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Quality of cooled semen of cold-blooded stallions evaluated with the use of apoptosis and DNA defragmentation markers

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Summary

The aim of this study was to determine the quality of semen collected from stallions and chilled to +4°C. Semen was collected from eight Polish cold-blooded stallions, aged 4-8 years. The ejaculate was examined macro- and microscopically to evaluate volume, concentration, viable sperm percentage, progressive motility, and morphology. The percentage of live/dead spermatozoa was determined by eosin and nigrosin staining. Flow cytometry was applied to detect phosphatidylserine translocation, whereas DNA defragmentation was determined with a commercial kit. The semen samples were examined immediately after collection and dilution (time 0) and at 6 storage times: 24, 48, 72, 96, and 120 hours later.

Time-related changes in all the investigated spermatozoa parameters were observed at 6 different times. Progressive motility was negatively correlated with the percentage of live spermatozoa with phosphatidylserine translocation, but positively correlated with DNA defragmentation ($P < 0.05$).

This study demonstrates that the evaluation of selected indicators of apoptosis is very useful in assessing the quality of chilled stallion semen. The results obtained show what significant changes occur in spermatozoa during storage at +4°C. Evaluation of the cell membrane integrity and DNA defragmentation of spermatozoa makes it possible to determine whether and for how long semen chilled to +4°C may be used for artificial insemination, which is extremely important during semen transportation. The methods of semen quality evaluation applied in this study should also be used in determining the usefulness of chilled semen for artificial insemination. This may lead to higher fertilization rates in mares.

Keywords: stallion, sperm, DNA defragmentation, apoptosis, flow cytometry

Semen evaluation provides useful data on spermatozoa fertilization ability, and can be used for the assessment of epididymis and testis functions (5, 26). Semen evaluation in most mammal species involves determining the concentration and progressive motility of spermatozoa, as well as their morphological characteristics and classification. All these indicators of semen quality are obtained with the use of the light microscopy technique. However, some reports point out the limitations of this diagnostic method in determining spermatozoa fertilization ability and in evaluating semen quality (20, 25).

To perform a deeper analysis of the spermatozoa functional state, novel diagnostic techniques based on fluorescence microscopy and flow cytometry have recently been introduced (10, 20). Both these techniques make it possible to analyze spermatozoa properties faster and much more precisely (10). These advantages are extremely important in the case of semen collected from stallions, especially when one considers the rarity of natural mating in horses and the constantly increasing number of artificial inseminations (AI) with frozen or fresh semen, diluted and cooled to a temperature of +4°C (12). Moreover, semen collected from stal-

lions is transported routinely over long distances, which involves a considerable time gap between semen collection and fertilization. Even though semen temperature change is required for the routine preservation procedure, it is not a physiological process to which spermatozoa is physiologically adapted. Thus, semen cooling or freezing has a negative impact on spermatozoa motility and limits their fertility rate (2, 8, 17, 27). As proven in previous investigations, semen cooling or freezing disturbs spermatozoa membrane integrity, which results in higher oxidative stress and the increased formation of reactive oxygen species (ROS) (2). It was confirmed in experimental studies that the longer the semen preservation time, the higher the number of DNA damages (8, 18).

The apoptosis process has been found to be strongly connected with fertility disturbances, and has been examined extensively in relation to the andrological examination of semen quality (1, 11, 15, 22, 23). The formation of apoptotic bodies in spermatozoa during apoptosis has been recognized as a major characteristic change. These vesicular structures feature phosphatidylserine (PS), located on the external surface of the spermatozoa membrane, which is important for recognition and phagocytosis by phagocyting cells (9, 11). The presence of phosphatidylserine on the external surface of spermatozoa may be considered as an initiation of cell membrane integrity loss, which leads to further apoptotic stages, such as the cascade caspase reaction and the degradation of cellular proteins (9). However, evaluation of spermatozoa chromatin is considered as the most important indicator of spermatozoa apoptosis (16, 19, 21). This evaluation indicates the susceptibility of spermatozoa to DNA defragmentation. To performer effective fertilization, spermatozoa must fulfill numerous physiological functions. Recent experimental data indicate that the integrity of the cellular membrane and the DNA defragmentation status in spermatozoa significantly reduce the fertility rate (17, 21). DNA damages may be a causative factor of morphological changes in spermatozoa and of their decreased survival, or may even lead to genetic defects of embryos, resulting in their death and the following resorption at early pregnancy stages (3, 6, 7, 9, 16). It was shown in previous studies that defragmentation of spermatozoa DNA exceeding 30% significantly reduces the fertility rate and doubles the rate of spontaneous abortion (16).

In the literature there is no detailed information concerning changes that occur in semen collected from heavy draft stallions and stored at +4°C for 120 h. Therefore, the aim of this study was to determine the quality of that semen using flow cytometry and apoptotic markers.

Material and methods

The study was carried out between the end of April and beginning of June 2012 at a horse-breeding farm in eastern Poland. Semen was collected from eight fertile Polish cold-blooded stallions, aged 4-8 years. The stallions were clinically healthy, and their sperm was regularly used for artificial insemination. The semen samples were collected with an artificial vagina (the Missouri model), 3 ejaculates from each stallion. After the separation of the gel fraction, part of the ejaculate was examined macro- and microscopically to evaluate the volume, concentration, viable sperm percentage, progressive motility, and morphology. The remaining ejaculate was diluted with the INRA 96 extender (IMV Technologies, L'Aigle, France) to the final concentration of 25×10^6 and chilled to + 4°C. The sperm concentration was assessed photometrically (SpermaCue – Minitüb Abfüll- und Laboretechnik GmgH, Tiefenbach, Germany). Progressive motility of spermatozoa was determined with a computer-assisted sperm class analyzer (SCA) (MICROPTIC S.L). The system consisted of an Olympus CX4 light microscope with a Basler A312 camera and a computer equipped with appropriate software. The motility of spermatozoa was assessed with 20-µl Leja slides (Leja Amsterdam, The Netherlands); 5 µl aliquots of spermatozoa warmed to 37°C were used for analysis. Evaluation of sperm morphology was performed with a Diff Quik kit (Sigma – Aldrich, Vienna, Austria). The percentage of live/dead spermatozoa was defined by staining the specimens with eosin and nigrosin. Flow cytometry (Epics XL Beckman-Coulter, Comesa CH-Werfen Company, Miami FL, USA) was applied to detect phosphatidylserine translocation (Annexin V-FITC Apoptosis Detection Kit I; BD Pharmingen 556547), whereas DNA defragmentation was determined with an APO BRDU™ Detection Kit. The semen samples were examined immediately after collection and dilution (time 0) and at 5 storage times: 24, 48, 72, 96, and 120 h.

Annexin V-FITC assay. Semen samples were washed twice in the HEPES buffer (NaCl, 0.01 M Hepes/NaOH, pH 7.0) and centrifuged at 500 × g for 10 minutes. Then 5 µl of Annexin V-FITC and 5 µl of propidium iodide (Pi) were added to 100 µl of sperm. The samples were gently mixed and incubated at room temperature in the dark for 15 min. Following the incubation, the samples were supplemented with 500 µl of the binding buffer solution and analyzed by flow cytometry. Flow cytometry histograms and Annexin V-FITC/Pi were used to assess the percentage of live (green fluorescence – A–/Pi–) and dead (red fluorescence – A–/Pi+) spermatozoa. Moreover, dead spermatozoa were determined with translocation of phosphatidylserine (A+/Pi+), and live spermatozoa were determined with translocation of phosphatidylserine (A+/Pi–).

TUNEL assay. Sperm DNA integrity was determined by the TUNEL method (terminal deoxynucleotidyl transferase (TDT)-mediated dUDP nick-end-labelling). Sperm samples were diluted in PBS (0.01 M NaH_2PO_4, pH 7.2 and 1.5 M NaCl) to the concentration of 2×10^6 sperm/ml, and the spermatozoa were fixed by adding 1% paraformaldehyde in PBS for 15 minutes. After fixation, the spermatozoa were washed in PBS twice, suspended in cold 70% ethanol and stored at –20°C for 24 h. Before analysis, the samples were centrifuged to remove alcohol and washed twice in PBS.

Then, 5 µl of proteinase K was added (20 µl/ml dissolved in 10 mM Tris-HCL, pH 7.6) to each 100 µl of thus processed sperm. The samples were incubated at room temperature for 20 min and washed in 1 ml of the wash buffer twice. Subsequently, 50 µl of the DNA-labelling solution containing 0.75 µl of TDT, 8.0 µl of BrdU, 10 µl of TDT, and 32.25 µl of distilled water were added to each sample, and the samples were incubated for 4 h in a water bath at 37°C, with gentle mixing every 30 minutes. After incubation, they were washed twice in 1 ml of the rinse buffer and 100 µl of the antibody solution containing 5 µl of fluorescein-labelled anti-BrdU. Next, 95 µl of the rinse buffer was added, and the samples were further incubated at room temperature in the dark for 30 minutes. The control samples were prepared in the same way, but without the addition of TDT to the DNA-labeling solution, and analyzed by flow cytometry. Green fluorescence (480-530 nm) and red fluorescence (580-630 nm) were measured in the FL-1 logarithmic and FL-linear channel, respectively. The results were presented as a percentage of spermatozoa with DNA defragmentation ApoBrDu (+). The flow cytometry histogram differentiates spermatozoa with DNA defragmentation ApoBrDu+ (red fluorescence) and those without DNA damage ApoBrDu – (green fluorescence).

Statistical analysis. Statistical analysis was performed by Statistica software (version 6.0, StatSoft, Inc., Tulsa, OK, USA). All data are presented as means ± SEM. The Kolomogorov-Smirnov test revealed a normal distribution of the collected data. Differences between the values obtained for several periods of time were tested for statistical significance by one-way ANOVA. Post hoc comparisons of the differences were performed by Duncan's test. For all comparisons, $P \leq 0.05$ was considered as statistically significant. Pearson's correlation coefficient (r) was determined for all the variables of semen investigated, and $P < 0.05$ was considered as statistically significant.

Results and discussion

Basic stallion semen characteristics are presented in Table 1. The mean volume of ejaculates amounted to 32.2 ± 8.75 ml, and sperm concentration was 353.4 ± 154 × 10^6. The percentages of live sperm, progressive sperm motility, and normal morphology of sperm reached the values of 74.0 ± 15.1%, 31.4 ± 7.4%, and 61.4 ± 14.0%, respectively.

Time-related changes of all the spermatozoa parameters at 6 different times are shown in Table 2. Compared to T-0, the percentage of live sperm (A–/Pi–) decreased significantly at T-3, T-4 and T-5 storage times ($P \leq 0.01$). The mean values of A–/Pi– were gradually reduced at T-4 and T-5, compared to T-3, and differences between all these periods were statistically significant ($P < 0.05$). The values of A–/Pi– assessed at T-4 and T-5 were significantly different from those obtained at T-1 and T-2 ($P < 0.01$). The percentage of live spermatozoa with phosphatidylserine translocation (A+/Pi–) significantly decreased at T-2, compared to T-0 ($P = 0.03$). The percentage of dead spermatozoa with phosphatidylserine translocation (A+/Pi+) was significantly lower at T-5 compared to all the other periods ($P < 0.01$). The percentage of dead spermatozoa (A–/Pi+) significantly increased at T-3, T-4, and T-5, compared to T-0 ($P \leq 0.01$). The mean values of A–/Pi+ rose gradually at T-4 and T-5, compared to T-3, and the differences between all these periods were statistically significant ($P < 0.05$). Statistically significant differences were also found between A-/Pi+ determined at T-4 and T-5, compared to T-1 and T-2 ($P < 0.001$). The percentage of spermatozoa with DNA defragmentation (ApoBrDu+) was significantly elevated at T-2 and T-3 when compared to the semen collected at T-0 and T-1. The percentage of spermatozoa with DNA defragmentation was the highest at T-4 and T-5, and significantly different from all the other periods ($P < 0.001$).

The values of Pearson's coefficient of correlation between all the spermatozoa parameters investigated at 6 different periods are presented in Table 3. A–/Pi– was positively correlated with A+/Pi–, but negatively correlated with all the other parameters, and the correlations were statistically significant (all $P < 0.05$). Statistically significant negative correlations of A+/Pi–

Tab. 1. Quantitative and qualitative properties of fresh semen collected from cold-blooded stallions (n = 8)

Stallions	Volume (ml)	Sperm concentration (× 10^6)	Viable sperm (%)	Progressive motility (%)	Normal morphology (%)
n = 8	32.2 ± 8.75	353.4 ± 154	74.0 ± 15.1	31.4 ± 7.4	61.4 ± 14.0

Explanation: Values are means ± SEM

Tab. 2. Results of apoptosis and DNA defragmentation in semen during storage at +4°C for 120 h (n = 8)

Parameter	T-0 (0 h)	T-1 (24 h)	T-2 (48 h)	T-3 (72 h)	T-4 (96 h)	T-5 (120 h)
A–/Pi– (%)	76.5^{a} ± 4.7	68.7^{ab} ± 5.2	62.6^{ab} ± 5.8	55.1^{b} ± 7.2	33.7^{c} ± 6.3	15.0^{d} ± 3.8
A+/Pi– (%)	1.437^{a} ± 0.350	0.691^{ab} ± 0.125	0.529^{b} ± 0.076	0.886^{ab} ± 0.236	0.703^{ab} ± 0.341	0.676^{ab} ± 0.263
A+/Pi+ (%)	1.368^{a} ± 0.397	1.056^{a} ± 0.259	1.191^{a} ± 0.332	1.597^{a} ± 0.327	2.100^{a} ± 0.600	3.853^{b} ± 0.479
A–/Pi+ (%)	20.6^{a} ± 4.4	29.0^{ab} ± 5.2	34.1^{ab} ± 5.2	43.4^{b} ± 7.7	63.6^{c} ± 6.4	80.4^{d} ± 3.7
ApoBrDu+ (%)	18.3^{a} ± 4.8	30.1^{a} ± 6.3	49.1^{b} ± 4.4	54.1^{b} ± 4.4	78.1^{c} ± 3.8	86.1^{c} ± 3.3

Explanations: Values are means ± SEM. a-d – mean values that do not share a common superscript letter in the same row differ significantly for $P < 0.05$ as compared by Duncan's multiple range test

and A–/Pi+, and ApoBrDu+ were observed ($P < 0.05$). ApoBrDu was significantly positively correlated with A+/Pi+ and A–/Pi+ ($P < 0.05$). Furthermore, statistically significant positive correlation was found between A+/Pi+ and A–/Pi+ ($P < 0.05$).

The values of Pearson's correlation coefficient for the basic parameters of semen and the investigated parameters of spermatozoa in stallions are presented in Table 4. Progressive motility was found to be significantly negatively correlated with A+/Pi–, but positively correlated with ApoBrDu (both $P < 0.05$).

Tab. 3. The values of Pearson's coefficient of correlation between the selected parameters of semen during storage at +4°C for 120 h (n = 8)

Parameter	A–/Pi–	A+/Pi–	A+/Pi+	A–/Pi+	ApoBrDu+
A–/Pi–	x	0.30*	–0.62*	–0.99*	–0.69*
A+/Pi–	0.30*	x	–0.17	–0.32*	–0.29*
A+/Pi+	–0.62*	–0.17	x	0.58*	0.49*
A–/Pi+	–0.99*	–0.32*	0.58*	x	0.68*
ApoBrDu+	–0.69*	–0.29*	0.49*	0.68*	x

Explanation: * $P < 0.05$

Tab. 4. The values of Pearson's coefficient of correlation between the selected parameters of semen during storage at +4°C for 120 h (n = 8)

Parameter	A–/Pi–	A+/Pi–	A+/Pi+	A–/Pi+	ApoBrDu+
Volume (ml)	0.09	0.08	–0.38	–0.07	0.10
Sperm concentration (× 10^6)	–0.53	0.19	0.15	0.53	–0.31
Viable sperm (%)	0.43	0.29	–0.55	–0.44	0.24
Progressive motility (%)	0.45	–0.69*	–0.57	–0.38	0.90*
Normal morphology (%)	–0.63	0.27	0.57	0.60	–0.53

Explanation: * $P < 0.05$

According to the recent findings, apoptosis is considered the main reason of spermatozoa damage during preservation at low temperatures (23, 24). Investigations performed on men and animals, such as bulls, boars and stallions, have revealed that the majority of spermatozoa are programmed during ejaculation to die by apoptosis (1, 15, 22, 23). Phosphatydylserine (PS) translocation from the internal membrane surface of live spermatozoa to the external cellular surface is one of characteristic processes occurring during apoptosis and apoptotic body formation (2, 22). Owing to this phenomenon, PS becomes available for annexin V – a protein characterized by high affinity for PS. Connection of annexin V with fluorescent stain such as Fluorescein isothiocyanate (FITC) makes it possible to detect PS translocation by flow cytometry (1, 9). Moreover, in order to differentiate between apoptotic and necrotic cells by this method, additional staining is applied with the use of propidium iodide (Pi) connecting to DNA. Propidium iodide penetrates the lipid barrier only in cells with a damaged cellular membrane (10). DNA condensation process is another extremely important indicator of apoptosis in spermatozoa (3, 18). At the late stage of apoptosis, the majority of ejaculated spermatozoa are characterized by DNA defragmentation. These changes may be detected by the terminal deoxynucleotidyl transferase-mediated dUDP nick end-labeling (TUNEL) method and by flow cytometry or fluorescence microscopy (20). As suggested by many authors, the analysis of DNA integrity is a crucial criterion to evaluate the usefulness of semen for preservation and its biological quality (5, 21).

Investigations performed by Love et al. (17) indicate that fresh semen collected from fertile stallions and chilled to +5°C should maintain optimal chromatin integrity for at least 46 hours. These results are in accordance with our previous studies on stallion semen characteristics (14) and reports provided by other researchers (5, 8, 27). However, some breed-related differences were found in the characteristics of semen chilled to +4°C. In the semen of Arabian stallions, nuclear chromatin integrity was maintained only up to 24 hours of storage (14). This may indicate time-differentiated sperm viability without spermatozoa cell membrane integrity disruptions in stallions of different breeds (13, 14).

In studies performed by Freitas-Dell'Aqua et al. (8) concerning the quality evaluation of cooled semen with the use of apoptotic markers, it was shown that semen may be stored at either +15°C or +5°C, maintaining optimal quality for up to 24 hours. For storage longer than 24 hours, a storage temperature of +5°C is recommended. It was experimentally confirmed that chilled semen retains optimal quality for up to 48 hours at a temperature of +5°C. In other studies performed by Gallardo-Bolanos et al. (9), it was shown that fresh diluted (FD) semen chilled to +5°C or subjected to a single layer colloidal centrifugation (CC) procedure may be characterized by high spermatozoa viability for up to 5 days. However, the percentage of viable sperm in CC semen was significantly higher than in the case of the FD procedure (9). Additionally, in studies with the use of Yo-Pro-1/Eth and Annexin V markers reflecting the stage of apoptosis and necrosis, the percentage of viable sperm with a non-disturbed cell membrane (Yo-Pro –) was significantly decreased on the 3rd day of the storage only for the CC procedure. However, in the case of fresh diluted semen, the percentage of viable sperm with non-disturbed cell membrane (Yo-Pro –) was significantly decreased on the 5th day of the storage. Using Annexin V, the percentage of live sperm with an intact cell membrane (A–/Pi–) was shown to

be significantly decreased on the 3rd day of storage in samples obtained with the use of both semen handling procedures. It is intriguing that in the study on cell membrane integrity assessment by the SYBR-14/Pi method, a higher percentage of live spermatozoa with an intact cell membrane was found on the 2nd day of storage, compared to the results obtained 24 hours earlier (9).

The results obtained in the present study and concerning apoptotic changes in spermatozoa during storage at +4°C are generally in accordance with previous reports (2, 8, 20, 27). However, a detailed analysis of apoptosis and the DNA defragmentation rate during the storage procedure has shown some differences. The assessment of cell membrane permeability with the use of propidium iodide and of phosphatydylserine translocation during spermatozoa apoptosis with the use of Annexin V-FITC, has revealed that the cell membrane integrity of spermatozoa without PS translocation (A–/Pi–) was unchanged until after 48 hours of storage. A significant decrease in the number of live spermatozoa (A–/Pi–) was noted after 72, 96, and 120 hours of storage time. A decreased number of live spermatozoa at those times was associated with an increased percentage of dead spermatozoa (A–/Pi+). The results obtained show statistically significant correlations between the apoptosis indices, which confirm time-dependent negative functional and structural changes in spermatozoa occurring as a result of long-term storage of chilled semen. These results confirm the typical course of the spermatozoa aging process related to the acceleration of negative changes in the cell membrane and DNA. The present study has also revealed correlations between parameters characterizing basic semen properties and the investigated apoptosis indices. Phosphatidylserine translocation (A+/Pi–) was negatively correlated with progressive motility, whereas a significant positive correlation was observed between sperm progressive motility and DNA defragmentation. This observation may be explained by the fact that during late apoptosis characterized by DNA damage, there is also a decrease in the mitochondrial membrane potential, resulting in depolarization of the mitochondrial cell membrane and cytochrome C release, inducing cell death (10). In our study, analogous changes in the DNA defragmentation index (ApoBrDu+) were observed, expressed as the percentage of spermatozoa with DNA defragmentation. Changes in the DNA defragmentation index are gradual, and the first significant decrease in ApoBrDu+ was observed after 48 and 72 hours of storage, and then after 96 and 120 hours of storage. However, some controversies exist about whether PS translocation without cell membrane integrity disruptions may be considered a meaningful indicator of semen quality.

Studies performed by Brum et al. (4) and Freitas-Dell'Aqua et al. (8) indicate a relatively poor usefulness of Annexin V as a marker for semen quality evaluation. It seems to be confirmed by the results obtained in the present study, since PS translocation assessment did not reveal important determinations of spermatozoa viability during long-term storage (4, 8). Certainly, the evaluation of caspases 3 and 9 would explain whether PS translocation in the cell membrane initiates the synthesis of caspases leading to spermatozoa death. It is worth mentioning here that PS translocation to the external cell membrane surface may result in antigen recognition and the subsequent phagocytosis of spermatozoa by phagocytes present in the uterus (9). Thus, a high percentage of spermatozoa with PS translocation in fresh semen may lead to a low fertility rate.

In conclusion, results obtained in this study on cold-blooded stallions have shown that the evaluation of selected indicators of apoptosis is very useful in assessing the quality of chilled stallion semen. This study has revealed significant changes that occur in spermatozoa during the storage of semen chilled to +4°C. The evaluation of cell membrane integrity and DNA defragmentation of spermatozoa makes it possible to determine whether and for how long semen chilled to +4°C can be used for the artificial insemination of mares, which is extremely important in the case of long-distance semen transportation. It is proposed that the methods of semen quality evaluation applied in this study should be additionally used in determining the usefulness of chilled semen for artificial insemination. This may lead to higher fertilization rates in mares.

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