Pferdeheilkunde 30 (2014) 5 (September/Oktober) 557-560

Horse Seminal Plasma proteins (HSP-1 and HSP-2) concentration: a possible marker for poor fertility?

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Summary: Seminal plasma (SP) proteins have been assessed in relation to reproductive fertility levels or infertility, in several species of mammals, particularly domestic animals. Horse seminal plasma proteins 1 (HSP-1) and 2 (HSP-2) are the most abundant proteins in equine seminal plasma. The aim of this study was to investigate in adult stallions the concentrations of seminal plasma HSP-1/2 and total protein in the breeding season and non-breeding season and to determine if these concentrations were related with fertility. Seminal plasma was obtained from 42 ejaculates of 11 adult stallions (3–25 yrs). Stallions were allocated into two groups (good and poor fertility) according to pregnancy rates of mares, and to their semen viability data in the first collection day. Seminal plasma HSP-1/2 concentrations (mg/mL) were measured and analyzed by an Ultra High Performance Liquid Cromatography using a UHPLC column. There were significant differences ($P < 0.05$) in total protein and HSP1/2 concentration (mg/mL, mean ± SD) in the ejaculates from good and poor fertility stallions. The HSP1/2 concentration did not show differences in the first and second ejaculates of good fertility stallions in both the non-breeding and breeding season. Seminal plasma of stallions classified as poor fertility showed significant difference ($P < 0.05$) in HSP-1/2 concentration between the first and second ejaculate in both the non-breeding and breeding season. In conclusion, the concentration of the major proteins of stallion seminal plasma HSP1/2 was higher in ejaculates from stallions with poor fertility, is not influenced by the season and could serve as biomarker for poor fertility in stallions.

Keywords: stallion / UHPLC / breeding season / seminal plasma / reproduction

Ist die Konzentration der Sperma-Plasmaproteine HSP-1 und HSP-2 des Pferdes ein möglicher Marker für mangelhafte Fruchtbarkeit?

Die Proteine des Seminalplasmas wurden im Zusammenhang mit dem Fruchtbarkeits- oder Unfruchtbarkeitslevel diverser Säugetiere, speziell der Haustiere, bewertet. Die "Horse Seminal Plasma" Proteine 1 (HSP-1) und 2 (HSP-2) sind die am meisten vorkommenden Proteine im Seminalplasma von Pferden. Ziel der Studie war es, die Konzentration des Seminalplasmas HSP-1/2 und die gesamten Proteine bei erwachsenen Hengsten, während und außerhalb der Decksaison zu untersuchen und festzustellen, ob diese Konzentrationen mit der Fruchtbarkeit zusammenhängen. Seminalplasma wurden aus 42 Ejakulaten von 11 erwachsenen Hengsten (3–25 Jahre) gewonnen. Die Hengste wurden in zwei Gruppen aufgeteilt (hohe und niedrige Fruchtbarkeit) gemäß der Trächtigkeitsrate der Stuten und der Viabilität des Samens in der Samenentnahme des ersten Tages. Samenplasma wurde aus 42 Ejakulaten gewonnen und die Konzentration von HSP-1/2 (mg/mL) wurden mit einer Hochleistungsflüssigkeitschromatographie unter Anwendungen einer UHPLC Trennsäule gemessen und analysiert. Es wurden signifikante Unterschiede ($P < 0.05$) in der Konzentration der gesamten und der HSP1/2 Proteine (mg/mL, Durchschnitt ± SD) im Ejakulat der Hengste mit hohen und niedrigen Fruchtbarkeit festgestellt. Die HSP1\2 Konzentration der Hengste mit hoher Fertilität zeigte keinen Unterschied im ersten und zweiten Ejakulat sowohl während als auch außerhalb der Decksaison. Seminalplasma, der mit niedriger Fertilität eingestuften Hengste, zeigten einen signifikanten Unterschied ($P < 0.05$) zwischen dem ersten und zweiten Ejakulat sowohl innerhalb als auch außerhalb der Decksaison. Abschließend wurde festgestellt, dass die HPS1/2 Konzentration, die am häufigsten vorkommende Proteine, höher im Ejakulat der Hengste mit niedriger Fertilität war, diese nicht durch die Saison beeinflusst wurde und kann als Biomarker geringer Fertilität eingesetzt werden.

Schlüsselwörter: Hengst / UHPLC / Decksaison / Seminalplasma / Reproduktion

Citation: L. A. D. Garcia, E. L. R. Brito, P. Serpa, J. Gregory, C Natalini, R. C. Mattos, M. I. M. Jobim (2014) Horse Seminal Plasma proteins (HSP-1 and HSP-2) concentration: a possible marker for poor fertility? Pferdeheilkunde 30, 557-560

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Introduction

Progress has been made in developing reliable indicators of ejaculate quality that allow exclusion of low-quality ejaculates for use in natural breeding or artificial insemination. Physical semen characteristics and sperm morphology measurements allow detection of the stallions most likely to be fertile. However, some of them are or became subfertile despite acceptable results of the conventional breeding soundness examination (*Barrier-Battut* et al. 2005). The ability to select these fertile stallions or predict fertility using biomarkers is a promising goal. Accurate or predictive genetic and protein markers are still needed.

The suggested functions of seminal plasma proteins include their involvement in several essential steps preceding fertilization, such as regulating sperm capacitation, establishment of the oviductal sperm reservoir, modulation of the uterine immune response and sperm transport in the female genital tract, as well as in gamete interaction and fusion (*Töpfer-Petersen* et al. 2005).

Seminal plasma (SP) proteins have been assessed in relation to reproductive fertility levels or infertility, in several species of mammals, particularly domestic animals. SP proteins have been identified as associated with high and low fertility in bulls (*Killian* et al. 1993) isolated as osteopontin (OPN) and

lipocalin-type prostaglandin D synthase respectively (*Cancel* et al. 1997, *Gerena* et al. 1998). The latter has been always present in the sperm-rich fraction of ejaculates in species with fractionated ejaculation. OPN has been related to fertility in pigs (*Hao* et al. 2006, *Hao* et al. 2008) and stallions (*Brandon* et al. 1999). *Jobim* et al. (2005) observed one protein (20–25 kDa, pI 8.5–8.7) present only in the ejaculates of high fertility stallions and another protein (25–30 kDa, pI 7.5–7.7) that had higher relative protein content in ejaculates of low fertility stallions. More recently, the abundance of cysteine-rich secretory protein 3 (CRISP3) was positively related to first cycle conception rate and the abundance of four seminal plasma proteins were identified as being negatively related to fertility; these were identified as kallikrein-1E2 (KLK2), clusterin, and seminal plasma proteins 1 (SP1) and 2 (SP2) (*Novak* et al. 2010).

Horse seminal plasma proteins 1 (HSP-1) and 2 (HSP-2); recently, renamed SP-1 and SP-2, respectively are the most abundant proteins in equine seminal plasma, accounting for 70–80% of the total proteins (*Calvete* et al.1994). They showed heparin-binding ability (*Calvete* et al. 1994) and were found to be associated to the sperm surface, indicating a potential role in fertilization (*Töpfer-Petersen* et al. 2005). They belong to the short seminal Fn-2 type proteins (*Calvete* et al. 1995 and *Ekhlasi-Hundrieser* et al. 2005) and are the equine orthologs to the major bovine heparin-binding proteins (BSP), which have been shown to be involved in early fertilization steps (capacitation).

The season of the year influences many of the physical and chemical characteristics of stallion semen as well as fertility (*Pickett* et al. 1975). The influence of season on the total protein concentration and the composition of seminal plasma of many species has been described in the ram (*Perez-Pé* et al. 2001, *Cardozo* et al. 2006), boar (*Trudeau* et al. 1986, *Strzezek* 2002) and horse (*Janett* et al. 2003), with significant differences between breeding and non-breeding seasons.

The aim of this study was to investigate in adult stallions the concentrations of seminal plasma HSP-1/2 and total protein in the breeding season and non-breeding season and to determine if these concentrations were related with fertility.

Material and methods

Animals and Samples Collection

Seminal plasma was obtained from 11 adult stallions (3-25yrs) from commercial herds in the State of Rio Grande do Sul, Brazil (30° 16' 57'' latitude south and 55° 53' 47'' longitude west at 145 meters above sea level). Data were collected during the non-breeding season (winter and spring months) and the breeding season (summer months). Stallions were maintained under similar handling and feeding conditions, kept free in pastures. They were allocated into two groups (good and poor fertility; *Giesecke* et al. 2010) according to pregnancy rates of mares assessed by veterinary and to their semen viability data in the first collection day. Stallions of good fertility (n = 6) had a minimum of 65% of pregnancy rates during the 2-year period and more than 1.5×10^9 viable sperm. Stallions classified as poor fertility (n = 5) had no more than 55% of pregnancy rates and less than 1.4×10^9 viable sperm. Sperm viability (SV) was calculated using the following formula: SV = progressive sperm motility * morphologically normal sperm * sperm concentration.

Two ejaculates were collected by artificial vagina from each stallion in the breeding season and non-breeding season with one hour of interval. One of the poor fertility stallion die during the experiment and was not collected in the breeding season. A total of 42 ejaculates from 11 stallions were used. After collection and analysis, a 2.0 mL aliquot of semen was centrifuged at 1,500 × g for 15 to 20 min to obtain seminal plasma. The supernatant seminal plasma was transferred to cryovials for storage in liquid nitrogen and subsequent laboratory analysis. Frozen samples were thawed, recentrifuged at 10,000 × g for 60 min at 4°C and 50 µL were taken from the supernatant and transferred to cryovials for storage at -80°C.

Semen Collection and Evaluation

After collection, gel-free semen was taken to evaluate for volume, sperm motility, sperm concentration, and percent of morphologically normal sperm, according to conventional semen analysis described by *Sieme* et al. (2001).

Total Protein Concentration

Protein concentration in seminal plasma from each sample was assessed according to the method described by *Lowry* et al. (1951), using bovine serum albumin (1 mg/mL) as a standard.

HSP-1/2 Concentrations

Seminal plasma HSP-1/2 concentrations (mg/mL) were measured according to the method described by *Calvete* et al. (1997) with modification (trifluoroacetic acid was replaced by trichloroacetic acid as one of the mobile phases for chromatography analyses). The samples were defrosted at room temperature, filtered in 0.22 µm filters (Biofil Syringe Filter) and analyzed by an Ultra High Performance Liquid Cromatography using a Thermo Fisher Scientific UHPLC column (Hypersil Gold AX 50 × 2.1 mm, 1.9 micron pore) eluted at 1 mL/min with a gradient of 0.1% (v/v) trichloroacetic acid in (A) water and (B) acetonitrile as follows: isocratically with 25% B for 5 min, followed by 25–30% B for 5 min, and 30–70% B for 160 min. Proteins were detected at 220 nm. Integration of the sample curves with the calibration curve was done with ChromQuest® and values for the HSP-1/2 protein concentration were attained. The calibration curve was obtained with HSP-1/2 purificated kindly provided by Dr. J. J. Calvete (Instituto de Biomedicina de Valencia, Spain).

Statistical analysis

Data were analyzed using Statistical Analysis Software (SAS®). Analysis of variance (GLM–General Linear Model) was performed to compare (among fertility groups) the HSP-1/2 and total protein concentration in the first and second ejaculate from

good and poor fertility stallions measured in the non-breeding and breeding season. The Tukey post hoc test was used to locate differences and $P<0.05$ was regarded as significant.

Results

Overall pregnancy rates ranged from 66–100% in stallions of good fertility and from 0–53% in stallions of poor fertility. In two samples from the good fertility group and in one sample from the poor fertility group HSP1/2 detection was not possible. HSP-1/2 (n = 39 ejaculates) and total protein concentration (n = 42 ejaculates) (mean ± SD) from stallion with good and poor fertility are shown in Figure 1. There were differences ($P < 0.05$) in total protein and HSP1/2 concentration (mg/mL, mean ± SD) in the ejaculates from good and poor fertility stallions. HSP-1/2 accounting for 80% of the total proteins in the samples of stallions of good fertility, while that HSP-1/2 accounting for 93% of the total proteins in stallions classified as poor fertility. Results of HSP-1/2 concentration (mg/mL, mean ± SD) in the first and second ejaculate from good and poor fertility stallions measured in the non-breeding and breeding season are shown in Table 1. There were no differences ($P > 0.05$) in HSP-1/2 concentration among adult stallion ages (3–25yrs).

Discussion

As a part of the fertilization process, seminal plasma proteins play an important role in sperm reservoir formation, sperm capacitation, and sperm-oocyte interactions (*Foxcroft* et al. 2008, *Rodríguez-Martínez* et al. 2008). Specific seminal plasma proteins have previously been identified as potential markers of male fertility in the bull (*Killian* et al. 1993) and stallion (*Brandon* et al. 1999, *Novak* et al. 2010). The present study investigated seminal plasma HSP-1/2 concentration to determine if these concentrations are related with stallion fertility in vivo, providing the basis to use them as a complementary tool to identify sires with high and low relative fertility that could have a considerable impact on reproductive efficiency.

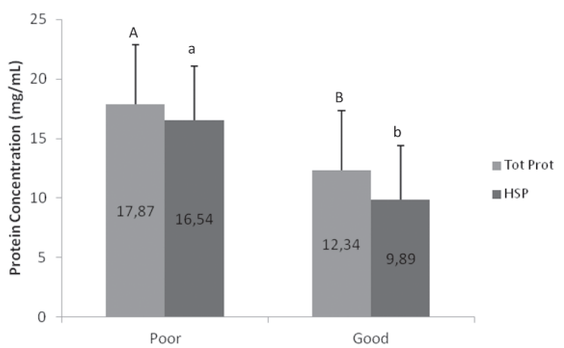

Fig. 1 HSP-1/2 (n = 39 ejaculates) and total protein (n = 42 ejaculates) concentration (mean ± SD) from stallion with good and poor fertility. Different superscripts indicate differences ($P < 0.05$).

The highest values of HSP-1/2 concentration were found in the ejaculates from stallions with poor fertility in the non-breeding and breeding season. This is consistent with previous findings of *Novak* et al. (2010) in the breeding season. HSP-1/2 shares significant homology with PDC-109 (*Calvete* et al. 1995) and this protein at higher concentrations induces membrane permeabilisation (*Gasset* et al. 1997), stimulates cholesterol and phosphatidylcholine efflux (*Therien* et al. 1999), and also acts in the perturbation of the membrane integrity (*Calvete* et al. 2007). Perhaps the greater amount of HSP-1/2 in seminal plasma, as detected in samples from stallions with poor fertility, had the same negative effect as PDC-109 above mentioned. HSP-1/2 exhibits chaperone-like activity and may protect other proteins of equine seminal plasma against misfolding, unfolding or aggregation (*Sankhal* et al. 2012). The structure of HSP-1/2 is largely unordered and it is likely that this structural plasticity helps it to interact with other seminal plasma proteins effectively and protect them under stress conditions. Probably the values observed in the poor fertility stallions were related with a stress condition with production of other proteins that stimulates the high concentration of HSP-1/2.

Additionally, the proteins HSP1/2 were hypothesized to be similar to a sperm motility inhibitor protein (SPMI, 18-22 kDa) originating from the seminal vesicles (*Brandon* et al. 1999). The HSP-1/2 comprised 80% of the total proteins in the samples of stallions of good fertility which agree with the results of *Calvete* et al. (1994) that observed two major proteins, the heparin-binding HSP-1 and HSP-2, accounted for 70–80% of the total seminal plasma protein. However an increase in HSP-1/2 concentration was observed in samples of seminal plasma of stallions classified as poor fertility. The findings of *Calvete* et al. (1994) come only from healthy reproductively active stallions while in this study were used stallions of good and poor fertility. Total protein concentration showed the same pattern found for HSP-1/2 concentration because these proteins together account for 70–80% of the total proteins in stallion seminal plasma (*Calvete* et al. 1994).

The HSP 1/2 show higher concentration in the ejaculates from stallions in the poor fertility group, in the first and second eja-

Table 1 HSP-1/2 concentration (mg/mL, mean ± SE) in the first and second ejaculate from stallions with good (n = 6) and poor (n = 5) fertility measured in the non-breeding and breeding season.

Fertility	non-breeding		breeding	
	Ejaculates			
	1	2	1	2
Good	10.97 ± 1.19^{Aa} n = 6	8.31 ± 0.82^{Aa} n = 6	10.98 ± 1.81^{Aa} n = 5	9.40 ± 2.04^{Aa} n = 5
Poor	19.99 ± 1.21^{Ba} n = 5	13.51 ± 0.79^{Bb} n = 4	23.99 ± 1.80^{Ba} n = 4	13.77 ± 1.33^{Ab} n = 4

^{A,B} Column values with different superscripts indicates significant difference ($P<0.05$).
^{a,b} Row values with different superscripts indicates significant difference ($P<0.05$).

culate in the non-breeding season and in the first ejaculate in the breeding season in comparison with the good fertility group. In contrast, the second ejaculate in breeding season not shown to vary between the fertility groups. Perhaps the increase in the ejaculations number of the stallions during the breeding season may have an effect in the amount of HSP1/2 without variation between fertility groups in the second ejaculate.

The HSP1/2 concentration did not show differences in the first and second ejaculates of good fertility stallions in both the non-breeding and breeding season, indicating uniformity in their concentrations. On the other hand, HSP-1/2 concentration observed in samples of seminal plasma of stallions classified as poor fertility showed difference between the first and second ejaculate in both the non-breeding and breeding season.

In conclusion, the concentration of the major proteins of stallion seminal plasma HSP1/2 was higher in ejaculates from stallions with poor fertility, is not influenced by the season and could serve as biomarker for poor fertility in stallions.

Acknowledgements

The authors are grateful to Dr. Juan J. Calvete (Instituto de Biomedicina de Valencia, Valencia, Spain) for supply the HSP-1/2 purified and to CNPq, CAPES and FAPERGS for funding this study.

Conflict of interest

None of the authors have any conflict of interest to declare.

Animal welfare statement

Statement 23850, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

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