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Matrix metalloproteinase (MMP)-2, MMP-9, semen quality and sperm longevity in fractionated stallion semen

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Abstract

Matrix metalloproteinase (MMP)-2 and MMP-9 are gelatinases that take part in several reproductive processes. The aim of this study was to measure levels of MMP-2 and MMP-9 in fractionated stallion ejaculates, and to evaluate the association between these components and semen quality, and sperm longevity during cooled storage. Semen quality were assessed separately for sperm-rich fractions (HIGH), sperm-poor fractions (LOW), and whole ejaculate samples (WE) from 33 stallions. After cooled storage with SP either present or removed, sperm motility and DFI were determined. The relative activity of the pro-form of MMP-2, active MMP-2 and total MMP-9 were evaluated using gelatin zymography, and all were present in all fractions of the stallion's ejaculate, with higher relative activity of the latent than active forms and the highest relative activity in the HIGH fraction. The relative activities of MMP-2 and MMP-9 were positively correlated to sperm concentration and total sperm count, but only in the HIGH fraction and not in LOW or WE. The relative activities of MMPs were not related to differences in sperm longevity during cooled storage, measured as sperm motility and DFI. There was a harmful effect of SP on DFI during storage, but this effect was not associated with differences in the relative activities of MMPs. In conclusion, the relative activities of MMPs are not useful as markers for semen quality (other than sperm concentration), or sperm survival during storage in horses.

Keywords:

Stallion; Seminal plasma; Sperm longevity; Ejaculate; Matrix metalloproteinase

1. Introduction

The stallion's epididymides and accessory glands produce a significant amount of seminal plasma (SP), which flushes spermatozoa out via the urethra during ejaculation, and into the mare's uterus.

The composition of semen changes during the ejaculation, with the first few jets of the ejaculate containing most of the spermatozoa. These jets can be collected separately as so-called sperm-rich fractions of the ejaculate [1,2,3]. The accessory glands contribute to different fractions of the ejaculate [4-7]. When semen has been deposited in the mare's uterus, most of the SP is expelled via the cervix because of intensified uterine contractions [8]. In natural mating, sperm are exposed to SP only briefly, but when insemination doses are prepared for cooling, freezing and storage, sperm are in contact with SP for a longer time. During this contact period, SP can have marked effects on sperm survival and fertility even though the proportion of SP is lowered in the insemination doses before storage.

Several studies have shown that when the amount of SP is lowered to less than 5 or 10% of the total volume, sperm motility is higher after cooled storage compared to samples containing a higher proportion of SP (10–30%) [9-11]. There is variability in the effect of SP on sperm survival depending on the stallion, as shown in studies where SP has been exchanged between stallions [12,13]. There are also differences between ejaculate fractions, and the sperm-rich fraction has been shown to tolerate cooled storage [3], and freezing and thawing [14] better than whole ejaculates.

Matrix metalloproteinase (MMP)-2 and MMP-9 are proteins that degrade protein components of extracellular matrix and basement membranes during tissue restructuring, in both physiologic and pathologic processes [15-18]. They are secreted as latent forms, pro-MMP-2 and pro-MMP-9, which are activated through cleavage of an inhibitory pro-peptide [19]. Seminal plasma of men [17], bulls [20], rams [21], and dogs [22] have been shown to contain MMP-2 and MMP-9, and these MMPs have been studied also in testicular and epididymal fluid of rams, boars and stallions

[23]. The levels of MMPs and their tissue inhibitors of matrix metalloproteinases (TIMPs) are correlated to sperm count, motility and sperm DNA fragmentation in men [18,24-26].

The cause of the variation in the effects of SP between stallions and between fractions is largely unknown, and the levels and effects of matrix metalloproteinases on sperm have not been studied in stallion ejaculates prior to this experiment. The aim of this study was to measure the relative activity levels of MMP-2 and MMP-9 in different fractions of stallion ejaculates, and to evaluate the association between these components and semen quality parameters, and sperm longevity during cooled storage.

2. Material and methods

The samples analyzed in this study are from a larger study published by Kareskoski et al. [27].

2.1. Animals

Semen was collected during one breeding season from 33 stallions (ages 4 to 23 years) residing at nine stud farms in Finland. One ejaculate was collected from each stallion. Fourteen stallions were Standardbred trotters (ages 4 - 23 years), 16 Finnhorses (ages 8 - 23 years), two warmblood riding horses (ages 9 - 19 years), and one Shetland pony (age 9 years). The breeding history and current use of the stallions was variable, with the stallions serving from 2 to 150 (median 14) mares during the study season. Most of the stallions (28 stallions) were used for collection of both fresh and transported semen, and four stallions were also used for natural breeding. Ethical approval was not required for this study according to the Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013).

92 **2.2. Semen collection**

93 The ejaculatory jets were collected as three to four fractions using either an open-ended artificial
94 vagina (AV), a modified closed AV described by Kareskoski et al. [27] or a computer-controlled
95 fractioning phantom with an integrated AV (Equidame phantom, Haico Oy, Loimaa, Finland),
96 depending on stallion preference and stud farm. The sperm concentration in different fractions
97 did not differ significantly between the collection methods. To provide comparable samples, only
98 the fractions with the highest (HIGH) and lowest (LOW) sperm concentrations within each
99 ejaculate were included in the statistical analyses.

100

101 **2.3. Semen processing and storage**

102 After semen collection, the gel was removed and the volume of the fractions measured. A sample
103 representing the whole ejaculate (WE) was formed by combining 10% of the volume of all
104 fractions. The sperm concentration in each fraction was determined using a Bürker counting
105 chamber. One drop of semen from the HIGH fraction was placed on two glass slides and smears
106 for morphological evaluation were prepared and air-dried. For a sperm chromatin structure assay
107 (SCSA), a sample of 2 - 10 x 10^6 spermatozoa from each fraction of raw semen was pipetted into
108 cryovials and TNE-buffer (9.48 g Tris-HCl, 52.6 NaCl, 2.23 g disodium-EDTA, aqua ster. ad 600 mL,
109 pH 7.4) was added (ad 1.5 mL in each vial). The SCSA samples were placed in liquid nitrogen vapor
110 (3cm above the liquid surface) for 10min, and then plunged into liquid nitrogen.

111

112 Each fraction was divided into two parts: one half was centrifuged for the preparation of SP (SP
113 samples), and the other half was processed for cooled storage (semen samples). For cooled
114 storage, semen samples were extended in a semen:extender ratio of 1:1 using skim milk extender
115 [28]. Each fraction of the semen samples was divided into two centrifuge tubes and centrifuged at

500 x g (10 min). After removal of the major part of the supernatant (i.e. extender and SP), leaving about 5%, the sperm pellet was extended in either: a) skim milk extender only (these samples stored without SP were named SP0 samples), or b) a combination of supernatant and skim milk extender (these samples stored with SP were named SP1). The final sperm concentration was 50 x 10^6 sperm/mL. The final SP to extender ratio in the SP1 samples was 1:2. The semen samples were stored in 1-mL vials at 5°C for 24h. After the 24-h cooled storage, 5 x 10^6 spermatozoa from each semen sample were pipetted into cryovials for SCSA, and TNE-buffer was added (ad 1.5 mL in each vial). The samples were placed in liquid nitrogen vapor (3cm above the liquid surface) for 10min and plunged into liquid nitrogen.

The SP samples were centrifuged at 4000 x g (15 min), and the supernatant was filtered using 0.45-µm filters (Millex-HV, Millipore, Billerica, MA, USA). The SP samples were stored frozen in 1-mL aliquots in -75°C until analyzed.

2.4. Sperm motility analyses after cooled storage

After cooled storage for 24 h, the semen samples were warmed in a water bath (37°C, 5 min). Motility parameters were evaluated using the SpermVision computer-assisted sperm analysis system with the equine-specific settings provided by the manufacturer (SpermVision, Minitube, Tiefenbach bei Landshut, Germany): area for cell identification: 14-80 μm^2; cells considered non-motile: average orientation change of head < 9.5º, average path velocity < 20 µm/s; local motile (i.e. non-progressive): distance straight line < 6 µm, average path velocity > 20 and < 30 µm/s, curvilinear velocity <9 µm/s; linear: straightness > 0.9 and linearity > 0.5; curvilinear: distance average path/radius > 3 and linearity < 0.5. A minimum of 500 cells or 7 fields were analyzed twice from each sample. In order to detect effects of SP on motility parameters during storage despite

individual variation in motility values before storage, we calculated the difference (DIFF) in motility between SP0 and SP1 samples as DIFF = motility(SP1) – motility(SP0). The effects of SP on sperm motility can be either positive or negative, depending on the individual stallion.

2.5. Sperm morphology staining and evaluation

The morphology smears were fixed and stained using the Giemsa method according to Watson [29] (1975), and 200 sperm were assessed in each sample. The morphological characteristics were classified in the following way: morphologically normal sperm, abnormal heads, abnormal acrosomes, abnormal midpieces, tailless heads, proximal cytoplasmic droplets, distal cytoplasmic droplets, bent tails and coiled tails.

2.6. Sperm chromatin structure assay

Sperm chromatin stability of sperm from both the raw and stored semen samples was measured as the susceptibility of sperm DNA to denaturation using the sperm chromatin structure assay (SCSA) as described by Evenson et al. [30]. In order to detect effects of SP on DFI during storage, we calculated the difference (DIFF) in DFI between SP1 and SP0 samples as DIFF = DFI(SP1) - DFI(SP0).

2.7. Gelatin zymography and densitometry

The relative activity levels of the pro-form of MMP-2, active MMP-2 and total MMP-9 were evaluated with gelatin zymography, using the methods described by Shimokawa et al. [17] with modifications. Samples were analyzed with 10% SDS-PAGE electrophoresis containing 0.7 mg/mL of gelatin (G1890, Sigma Aldrich, Missouri, USA). The samples were diluted in a sample buffer (40 mg bromophenol blue, 6 g SDS, 87% glycerol/ 100 mL deionized water) in a ratio of 1:40. Human

recombinant protein MMP-2 (902-MP, R &D Systems, Minneapolis, USA), and human recombinant protein MMP-9 (911-MPN-010, R &D Systems, Minneapolis, USA) were used as controls. After the electrophoresis, gels were incubated one hours at room temperature with the renaturing buffer (2.5% Triton X-100/ deionized water; Sigma-Aldrich, Missouri, USA). Thereafter, the gels were washed 30 minutes at room temperature with the developing buffer (50mM Tris-HCl [pH, 7.5], 200mM NaCl, 5mM CaCl2, and 0.02% Brilj-L23 solution; B4184, Sigma-Aldrich, Missouri, USA) followed by a 19-hour incubation at 37^{o}C with the developing buffer. After the incubation, the gels were washed three times with deionized water and stained with Coomassie Brilliant Blue G-250 (Sigma-Aldrich, Missouri, USA), which revealed clear bands against a blue background. The gels were scanned (EPSON Expression 1640 XL; Epson, Suwa, Japan), and density of the bands was measured with a spot-density tool of an imaging system (Alpha Image HP; Alpha Innotech Corp, California, USA). The intensity of the band was quantified by the area mode of the imaging system program. The area of each sample was normalized to the area of the band of human recombinant MMP controls. The activity of each sample was reported as the mean of two parallel measurements.

2.8. Statistical analyses

The HIGH and LOW semen fractions were included in all statistical analyses, but the WE fraction was excluded from the linear regression analysis due to missing samples. Because of a few missing samples, the number of stallions varies between 25 and 33 stallions depending on the analysis. The following sperm motility parameters were included in the analyses: total sperm motility (TMOT, %), progressive motility (PMOT, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), straightness (STR), and linearity (LIN). The differences between fractions in the relative activity levels of pro-MMP-2, active MMP-2, total

MMP-9, and in the mean difference (DIFF) in sperm motility parameters between SP1 and SP0 samples were evaluated using a two-sample t-test. The t-test was also used to compare mean values in sperm motility and DFI between fractions within the storage groups (SP1 and SP0), and between the storage groups within fractions. Because of the skewed distribution of the relative activity of MMPs and the mean DIFF in DFI, the nonparametric Mann-Whitney U-test was used for fraction comparisons.

The sperm longevity parameters included in the correlation analyses were the DIFF in motility between SP1 and SP0 samples, and the DIFF in DFI between samples before storage and after storage (separately for SP0 and SP1 samples). The semen quality parameters were sperm concentration, total number of sperm, total and progressive motility before storage, and the percentage of morphologically normal sperm. . The correlation of the sperm longevity and semen quality parameters with the relative activity of the MMPs was evaluated using the Pearson correlation coefficient.

The dependent variables (ie. the sperm longevity and semen quality parameters) that correlated significantly with any MMP component were included in a stepwise linear regression analysis for assessment of the association of the relative activity of these SP components (explaining variables) with each dependent variable.

3. Results

The differences in the relative activity levels of MMPs, sperm quality and sperm longevity parameters between HIGH and LOW fractions of the ejaculate are shown in Table 1. Pro-MMP-2, active MMP-2 and total MMP-9 were detected in stallion seminal plasma, with the highest relative

activity of active MMP-2 and total MMP-9 found in the sperm-rich fraction. In both HIGH and LOW fractions, the relative activity of pro-MMP-2 were significantly higher than the relative activity of active MMP-2 and total MMP-9, which did not differ from each other. The results on the effects of SP on sperm longevity have been reported earlier [27]. In short, there were no significant differences between HIGH and LOW fractions in any sperm motility parameters within the two storage groups SP0 and SP1. The sperm velocity parameters VAP and VCL were significantly higher in SP1 than in SP0 in all fractions, whereas total and progressive motility did not differ between SP1 and SP0 (Fig. 1). The DFI was higher in SP1 than in SP0 in both HIGH and LOW fractions (Fig. 2).

In the HIGH fraction, the relative activity of active MMP-2 was correlated to sperm concentration ($r=0.51$, $p=0.007$) and the number of sperm ($r=0.48$, $p=0.013$). The relative activity of total MMP-9 was correlated to sperm concentration ($r=0.616$, $p=0.001$) and the number of sperm ($r=0.47$, $p=0.015$).

In the LOW fraction, the relative activity of active MMP-2 was positively correlated to sperm concentration ($r=0.51$, $p=0.007$), and the number of sperm ($r=0.48$, $p=0.013$). The relative activity of pro-MMP-2 was positively correlated to sperm concentration ($r=0.78$, $p=0.001$) and the number of sperm ($r=0.56$, $p=0.008$).

In HIGH, LOW and WE, the relative activity of MMPs was not significantly correlated to DFI before storage, DFI after storage in SP1 or SP0 samples, sperm morphology, any of the sperm motility parameters, nor any of the DIFF values (DIFF in motility parameters, DIFF in DFI). Therefore, only sperm concentration and the number of sperm were included in the linear regression analysis.

236 The results of the linear regression analyses are shown in Table 2. In the HIGH fraction, higher
237 sperm concentration and a higher number of sperm were associated with higher relative activity
238 of active MMP-2 and total MMP-9. In the LOW fraction, higher sperm concentration and a higher
239 number of sperm were associated with higher relative activity of pro-MMP-2, but not active MMP-
240 2 or total MMP-9.
241
242 **4. Discussion**
243 Latent pro-MMP-2, active MMP-2 and total MMP-9 were present in all fractions of the stallion's
244 ejaculate. This was expected based on studies in other species, namely in men [15-18], bulls [20],
245 rams [21,23], boars [23], and dogs [22], where MMP-2 and MMP-9 were the forms of MMP
246 gelatinases commonly detected in semen or epididymal fluid. Stallion SP contained more latent
247 pro-MMP-2 than active MMP-2 in both HIGH and LOW fractions. The latent forms of MMP-2 and
248 MMP-9 are more abundant than the active forms also in human SP [18]. Latent pro-MMP-2 is
249 activated through cleavage of the inhibitory pro-peptide by membrane-type matrix
250 metalloproteinases, and this is part of the regulation of MMP levels [19]. It has been suggested
251 that prostate specific antigen (PSA) is involved in the activation of MMP-9 in seminal plasma in
252 men [17]. Little is known about the physiological significance of prostate secretions in stallions.
253
254 In stallion semen, the highest relative activity levels of active MMP-2 and total MMP-9 were found
255 in the HIGH fraction. These first sperm-rich fractions contain secretions from the testes,
256 epididymides, ampullae and prostate gland, while the latter fractions are composed of fluid from
257 the seminal vesicles and have low sperm concentration [4-7]. Matrix metalloproteinases are
258 presumably secreted to some degree throughout the male reproductive tract, as these gelatinases
259 are quite ubiquitously expressed in the body. There is evidence of MMP secretion from Sertoli

cells in association with FSH stimulation in rats [31-33] and humans [34]. Métayer et al. [23] could not find other MMPs than pro-MMP-2 in detectable amounts in testicular fluid of rams, boars or stallions, but pro-MMP-2, active MMP-2 and MMP-9 were all detected in epididymal fluid, where they were suggested to participate in sperm maturation or epididymal plasticity. The seminal vesicles and prostate gland are also sites of MMP secretion in men [24,35]. Latent pro-MMP-2 and active MMP-2, but not MMP-9, has also been found bound to human sperm [24].

The relative activity level of MMP-9 was positively correlated to sperm concentration and total sperm count, but only in the HIGH fraction and not in LOW or WE. Earlier studies have given somewhat conflicting results regarding the relationship between MMP levels and semen quality (mainly sperm concentration, total sperm count, and sperm morphology). Levels of latent and active forms of MMP-2 and MMP-9 in human SP have been shown to be inversely correlated to sperm concentration, total sperm count, sperm motility and the percentage of morphologically normal sperm [18,36], while Baumgart et al. [24] found no significant correlations. Similar inverse correlations have been reported also in canine semen samples, where pro-MMP-2, pro-MMP-9 and active MMP-9 levels were higher in samples with lower sperm count, sperm viability and percentage of morphologically normal sperm [22,37]. Results may vary between studies due to different methods and small sample sizes. Our study partly differs from earlier studies because we have focused on fractionated ejaculates, but WE samples were also included in the analyses and significant correlations were not found. Some of the other studies [22,36,37] have compared MMP levels between samples that have been categorized as normal or abnormal based on a set of parameters, such as sperm concentration, sperm count, and motility or morphology, whereas in our study the semen parameters were evaluated separately.

Sperm concentration and number of sperm were significantly correlated to the relative activity of active MMP-2 in HIGH, and pro-MMP-2 in the LOW fraction. A similar positive correlation between MMP-2 and sperm count has been reported in human semen samples [24], but data on the association between MMPs and sperm quality vary between studies and animal species. The correlation between MMP-2 and MMP-9 and sperm concentration suggests that these MMPs are derived from the glands contributing to the sperm-rich part of the ejaculate. Follicle-stimulating hormone stimulates MMP-2 production in Sertoli cells and initiates structural changes in these cells in rats [38], supporting spermatogenesis and increased sperm production [39]. Saengsoi et al. [22] showed that the levels of active MMP-2 were positively correlated with sperm motility and morphology in dogs, while active MMP-2 and pro-MMP-2 levels did not differ between normal and abnormal human semen samples in a study by Buchman-Shaked et al. [36]. Matrix metalloproteinase-2 and MMP-9 participate in spermatogenesis by remodeling extracellular matrix to enable germ cell migration [40]. They can also affect spermatogenesis through regulating apoptosis, as pro-apoptotic effects of MMP-9 have been detected [41]; these effects could be involved in testicular degeneration and declining fertility. Testicular degeneration in stallions can be related to either aging or some insult to the testes, such as heat stress [42], which has been shown to increase MMP-9 production in cumulus cells in cattle [43]. In dogs, the levels of active MMP-2 in sperm do not change after spermatogenesis is completed, and remain stable during transit through the epididymis and caudal reproductive tract [37]. Sperm-bound MMP-2 is localized in the acrosome region in human sperm [36], and functions in sperm-egg penetration [44], but possibly also in other processes.

The relative activity levels of MMPs were not related to differences in sperm longevity during cooled storage, measured as sperm motility and DFI. There was a harmful effect of SP on DFI

during storage, but this effect could not be attributed to differences in MMP relative activity in the ejaculate. In human semen, expression and activity of MMPs is regulated by oxidative stress [45], which can be a significant factor affecting sperm survival during semen storage. There is a correlation between sperm MMP-2 activity and the total antioxidant capacity of SP in men [46]. Extenders are used for semen storage partly to protect spermatozoa against oxidative damage, and this could explain why MMP relative activity were not a significant factor affecting sperm longevity in this study. Skim milk extenders themselves are also likely to contain variable amounts of MMPs, as milk contains various MMPs and other proteolytic compounds [47]. In our study, MMP levels were measured from SP frozen directly after semen collection, but the sperm samples were stored in skim milk extender.

Regulation of MMP secretion occurs at the level of gene expression, activation of latent forms, and inhibition of active MMPs by endogenous inhibitors, primarily tissue inhibitors of metalloproteinases (TIMPs) [19]. Even though we could not identify any effects of the relative activity of MMPs on DFI, we recently reported that higher DFI immediately after semen collection and less chromatin damage during semen storage were associated with upregulation of TIMP-2 [48].

5. Conclusions

Latent pro-MMP-2, active MMP-2 and total MMP-9 were present in all fractions of the stallion's ejaculate, with higher relative activity levels of the latent than active forms and the highest relative activity in the HIGH fraction. Because these MMPs are associated with sperm concentration and number of sperm, and they are emitted into the first sperm-rich fractions of the ejaculate, the glands contributing to these fractions are probably their main source. Based on

current evidence, the relative activity levels of MMPs are not useful as markers for semen quality other than sperm concentration and sperm count, or for sperm survival during storage. Further research could be directed at studying the regulation of MMP and TIMP expression and activation especially in the context of testicular degeneration, oxidative damage and environmental endocrine disruptors related to oxidative damage in sperm.

Conflict of interest statement

None to declare.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the corresponding author. The data are not publicly available due to privacy restrictions, e.g. their containing information that could compromise the privacy of research participants.

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Table 1. Mean (± SEM) values of the relative concentration of pro-matrix metalloproteinase (MMP)-2, active MMP-2, total MMP-9, and sperm quality parameters in ejaculate fractions with high (sperm rich fractions; HIGH) or low (sperm-poor fractions; LOW) sperm concentration, and in the whole ejaculate (WE). N=25-33

	HIGH	Range (min-max)	LOW	Range (min-max)	WE	Range (min-max)
Active MMP-2	0.34 ± 0.03[a,1]	0.10 – 0.65	0.22 ± 0.03[b,1]	0.08 – 0.65	0.22 ± 0.02[b,1]	0.11 – 0.40
Pro-MMP-2	0.45 ± 0.03[2]	0.09 – 0.70	0.49 ± 0.03[2]	0.32 – 0.68	0.53 ± 0.03[2]	0.31 – 0.84
Total MMP-9	0.36 ± 0.04[a,1]	0.06 – 0.74	0.21 ± 0.03[b,1]	0.05 – 0.48	0.22 ± 0.03[b,1]	0.09 – 0.48
Sperm motility parameters (DIFF = SP0-SP1):						
DIFF in total motility	0.1 ± 3.3	-25.3 – 33.75	-4.1 ± 2.8	-23.6 – 25.6	-8.6 ± 2.2	-25.3 – 4.0
DIFF in progressive motility	-0.2 ± 3.1	-29.3 – 28.6	-4.4 ± 2.8	-28.7 – 22.7	-6.9 ± 3.1	-30.6 – 13.5
DIFF in VAP	-10.5 ± 3.4	-43.0 – 23.0	-13.1 ± 3.6	-49.9 – 9.5	-12.5 ± 5.1	-38.0 – 32.6
DIFF in VCL	-20.9 ± 6.9	-81.9 – 58.6	-26.3 ± 7.3	-91.6 – 29.0	-22.3 ± 11.2	-78.1 – 88.7
DIFF in VSL	-5.8 ± 2.5	-29.1 – 14.4	-5.2 ± 2.5	-24.5 – 15.8	-6.1 ± 3.6	-24.8 – 21.0
Sperm concentration (x 10^6/mL)	275.3 ± 33.0	24.0 – 656.0	67.6 ± 16.1	7.0 – 280.0	127.9 ± 24.1	15.0 – 289.0
Number of sperm (x 10^9)	4.2 ± 0.6[a]	0.3 – 11.8	2.0 ± 0.6[b]	0.1 – 12.3	3.9 ± 6.3[a]	0.7 – 18.7
Morphologically normal sperm (%)	49.9 ± 3.4	22.0 – 68.5	39.9 ± 8.8	2.5 – 75.5	62.8 ± 1.2	20.5 – 64.0
DNA fragmentation index (DFI):						
DFI before storage	16.1 ± 3.3[1]	4.0 – 95.0	18.6 ± 4.0[1]	4.4 – 93.6	14.3 ± 1.9[1]	5.7 – 35.8
DFI after storage with SP	24.5 ± 2.3[2]	5.8 – 48.6	30.5 ± 4.1[2]	2.9 – 72.6	26.6 ± 2.5[2]	13.5 – 46.5
DFI after storage without SP	17.5 ± 1.8[a,3]	6.1 – 36.9	26.2 ± 2.9[b,3]	9.4 – 55.5	21.2 ± 1.9[b,3]	10.8 – 33.3
DIFF in DFI	5.8 ± 1.4	-12 – 21.5	2.7 ± 2.8	-32.6 – 31.1	5.4 ± 1.1	-0.1 – 13.5

[a,b]Values with different superscripts within the same row differ significantly (P<0.05).

[1,2]Values with different superscripts within the same column differ significantly (P<0.05).

DIFF in motility = the difference in sperm motility between samples stored with seminal plasma present and samples stored without seminal plasma; SP0 = samples stored without SP; SP1 = samples stored with SP; DFI = DNA fragmentation index; DIFF in DFI = the difference in DFI between samples stored without seminal plasma and samples stored with seminal plasma; VAP = average path velocity of sperm, VCL = curvilinear velocity; VSL = straight-line velocity.

Table 2. Linear regression results for pro-matrix metalloproteinase (MMP)-2, active MMP-2, total MMP-9 and sperm concentration and number of sperm. The results are shown separately for ejaculate fractions with high (sperm-rich fractions; HIGH) or low (sperm-poor fractions; LOW) sperm concentration. N=25.

		Regression coefficient β	R^2	p
Sperm concentration				
HIGH	**Active MMP-2**	**0.51**	**0.26**	**0.007**
	Pro-MMP-2	-0.09	0.01	0.632
	Total MMP-9	**0.62**	**0.38**	**0.001**
LOW	Active MMP-2	0.06	0.004	0.796
	Pro-MMP-2	**0.78**	**0.61**	**0.001**
	Total MMP-9	0.32	0.11	0.151
Number of sperm				
HIGH	**Active MMP-2**	**0.48**	**0.23**	**0.013**
	Pro-MMP-2	-0.17	0.03	0.412
	Total MMP-9	**0.47**	**0.22**	**0.015**
LOW	Active MMP-2	-0.02	0.00	0.936
	Pro-MMP-2	**0.56**	**0.31**	**0.008**
	Total MMP-9	0.11	0.01	0.620

Figure legends

Figure 1. Mean (± SE) values of total motility (TMOT, %), progressive motility (PMOT, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), and straight-line velocity (VSL, µm/s) in two cold-storage groups (with seminal plasma, SP1, and without seminal plasma, SP0) and in ejaculate fractions with high (HIGH) and low (LOW) sperm concentration, and in whole ejaculates (WE) [27].

*Asterisks indicate significant differences between SP1 and SP0 groups.

Figure 2. Mean (± SE) values of the DNA fragmentation index (DFI) in ejaculate fractions with high (HIGH) and low (LOW) sperm concentration, and in whole ejaculates (WE), in samples taken before storage, and in two cold-storage groups (with seminal plasma, SP1, and without seminal plasma, SP0) [27].

[abc]Different letters indicate significant difference between storage groups (before storage, SP1 and SP0) within an ejaculate fraction.

*Asterisks indicate significant differences between fractions within a storage group (SP1 and SP0).