this study was to evaluate the effect of lyophilized seminal plasma (LSP)

supplementation and relationship of its antioxidant effect on stallion semen freezability.

MATERIALS AND METHODS

Thirty five Colombian Creole horses (*Equus caballus*) located in Antioquia (Colombia) were used. The age of the animals ranged from 5 to 8 years and their fertility was confirmed by their living offspring. The body condition score of the horses ranged from 6 to 7 with scoring scale of 1–9, and they were subjected to the similar animal husbandry and feeding conditions. Three semen ejaculates each from five stallions were collected at 7 days interval to obtain a total of 15 ejaculates. A Missouri model of artificial vagina (Minitube, Germany) lubricated with non-spermicidal gel and a mare were used for collection of semen. The gel fraction of the ejaculate was separated by filtration. Spermatozoa concentration was assessed using a photometer (Spermacue[®], Minitube, Germany). Semen was diluted 1:1 with EquiPlus[®] (Minitube, Germany) and was transported at 5°C in an Equitainer[®] (Minitube, Germany).

The semen from other 30 stallions was collected to obtain a pool of LSP. Thirty gel-free ejaculates were centrifuged for 15 minutes at $800 \times g$. Then, 20 ml of seminal plasma (supernatant) from each sample were centrifuged for 10 minutes at $3,400 \times g$ and stored at -20°C for a minimum of 24 hours before freeze-drying. Freeze-drying of seminal plasma was carried out using a modified protocol described by Gianaroli *et al.* (2012). Samples were put inside a Freeze Dry System (Labconco, Freezone, USA) and exposed to a

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30 h lyophilization cycle with a condenser temperature of -50°C and vacuum of 25×10^{-3} mbar. Lyophilized samples were mixed and stored at room temperature.

TAC of LSP pool was evaluated by the ORAC assay, as reported by Ou *et al.* (2001). Fluorescein and AAPH (2,2'-azobis 2-amidino-propane) solutions were prepared in PBS at 1×10^{-5} M (75 mM—pH 7.4) and 0.6 M (75 mM, pH 7.4), respectively. Each reaction tube was prepared with 21 μL of fluorescein, 2.899 μL of PBS, 50 μL of AAPH (Sigma-Aldrich, USA) and 30 μg of LSP. Controlled conditions of temperature at 37°C and pH 7.4 were used; additionally Trolox[®] (Merck, German) was used as a reference antioxidant. Counts were performed by spectrofluorimetry (LS 55 spectrofluorometer, Perkin Elmer, USA). An ORAC unit was defined as 1 μmol Trolox 100/g of LSP.

Each of the 15 ejaculates was centrifuged for 15 minutes at $800 \times g$, and the supernatant was discarded. The precipitate was extended to a final concentration of 100×10^6 sperm/mL, in EquiPlus[®] (Minitube, Germany), supplemented with 5% of egg yolk and 5% of dimethylformamide (Sigma-Aldrich, USA). Each extended semen sample was divided into four aliquots, three of which were supplemented with LSP, 1.44 mg/mL (LSP1), 5.04 mg/mL (LSP2) and 8.68 mg/mL (LSP3), respectively, keeping one aliquot as control with no supplementation of LSP. Subsequently, semen samples were maintained in refrigeration at 5°C for 30 minutes and then packed in straws of 0.5 mL (V2 Dual MRS1, IMV Technologies, France). A controlled curve (Crysalis Cryo controller PTC-9500, Biogenics Inc., USA) was used to provide a cooling rate of $-8^{\circ}\text{C}/\text{min}$ between 5°C and -6°C , followed by a cooling rate of $-6^{\circ}\text{C}/\text{min}$ for 43.3 min down to -32°C . Finally semen was stored in liquid nitrogen at -196°C .

Fresh and post-thaw semen quality was evaluated by the methods described below. Seminal motility and kinetics was assessed using the Sperm Class Analyzer (SCA[®]) System (Microptic SL., Spain), and the parameters analyzed were: total motility (TM), progressive motility (PM), straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF) (Usuga *et al.* 2017). Particles with a size between 15 and $70 \mu\text{m}^2$ were evaluated. Normal morphology (NM) was assessed using the modified eosin-nigrosin test (Brito *et al.* 2011). Functional integrity of the cell membrane (MI) of the spermatozoa was assessed using the hypo-osmotic swelling test (Neild *et al.* 1999). Sperm viability (SV) was determined using the Live/Dead kit (Molecular Probes Inc., USA) (Gamboa *et al.* 2010). For each of the 15 ejaculates, five straws of frozen-thawed semen samples were evaluated per treatment, thus, a total of 300 straws were thawed to evaluate four treatments.

Completely randomized mixed models were fitted for statistical analyses of data. Semen samples were divided according to their freezability; samples with post-thaw motility (PM) $\geq 35\%$ were considered as good freezability and samples with PM $< 35\%$ were considered as poor freezability (Hoffmann *et al.* 2011). Data normality was

assessed with the Shapiro-Wilk test and means were compared using Tukey's test with significance level $p < 0.05$. Statistical analyses were conducted using SAS version 9.2 software (SAS Inst. Inc., USA).

RESULTS AND DISCUSSION

Seminal plasma has been used as an alternative semen additive to reduce the oxidative stress of equine spermatozoa during freezing (Al-Essawe *et al.* 2018). Raw SP has been commonly used in liquid state for this purpose; however, the use of lyophilized SP allows more precise supplementation in form of solids (Usuga *et al.* 2017). In addition, lyophilization allows long-term preservation of seminal plasma in form of solid state and has advantages for the convenient transport of samples (Olaciregui and Gil 2017). In the present work, the TAC found for seminal plasma pool was $13679.4 \pm 911.6 \mu\text{mol}$ Trolox 100/g of LSP (ORAC units). There are few reports of measuring TAC on equine seminal plasma. Restrepo *et al.* (2015) obtained a mean of ORAC of $4049.4 \mu\text{mol}$ Trolox/L of raw seminal plasma from 24 stallions. On the other hand, Barranco *et al.* (2015) reported that measurement of seminal plasma TAC could be a discriminative tool for prognosis of fertility in breeding boars.

Results for quality parameters of fresh semen assessment are presented in Table 1. The fixed effect of the treatment was significant ($P < 0.05$) for all variables of seminal quality, except for VCL and NM.

Table 1. Fresh stallion semen quality.

	Mean	SD	CV	SEM	Min	Max
Volume (mL)	29.6	24.7	83.4	6.4	7.5	100.0
Concentration	254.1	110.2	43.4	28.4	101.0	428.0
($10^6/\text{mL}$)						
TM (%)	83.8	13.0	15.5	3.3	60.1	98.9
PM (%)	57.2	17.2	30.1	4.4	29.3	86.4
VCL ($\mu\text{m}/\text{s}$)	106.4	18.4	17.3	4.8	79.8	156.4
VSL ($\mu\text{m}/\text{s}$)	42.0	13.4	31.8	3.4	21.5	63.4
VAP ($\mu\text{m}/\text{s}$)	78.5	17.0	21.6	4.4	49.1	113.3
ALH ($\mu\text{m}/\text{s}$)	3.3	0.9	27.9	0.2	2.2	5.3
BCF ($\mu\text{m}/\text{s}$)	7.7	0.8	10.5	0.2	6.5	9.1
SV (%)	80.9	9.5	11.8	2.5	63.0	95.0
NM (%)	76.6	8.0	34.2	2.1	12.0	44.0
MI (%)	58.4	11.6	19.9	3.0	41.0	79.0

SD, Standard deviation; CV, coefficient of variation (%); SEM, standard error of mean; TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; SV, sperm vitality; NM, normal morphology; MI, functional integrity of the cell membrane.

Supplementation of semen extender with either enzymatic or non-enzymatic antioxidants has proven to be effective for scavenging excessive ROS generation and thereby preventing oxidative stress (Restrepo and Rojano 2018). The results of this study show that supplementing

Table 2. Post-thaw semen quality with different treatments of LSP

Treatment	TM (%)	PM (%)	VCL ($\mu\text{m/s}$)	VSL ($\mu\text{m/s}$)	VAP ($\mu\text{m/s}$)	ALH (μm)	BCF (Hz)	SV(%)	NM (%)	MI(%)
Control	54.3 $\pm$ 1.63 ^b	33.9 $\pm$ 1.56 ^{ab}	78.1 $\pm$ 1.82 ^a	45.0 $\pm$ 1.23 ^a	58.0 $\pm$ 1.81 ^{ab}	2.9 $\pm$ 0.04 ^b	9.6 $\pm$ 0.14 ^a	55.1 $\pm$ 1.03 ^a	77.2 $\pm$ 0.63 ^a	38.6 $\pm$ 1.09 ^b
LSP1	58.9 $\pm$ 1.88 ^a	35.9 $\pm$ 1.57 ^a	77.1 $\pm$ 1.60 ^a	44.9 $\pm$ 1.02 ^a	59.1 $\pm$ 1.53 ^a	2.8 $\pm$ 0.04 ^c	8.8 $\pm$ 0.12 ^b	55.1 $\pm$ 0.82 ^a	77.8 $\pm$ 0.5 ^a	46.5 $\pm$ 1.01 ^a
LSP2	57.9 $\pm$ 1.69 ^a	32.7 $\pm$ 1.44 ^b	77.6 $\pm$ 1.92 ^a	41.8 $\pm$ 1.04 ^b	58.0 $\pm$ 1.53 ^{ab}	3.1 $\pm$ 0.05 ^{ab}	8.5 $\pm$ 0.09 ^{bc}	51.9 $\pm$ 1.21 ^b	77.5 $\pm$ 1.06 ^a	44.8 $\pm$ 0.98 ^a
LSP3	49.2 $\pm$ 1.91 ^c	26.0 $\pm$ 1.46 ^c	74.6 $\pm$ 1.66 ^a	37.7 $\pm$ 0.96 ^c	54.6 $\pm$ 1.27 ^b	3.2 $\pm$ 0.08 ^a	8.5 $\pm$ 0.08 ^c	52.0 $\pm$ 1.10 ^b	76.5 $\pm$ 1.08 ^a	37.2 $\pm$ 1.11 ^b

LSP, lyophilized seminal plasma; Control, treatment without LSP; LSP1, supplementation with 1.44 mg/mL of LSP; LSP2, supplementation with 5.04 mg/ml of LSP; LSP3, supplementation with 8.68 mg/ml of LSP; TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; SV, sperm vitality; NM, normal morphology; MI, functional integrity of the cell membrane. Results are presented as mean $\pm$ standard error of mean (SEM). Different letters within columns indicate statistically significant difference ($P < 0.05$).

stallion semen with LSP1 and LSP2, has a positive effect on the post-thaw TM and MI of spermatozoa (Table 2). These amounts of LSP used, would be contributing 4,000 and 14,000 ORAC units before freezing, respectively. These results suggest that antioxidant activity obtained from LSP would generate less oxidative stress in spermatozoa during cryopreservation, resulting in better sperm cryosurvival. Similar results were reported by Li *et al.* (2018), who showed that certain SP antioxidants are positively involved in boar sperm cryotolerance. Additionally, SP TAC in bulls has been negatively correlated with lipid peroxidation of cryopreserved sperm (Guthrie and Welch 2012). In the equine species, Restrepo *et al.* (2015) found that TAC had a significant effect on the sperm motility and normal morphology of stallion semen. However, different investigations have reported opposite results regarding the addition of seminal plasma in the equine semen (Al-Essawe *et al.* 2018, Neuhauser *et al.* 2019), being possible the presence of factors that alter the antioxidant components of seminal plasma (Chand *et al.* 2019).

Supplementation with LSP2 and LSP3 showed a decrease on VSL and SV, while LSP3 produced a decrease in post-thaw TM and PM (Table 2). This can be attributed to presence of antioxidants, viz. α -tocopherol, vitamin C and phenolic acids in SP that can become pro-oxidants depending on its quantity and its neighboring molecules (Carocho and Ferreira 2013); because in the presence of transition metals, they make radicals highly reactive and more destructive, generating more free radicals (Vasconcelos *et al.* 2013). Additionally, some changes that

are considered as descriptive of stallion sperm hyperactivity, as VSL, MP and BCF decrease and ALH increase (McPartlin *et al.* 2009; Ortgies *et al.* 2012) were observed with LSP3, being the highest amount of supplementation in present experiment, which represents a contribution of 24,000 ORAC units.

Variability in sperm freezability among stallions is a widely known issue (Prien and Iacovides 2016); and even, variations in semen quality and cryosurvival has been described within individual animal (Sieme *et al.* 2015). Different researchers have used post-thaw motility (PM) as a criterion for the definition of stallion semen freezability (poor $< 35\%$ or good $\geq 35\%$) (Vidament *et al.* 1997; Hoffmann *et al.* 2011). Recently, Vilagran *et al.* (2015) demonstrated that quantitative differences in SP composition play an important role in such variability, between ejaculates from boar with good and poor sperm freezability. In this species, ejaculate portions with better or worse sperm cryosurvival were clearly related to those with higher or lower TAC values of SP (Mehrotra *et al.* 2013). This could explain the results obtained in the present study, because samples with good freezability, and therefore with possible high TAC, would not need LSP supplementation to increase its antioxidant capacity, showing no significant effect or even a deleterious effect by excess of it (Fig.1).

In contrast, poor freezability samples, supplemented with LSP1 and LSP2, showed comparatively better results for post-thaw TM and MI ($P < 0.05$), which might be due to presence of low TAC in these samples and its

Table 3. Post-thaw sperm kinetics with different treatments of LSP and semen freezability

Treatment	VCL ($\mu\text{m/s}$)		VSL ($\mu\text{m/s}$)		VAP ($\mu\text{m/s}$)		ALH (μm)		BCF (Hz)	
	Good	Poor	Good	Poor	Good	Poor	Good	Poor	Good	Poor
Control	92.0 $\pm$ 2.75 ^a	69.8 $\pm$ 1.38 ^a	54.1 $\pm$ 1.93 ^a	39.5 $\pm$ 0.94 ^a	72.3 $\pm$ 2.79 ^a	49.5 $\pm$ 1.21 ^a	3.04 $\pm$ 0.08 ^c	2.95 $\pm$ 0.05 ^a	9.14 $\pm$ 0.23 ^a	9.99 $\pm$ 0.16 ^b
LSP1	85.8 $\pm$ 2.04 ^a	70.3 $\pm$ 1.78 ^a	49.9 $\pm$ 1.30 ^{ab}	40.9 $\pm$ 1.23 ^a	66.5 $\pm$ 2.14 ^a	53.2 $\pm$ 1.68 ^a	2.98 $\pm$ 0.06 ^c	2.71 $\pm$ 0.05 ^b	9.17 $\pm$ 0.21 ^a	8.62 $\pm$ 0.12 ^a
LSP2	91.1 $\pm$ 1.47 ^a	69.6 $\pm$ 2.24 ^a	47.8 $\pm$ 1.18 ^b	38.2 $\pm$ 1.24 ^{ab}	67.8 $\pm$ 1.36 ^a	52.2 $\pm$ 1.85 ^a	3.41 $\pm$ 0.07 ^b	2.94 $\pm$ 0.07 ^a	8.73 $\pm$ 0.16 ^a	8.51 $\pm$ 0.11 ^a
LSP3	92.5 $\pm$ 2.03 ^a	69.7 $\pm$ 1.51 ^a	47.2 $\pm$ 1.34 ^b	35.2 $\pm$ 0.92 ^b	66.8 $\pm$ 1.62 ^a	51.2 $\pm$ 1.25 ^a	3.71 $\pm$ 0.20 ^a	3.08 $\pm$ 0.08 ^a	9.25 $\pm$ 0.09 ^a	8.33 $\pm$ 0.09 ^a

LSP, lyophilized seminal plasma; Control, treatment without LSP; LSP1, supplementation with 1.44 mg/ml of LSP; LSP2, supplementation with 5.04 mg/ml of LSP; LSP3, supplementation with 8.68 mg/ml of LSP; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; Good freezability, samples with PM $> 35\%$; Poor freezability, samples with PM $\leq 35\%$. Results are presented as mean $\pm$ standard error of mean (SEM). Different letters within columns indicate statistically significant difference ($P < 0.05$).

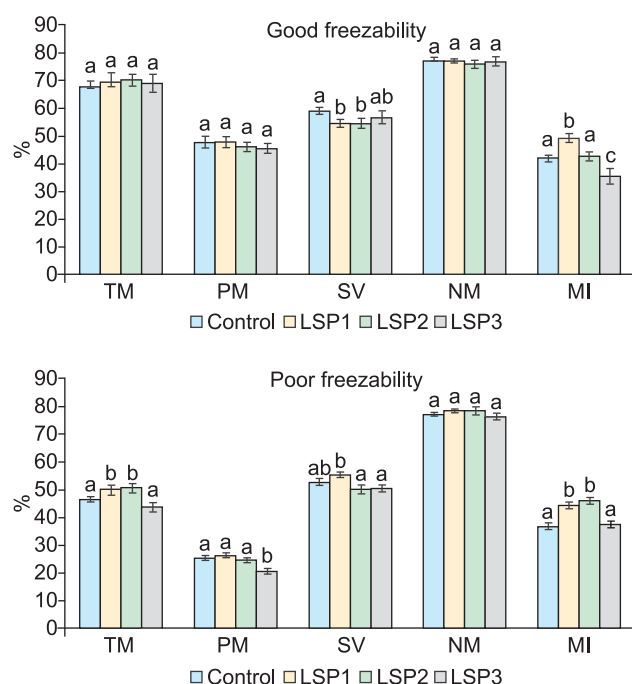


Fig. 1. Post-thaw semen quality by treatment and semen freezability.

LSP, lyophilized seminal plasma; control, treatment without LSP; LSP1, supplementation with 1.44 mg/mL of LSP; LSP2, supplementation with 5.04 mg/mL of LSP; LSP3, supplementation with 8.68 mg/mL of LSP; TM, total motility; PM, progressive motility; SV, sperm vitality; NM, normal morphology; MI, functional integrity of the cell membrane; Good freezability, samples with PM $\geq 35\%$; Poor freezability, samples with PM $\leq 35\%$. Results are presented as mean $\pm$ standard error of mean (SEM). Different letters within bars indicate statistically significant difference ($P < 0.05$).

supplementation with LSP would have resulted in beneficial effects on post-thaw semen parameters by reducing ROS. However, supplementation with greater amounts of LSP (LSP3) could generate deleterious effects due to presence of excess of some antioxidant compounds and therefore, a pro-oxidant effect. Likewise, changes in kinetic parameters such as VSL and ALH of sperm were observed for good and poor freezability samples due to the effect of LSP supplementation (Table 3).

In conclusion, supplementing stallion semen with LSP, before the freezing process, can improve its freezability owing to its TAC contribution. Post-thaw seminal quality of poor freezability semen samples can benefit more from LSP supplementation than good freezability semen samples.

ACKNOWLEDGEMENTS

The authors would like to thank Politécnico Colombiano Jaime Isaza Cadavid for its financial support.

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