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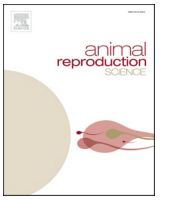

Deciphering sperm chromatin properties to predict stallion sperm fertility

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ABSTRACT

Although previous studies have examined the relationship between the sperm DNA fragmentation index and fertility in stallions, other aspects of chromatin structure or packaging and fertility have not been explored. In the present study, relationships between fertility and DNA fragmentation index, protamine deficiency, total thiols, free thiols and disulfide bonds in stallion spermatozoa were investigated. Ejaculates (n = 36) were collected from 12 stallions and extended to prepare semen doses for insemination. One dose from each ejaculate was sent to the Swedish University of Agricultural Sciences. Aliquots of semen were stained for flow cytometry with acridine orange for the Sperm Chromatin Structure Assay (DNA fragmentation Index, %DFI), with chromomycin A3 (CMA) for protamine deficiency, and with monobromobimane (mBBr) for detection of total and free thiols and disulfide bonds. Per season pregnancy rates after insemination were obtained. Mixed linear models were used to analyze data. Negative correlations were found between pregnancy rate and %DFI ($r = -0.35$, $P < 0.03$) and pregnancy rate and free thiols ($r = -0.60$, $P < 0.0001$). Furthermore, there were positive correlations between total thiols and disulfide bonds ($r = 0.95$, $P < 0.0001$), and protamine and disulfide bonds ($r = 0.4100$, $P < 0.01986$). Since chromatin integrity, protamine deficiency and packaging were all associated with fertility, a combination of these factors could be used as a biomarker of fertility when assessing ejaculates.

1. Introduction

Pregnancy rates per cycle in mares bred using cooled semen lie at approximately 67% in many countries e.g., Italy (Rota et al., 2004), Norway (Haadem et al., 2015), which means that one third of mares do not become pregnant following insemination in any one cycle. Both stallion and mare factors influence whether a pregnancy occurs. One of the causes of sub-fertility in stallions is thought to be sperm chromatin damage. Previous studies showed that the DNA fragmentation index is linked to subfertility or even infertility in stallions (Kenney et al., 1995). There may be a link between DNA damage and subsequent embryonic development (Katari et al., 2009; Johnson et al., 2011).

Sperm chromatin is important in transporting the genetic material from male to female. Many factors can contribute to sperm DNA fragmentation, such as the time elapsed since ejaculation, semen collection methods, sperm preparation techniques, storage temperature, cryopreservation, oxidative stress, abnormal spermatid maturation, testis temperature, age, and bacterial infections (González-Marin et al., 2012; McSwiggin and O'Doherty, 2018). However, aspects of sperm chromatin structure other than

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fragmentation, such as how tightly the DNA strands are packed, the presence of free thiols, and conversion of histones to protamines, also affect chromatin integrity and may be linked to fertility in some species. During spermatogenesis, most histones are replaced by protamines (Champroux et al., 2018), but a small proportion of histones may be retained. These retained histones are associated with subfertility in some species e.g. bulls (Boe-Hansen et al., 2018), and can be evaluated with chromomycin A3 (CMA3) staining. In addition, disulfide bond formation between and within DNA strands compacts the chromatin, helping to prevent damage to the DNA; the number of disulfide bonds may be linked to fertility (Oliva, 2006). Evaluation of disulfide bonds and free thiols by staining with monobromobimane (mBBr) could thus be used as a marker of fertility (Zubkova et al., 2005; Martínez-Pastor et al., 2010) but are not routinely included in sperm quality evaluation. In the mBBr-assay, the staining is performed with and without dithiothreitol (DTT); DTT breaks disulfide bonds to enable total thiols in the spermatozoa to be measured. Staining without DTT provides a measure of free thiols. The amount of disulfide bonds broken by DTT can thus be calculated from the results of these two measurements.

Some studies showed a relationship between chromatin packaging and male fertility; males of lower fertility had a higher amount of damaged DNA and less compact chromatin packaging than males of good fertility in human and bovine spermatozoa (Zini et al., 2001; Lewis, 2013; Narud et al., 2021). To our knowledge, such analyses have not been carried out in stallion spermatozoa. Therefore, the aim of the present study was to investigate the relationship between various aspects of sperm chromatin packaging and fertility in stallions.Protamine deficiency, and the presence of total and free thiols and disulfide bonds in chromatin (hereafter referred to collectively as “chromatin packaging”), were analysed, as well as the DNA fragmentation index, in semen doses from breeding stallions, and correlated with the per season pregnancy rate in inseminated mares. Our hypothesis was that these DNA strand breaks, protamine deficiency and chromatin packaging could be used as a biomarker for fertility in stallions.

2. Material and methods

2.1. Animals

Semen was collected from 12 privately owned adult stallions, 6 trotters and 6 warmblood sport horses, (36 ejaculates) during the breeding season. Their ages ranged from 5 to 21 years (mean 10.6 years). The animals were housed at stud farms in the vicinity of Uppsala, Sweden, in accordance with national and international regulations for housing and care of breeding animals. No ethical approval is required in Sweden for collection of semen from stallions using an artificial vagina. Pregnancy rate per season was calculated for the sampling year; excluding stallions with fewer than 10 inseminated mares . Pregnancy rate was calculated as the number of pregnant mares/number of inseminated mares * 100. The number of inseminated mares per stallion ranged from 15 to 205.

2.2. Semen

No ethical approval is required in Sweden for semen collection from stallions by artificial vagina, which is regarded as a normal husbandry practice. Semen was extended using one of three extenders: Equiplus (Minitüb, Tiefenbach, Germany), INRA96 (IMV-Technologies, l’Aigle France) or Botugold (Butopharma, USA), depending on which extender the stud had determined to be the best for that particular stallion in terms of preserving sperm motility during transport. The ejaculates were collected using a sterilized artificial vagina after the stallion had mounted a phantom. Each ejaculate was extended according to the standard protocol used to prepare semen doses at that stud. The semen doses were sent to other studs in an insulated box at 6 °C for insemination approximately 24 h after semen collection, and one dose was transported to SLU under the same conditions. The concentration (mean±SD) was $77 \pm 26 * 10^6$ spermatozoa/mL, as determined with a Nucleocounter (Chemometec, Allerød, Denmark). The total motility (mean±SD) was 66 ± 17%, as determined with the SpermVision motility analyser (Minitüb, Tiefenbach, Germany). Aliquots of extended semen were diluted 1:1 with TNE-buffer (see next section) and snap-frozen in liquid nitrogen. The samples were kept at − 80 °C until analysis.

2.3. Sperm analysis

The aliquots were thawed on crushed ice, and the sperm concentration was adjusted to $2 * 10^6$/mL using TNE-buffer.

2.3.1. Sperm Chromatin Structure Assay (SCSA)

The method of Evenson and Jost (2000) was used to determine the DNA fragmentation index (%DFI). To 20 μL semen, 80 μL of TNE were added. A detergent solution (200 μL) of a low pH containing 0.17% Triton X-100, 0.15 M NaCl and 0.08 M HCl (pH 1.2) was added. After 30 s, 600 μL acridine orange (AO) (6 μg mL−1 in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0) were added. Analysis was performed within 5 min of staining.

For each sample, a minimum of 10,000 events was analyzed at a speed of 200 cells/second after excitation with a blue laser (488 nm) using a FACSVerse flow cytometer (BDBioscieces, Franklin Lakes NJ, USA). The instrument was calibrated weekly using CS&T calibration beads (BDBiosciences). Reference samples were run at the start of each day. The forward scatter (FSC) and side scatter (SSC) as well as green and red fluorescence were collected, and a gate was applied in the FSC/SSC-distribution to restrict analysis to spermatozoa. The majority of the events were spermatozoa. The FL1 (green fluorescence) was collected through a band-pass filter (527/32 nm) and FL3 (red fluorescence) was collected using a band-pass filter (700/54 nm). The spermatozoa with higher red fluorescence as well as spermatozoa with high DNA staining were identified in the FL1/FL3 dot-plot. The DNA – Fragmentation Index (%DFI – the ratio of cells with denatured, single-stranded DNA to total cells acquired*100) was calculated for each sample using FCS Express version 5 (De Novo Software, Pasadena, CA, USA).

2.3.2. Protamine deficiency

After thawing, the samples (500 μL) were centrifuged at 300 g for 10 min. The supernatant was then removed, 500 μL PBS was added, the tubes were centrifuged at 300 g for 10 min and the supernatant was removed. After adding 100 μL McIlvanes buffer (17 mL 0.1 mol/L citric acid mixed with 83 mL 0.2 mol/L Na_2HPO_4 and 10 mmol/L $MgCl_2$, pH 7.0) containing 0.25 mg/mL CMA3 (Sigma-Aldrich) the samples were incubated for 20 min at 37 °C in the dark. After that, 500 μL PBS were added. The samples were centrifuged at 300 g for 10 min, the supernatant was removed and the pellet was resuspended in 500 μL PBS; 4 μL Propidium Iodide (PI) were added (sperm viability kit, L-7011; Invitrogen, Eugene, OR, USA). A flow cytometer (FACSVerse) equipped with a blue laser (488 nm) was utilized for analysis of the samples. The instrument was calibrated weekly using CS&T calibration beads (BDBiosciences). The same gains and voltages were used for all samples. Gating to restrict the sperm cell population was performed using a FSC/SSC-dotplot, and sperm cells were further identified by PI positive signal collected via a 586/42 bandpass filter. The PI signal was thus used for gating purposes only; PI fluorescence was not quantified. After excitation with a violet laser (405 nm), the arithmetic mean of CMA3 fluorescence from gated cells was collected through a 528/45 bandpass filter. Results were plotted on log-scale, although calculations were performed on actual values without log-transformation.

2.3.3. Free and total thiols (SH- groups) and disulfide bonds

The sample was divided into two aliquots (500 μL) in two tubes: to one of the tubes 1,4-dithiothreitol solution (DTT, Sigma-Aldrich) was added to a final concentration of 1 mM while the other was used as a control. Both tubes were incubated at 37 °C for 10 min; after the incubation both samples were centrifuged at 300 g for 10 min and supernatant was removed. Monobromobimane (100 μL, mBBr; Thermo-Fisher) at a final concentration of 0.5 mM was added to both samples, which were then incubated at 37 °C in the dark for 10 min. The samples were centrifuged at 300 g for 10 min. The supernatant was removed, 500 μL PBS were added to the tubes and they were analysed with a FACSVerse flow cytometer after excitation of mBBr by a 405 nm violet laser. The instrument was calibrated weekly using CS&T calibration beads (BDBiosciences). The same gains and voltages were used for all samples. The mBBr fluorescence was collected by a 528/45 bandpass filter, and gating of the sperm cell population was done using the FSC/SSC dot-plot. Results were plotted on log-scale, although calculations were performed on actual values without log-transformation. In order to calculate disulfide concentrations, the arithmetic means of the fluorescence signals of free thiols (mBBr fluorescence from non-DTT-treated sample) were subtracted from fluorescence signals of total thiols (mBBr fluorescence from DTT-treated sample); thereafter that value was divided by two, because one SH-bond yields two free thiol groups when broken by DTT.

2.4. Statistical analysis

All statistical analyzes were performed using SAS® software (version 9.4; SAS Institute Inc., Cary, NC). Data were tested for homogeneity of variance by Bartlett's test and for normal distribution by Shapiro Wilk test. Descriptive statistics (mean, standard deviation, median, boxplots) were calculated using the MEANS and SGPLOT procedure in the software.

Data on sperm chromatin properties were analyzed using PROC MIXED in the SAS software. The model also included the fixed effect of stallion. The data for protamine deficiency deviated from a normal distribution and were log transformed. However, to improve clarity and facilitate interpretation, the respective log-transformed value is presented as an untransformed value throughout the paper.

Least squares means (LSM ± SEM) estimated by the models were adjusted using the Scheffé adjustment for multiple post-ANOVA comparisons and compared.

The CORR procedure was used to compute Pearson correlation coefficients for the different sperm chromatin properties.

The alpha value for this experiment was selected to 5% and the p-values were compared based on the selected alpha value.

Table 1
Properties of stallion sperm chromatin (%DFI, protamine deficiency, total thiols and free thiols, and disulfide bonds; mean ± SD) and pregnancy rate (n = 12 stallions).

Stallion	ejaculate	%DFI	Protamine deficiency (mFL)	Total thiols (mFL)	Free thiols (mFL)	Disulfide bonds (mFL)	Pregnancy rate (%)
1	3	18.0 ± 2.0	1882.0 ± 213	40506 ± 7356	4183 ± 466	18162 ± 478	83
2	5	17.3 ± 2.1	2125.0 ± 150	28907 ± 6242	3779 ± 94	12564 ± 3130	73
3	3	32.1 ± 5.2	2561.0 ± 620	32288 ± 5551	8377 ± 592	11956 ± 2856	57
4	2	27.5 ± 2.0	2792.0	41996 ± 1583	6923 ± 1467	17536 ± 58	30
5	3	11.0 ± 2.3	3349.0 ± 1324	39547 ± 1576	4568 ± 536	17490 ± 954	79
6	3	15.2 ± 1.2	1816.0 ± 401	39790 ± 4099	4118 ± 1082	17836 ± 2050	85
8	4	16.7 ± 8.2	2016.0 ± 398	34324 ± 5876	5321 ± 741	14501 ± 2889	93
9	4	19.1 ± 5.4	3345.0 ± 174	40162 ± 1321	7713 ± 330	16224 ± 681	57
10	3	24.7 ± 9.7	2430.0 ± 483	42583 ± 897	6794 ± 318	17894 ± 290	20
11	3	26.3 ± 6.7	2807.0 ± 39	41755 ± 6376	3064 ± 587	19346 ± 2987	77
12	3	25.1 ± 4.5	1958.0 ± 318	33436 ± 3508	4553 ± 155	14442 ± 1804	70
13	2	32.8 ± 12.7	4350.0 ± 688	42821 ± 961	5254 ± 491	18784 ± 726	79

Note. mFL = mean fluorescence; %DFI = DNA fragmentation Index; %DFI was evaluated with the Sperm Chromatin Structure Assay (SCSA), protamine deficiency with chromomycin A3 staining, and thiols /disulfide bonds with monobromobimane staining.

3. Results

Sperm chromatin values (mean ± SD) and pregnancy rate for each stallion are given in Table 1. There was considerable variation among stallions for all variables: %DFI ($P < 0.0038$), protamine deficiency ($P < 0.0036$), total thiols ($P < 0.003$), free thiols ($P < 0.0001$), disulfide bonds ($p < 0.0014$) (Fig. 1).

Correlations were seen between pregnancy rate and %DFI ($r = -0.35$, $p < 0.034$) and between pregnancy rate and free thiols ($r = -0.603$, $P < 0.0001$) (Fig. 2). There were no correlations between pregnancy rate and protamine deficiency ($r = -0.2$; $P = 0.25$), pregnancy rate and total thiols ($r = -0.11$; $P < 0.39$), or pregnancy rate and disulfide bonds ($r = -0.11$, $P < 0.18$). Correlations were

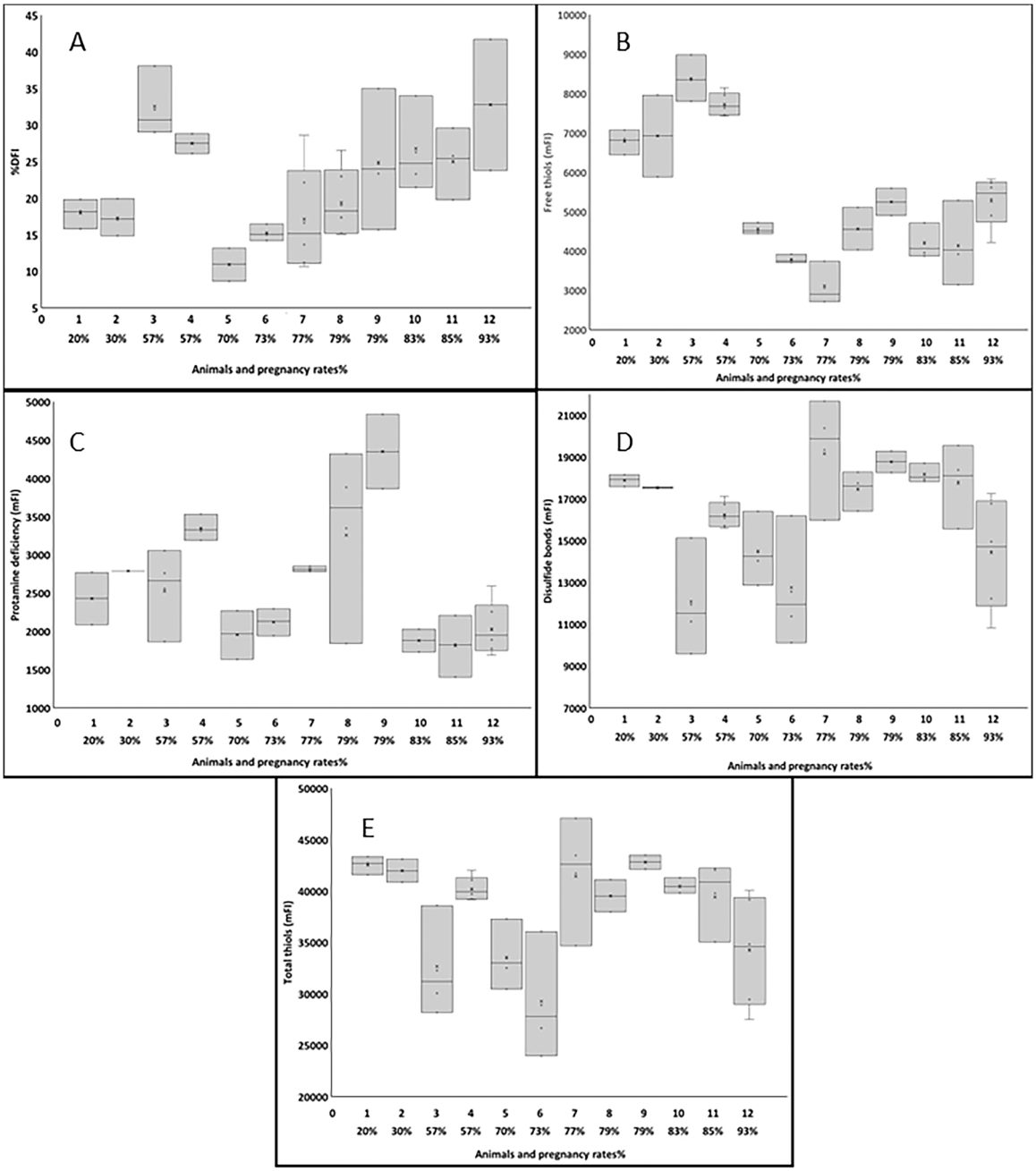

Fig. 1. Variation among stallions (A) DNA fragmentation index (%DFI); (B) protamine deficiency; (C) free thiols; (D) total thiols; and (E) disulfide bonds ($n = 12$). Note: Boxplot displays the distribution of data based on a five-number summary: 1. minimum, 2. first quartile (Q1), 3. median, 4. third quartile (Q3), and 5. maximum. %DFI was evaluated with the Sperm Chromatin Structure Assay, protamine deficiency with chromomycin A3 staining and total /free thiols and disulfide bonds with monobromobimane staining.

found between protamine deficiency and disulfide bonds (r = 0.13, $P < 0.026$) (Fig. 2), and between total thiols and disulfide bonds (r = 0.95, $P < 0.0001$) (Fig. 2).

4. Discussion

Several studies in humans and animals have linked sperm DNA integrity with fertility. The objective of the present study was to investigate the possible role of DNA protamine deficiency and disulfide bond formation in stallion fertility, in addition to the %DFI. Significant negative correlations were observed between pregnancy rate and free thiols, and between pregnancy rate and %DFI, although the association between pregnancy rate and protamine deficiency was not significant.

The negative relationship between DNA fragmentation and fertility seen in the present study is in agreement with previous studies

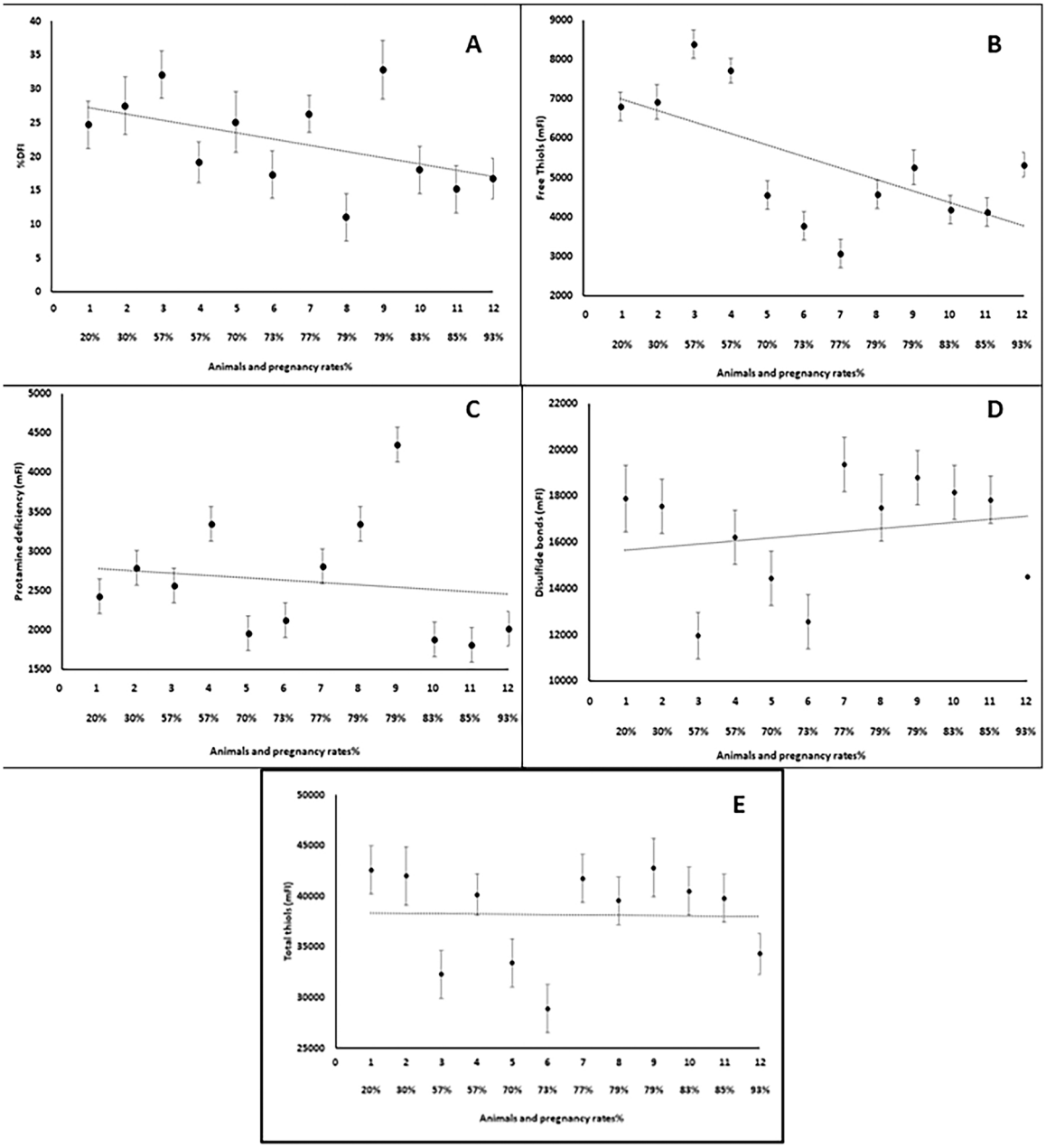

Fig. 2. Relationships between per season pregnancy rate and A: DNA-fragmentation index, r = - 0.35, $P < 0.03$; B: free thiols, r = - 0.60, $P < 0.0001$; C: protamine deficiency, r = - 0.2, $P < 0.25$; D: disulfide bonds, r = 0.23, $P < 0.18$; total thiols r = −0.04, $P < 0.81$), in stallion spermatozoa (n = 12 stallions). Notes: %DFI = DNA fragmentation index.

in stallion (Love and Kenney, 1998; Morrell et al., 2008), human (Waterhouse et al., 2006; Urdinguio et al., 2015; Kumaresan et al., 2017a), bull (Boe-Hansen et al., 2008; Kropp et al., 2017; Kumaresan et al., 2017b; Narud et al., 2021), and boar (Didion et al., 2009; Paradowska-Dogan et al., 2014). Love (1998) showed a relationship between SCSA and fertility in several species, including stallions. Kumaresan et al. (2017a) reported that human patients with unknown infertility based on conventional semen analysis had high sperm DNA damage. Furthermore, spermatozoa from below-average fertility bulls had worse DNA integrity than spermatozoa from higher fertility bulls (Kropp et al., 2017). A significant association was found between DNA integrity and bull field fertility, as well as between DNA integrity and cleavage rate in an in vitro fertilization study (Narud et al., 2021).

In our study, there was a correlation between protamine deficiency and free thiols, the latter being negatively associated with fertility. In a previous study in stallion spermatozoa (Paradowska-Dogan et al., 2014), the ratio of protamine 1 and 2 was analysed and found to be associated with infertility (Paradowska-Dogan et al., 2014). Kadivar et al. (2019) observed that three variants of protamine occur in stallion spermatozoa; if the ratios of the variants are incorrect, infertility ensues. These studies show the importance of protamine in male fertility in general, not just in stallions. In mammal sperm chromatin, the conversion of histones to protamines is essential for the final stages of maturation and chromatin condensation. Without this conversion, or if protamine deficiency is present, subfertility or even infertility is likely to occur (Akmal et al., 2016). Moreover, the latter authors reported that spermatozoa with protamine deficiency often show abnormal morphology and decreased motility.

A negative association between fertility and free thiols was observed in the present study. In a similar study using boar sperm samples, a significant correlation between %DFI and free thiols was reported (Khezri et al., 2019), although no such correlation was found in the present study. Another important finding in the study on stored boar sperm samples was that total thiols and free thiols decreased whereas the DNA fragmentation and protamine deficiency increased during storage at 18 °C (Khezri et al., 2019). Since stallion semen is usually cooled and stored at 6 °C for up to 36 h prior to insemination, a general loosening of the highly condensed DNA with time could make the DNA more prone to damage during cooled storage, for example by attack from reactive oxygen species. However, in the present study, the evaluation was made at only one-time point, on the day after semen collection, which is when insemination of cooled semen doses typically occurs. Therefore, the effect of storage on DNA packaging remains to be investigated.

In studies with human spermatozoa, there was a correlation between free thiols and DNA denaturation; the levels of free thiols and DNA denaturation were higher in infertile men than in fertile men (Zini et al., 2001). This observation could fit with the theory that the formation of disulfide bonds helps to pack the DNA more tightly, protecting it from damage. The more free thiols that are present, the fewer disulfide bonds there are and therefore the looser the DNA packaging. In another study in men suffering from fertility problems, there was a positive correlation between free thiols and oxidative stress (Bergsma et al., 2022). Thiol oxidation was observed to be related to reduced sperm motility (Cabrillana et al., 2016).

Although chromatin assays provide indirect information on subfertility or fertility in stallions, many factors (both male and female) are involved in whether a pregnancy can be established in any particular female or not. Therefore, to test male fertility in vivo, many females should be inseminated (Amann, 2005). However, this is not practical in horse breeding where relatively few mares are inseminated with semen from each stallion, compared to cattle where several hundred cows can be inseminated from each ejaculate.

Some causes of sub- or infertility are known (Krausz et al., 2015), but other causes are still unknown (Urdinguio et al., 2015). The inability to identify subfertile males and remove them from the breeding pool has economic repercussions for livestock production and for horse breeding. The results of the present study suggest that evaluating the presence of free thiols, disulfide bonds and protamine deficiency, in addition to the %DFI, would facilitate identification of sub-fertile stallions, to allow sub-standard ejaculates to be discarded. However, a larger study is required to confirm these preliminary findings.

5. Conclusion

There was considerable variation in %DFI, protamine deficiency, free thiols, total thols and disulfide bonds among stallions. Pregnancy rate was correlatied with %DFI and free thiols in our study population; in addition there was a correlation between free thiols and protamine deficiency. Therefore, it might be beneficial to include measurement of free thiols and protamine deficiency in routine evaluation of sperm quality in stallion sperm samples, and to investigate the changes occurring during storage of such samples. Chromatin packaging in general should be investigated in a larger number of stallions of known fertility. Free thiols and %DFI can be used as predictive biomarkers for fertility in stallions.

Conflict of Interest

The authors have no conflict of interest to declare.

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