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Original article

Applicability of 2,4-dinitrophenylhydrazine (DNPH) method of protein oxidative damage measurement in the seminal plasma of canine (*Canis lupus familiaris*) and stallion (*Equus caballus*)

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Abstract

Seminal plasma (SP) proteins are responsible for sperm functional quality. Developing a reliable method to determine the degree of oxidative damage of these proteins is important for establishing semen fertilizing ability. The main aim of the study was to verify the applicability of protein carbonyl derivatives measurement in the SP of canine and stallion, using a method with 2,4-dinitrophenylhydrazine (DNPH). The research material consisted of ejaculates obtained from eight English Springer Spaniels, and from seven half-blood stallions during the breeding and non-breeding season. The content of carbonyl groups in the SP was measured on the basis of the reactions with DNPH. The following reagent variants were used to dissolve protein precipitates: Variant 1 (V1) – 6M Guanidine solution and Variant 2 (V2) – 0.1M NaOH solution. It has been shown that to obtain reliable results for the measurement of protein carbonylated groups in the dog and horse SP, both 6M Guanidine and 0.1M NaOH may be used. A correlation was also found between the number of carbonyl groups and the total protein content in the canine (V1: r = -0.724; V2: r = -0.847) and stallion (V1: r = -0.336; V2: r = -0.334) SP. Additionally, the study showed a higher content ($p \leq 0.05$) of protein carbonyl groups in the stallion SP in the non-breeding season compared to the breeding season. The method based on the reaction with DNPH, due to its simplicity and cost effectiveness, appears to be suitable for large-scale application in the determination of the SP proteins oxidative damage in dog and horse semen.

Keywords: 2,4-dinitrophenylhydrazine, canine, protein carbonylation, seminal plasma, stallion

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Introduction

Several authors have shown that semen cryopreservation causes an increase in reactive oxygen species (ROS) production (Li et al. 2010, Klaiwattana et al. 2016, Mostek et al. 2018, Figueroa et al. 2019). Conversely, when the balance between ROS production and detoxification by antioxidants is disrupted, an excess of ROS creates oxidative stress. Scientific research has demonstrated that long-term oxidative stress leads to the irreversible modification of proteins and the gradual accumulation of their oxidatively modified forms in the body (Huang et al. 2018). Mechanisms of oxidative protein modifications are based on: oxidation of sulfur-containing amino acids, oxidation of aromatic moieties, glycoxidation, lipoxidation, carbonylation and nitration (Kehm et al. 2021).

The proteins found in the seminal plasma (SP) are essential for the normal course of reproductive processes, and their oxidative modifications can be responsible for male infertility (Huang et al. 2018). Elevated ROS levels in semen contribute to increased lipid peroxidation as well as mitochondrial and DNA damage, which consequently reduces sperm motility and may lead to impaired fertilization capacity (Ferramosca et al. 2013, Majzoub et al. 2018, Moraes and Meyers 2018). Reduced animal fertility due to deterioration in semen quality adversely affects the profitability of their breeding. Therefore, it appears important to study oxidative modifications of SP proteins in order to improve the diagnosis of oxidative stress disorders in breeding animals.

To determine the quality of fresh, refrigerated and cryopreserved semen, it is necessary to use biological markers correlated with its quality (Aquino-Cortez et al. 2016). Knowledge of protein markers enables the improvement of processes related to the storage and transport of semen and helps in the diagnosis and treatment of pathological conditions of the male reproductive system (Ponglowhapan et al. 2004, Niżański et al. 2016). The most important biomarkers commonly used in the assessment of oxidative damage include carbonyl derivatives (Shacter 2000). Due to their very high persistence in the body, they enable the assessment of the severity of oxidative processes in proteins (Dalle-Donne et al. 2005). The method employed to determine the carbonyl group content in tested material plays a key role in the quality, reliability and time needed to obtain reliable results (Alomari et al. 2018). Spectrophotometric measurement using 2,4-dinitrophenylhdrazine (DNPH), developed by Levine et al. (1990), is indicated as the most useful method for determining the carbonylated protein content.

The influence of oxidative stress inducers on the carbonylated protein content has been proven in several studies. It was indicated in Holstein-Friesian bulls that the cryopreservation process contributed to an increased proportion of carbonyl groups in their semen (Mostek et al. 2017). This suggests that a measurement of the carbonylated protein content can be used to assess the semen quality.

Importantly, there is no available literature detailing the analysis parameters required to obtain reliable measurement results for the canine and stallion SP.

The main aim of the study was to verify the applicability of 2,4-dinitrophenylhydrazine (DNPH) method of protein carbonyl derivatives content analysis in canine and stallion SP using two reagents for protein dissolving: 6M Guanidine and 0.1M NaOH. The second aim was to elucidate the relationship between the content of protein carbonyl derivatives and the stallion breeding/non-breeding season.

Materials and Methods

Animals

The research material was seminal plasma (SP) collected from eight English Springer Spaniel dogs (age range 1–5 y). One ejaculate was obtained from each individual. Seventeen ejaculates from seven half-bred stallions (age range 10–22 y) were collected. Three ejaculates originated from each of three stallions, and two ejaculates from each of the other four stallions. Dog ejaculates were collected manually by a qualified veterinarian, and stallion ejaculates were obtained using an artificial vagina during the breeding season (April – June, 7 ejaculates) and non-breeding season (September – December, 10 ejaculates). The tested dogs came from the "Spotted with Charm FCI" kennel in Warsaw, and the stallions were from Stack Stallions in Lack in Mazowieckie Voivodeship and from the stable of Marek Romanowski in Wozlawki. Although dog and stallion semen collection procedures do not require the approval of the Local Ethics Committee, exclusion letter no. LKE 1/22 was obtained.

Sample preparation

Each stallion ejaculate was centrifuged at 10000 × g for 10 min (room temperature) according to the procedure described by Mogielnicka-Brzozowska et al. (2019). Dog semen was centrifuged at 15000 × g for 15 min at 4°C. The obtained SP was transferred to clean Eppendorf tubes and centrifuged again using the above parameters in order to remove sperm residues. A protease inhibitor cocktail (P8340 Sigma-Aldrich) was added to each sample to decrease the proteolytic activity of enzymes. Samples were kept at 4°C in a thermobox

Table 1. Content of total protein and protein carbonyl groups in dog seminal plasma depending on the variant of reagents used for its measurement: V1 – 6M Guanidine, V2 – 0.1M NaOH. Different letters (a, b) indicate statistically significant differences between different variants (V1, V2) of samples ($p \leq 0.05$).

Sample	Total protein content (mg/mL)	Protein carbonyl group content [nmol/mg protein]		p value
		V1 6M Guanidine	V2 0.1M NaOH	
1	51	32.12 ± 7.83[a]	13.93 ± 0.45[b]	0.0159
2	85	5.72 ± 2.66	6.10 ± 2.61	0.8704
3	36.5	41.10 ± 4.57	31.30 ± 6.76	0.1059
4	35	23.07 ± 2.29	31.39 ± 5.58	0.0756
5	41	37.80 ± 4.38	34.55 ± 4.78	0.4338
6	93	18.93 ± 3.35[a]	5.47 ± 3.85[b]	0.0103
7	47.5	24.88 ± 0.67[a]	17.99 ± 3.56[b]	0.0301
8	69	11.79 ± 1.10[a]	4.30 ± 0.82[b]	0.0074
Av.	57.25 ± 21.40	24.42 ± 12.21	18.13 ± 12.63	

and transported to the laboratory of the Department of Animal Biochemistry and Biotechnology. The SP samples were then immediately frozen and stored at -80°C until further analysis.

Protein content determination

Total protein content was measured in the dog and stallion SP using the Lowry et al. (1951) method. Bovine serum albumin (BSA, IBSS BIOMED S.A., Poland) was used as the standard.

Protein carbonyl group content determination using DNPH

The content of carbonyl groups in SP proteins was measured based on the method described by Levine et al. (1990) and Fagan et al. (1999), which uses the reaction of the carbonyl group of proteins with DNPH, in which colored dinitrophenyl hydrazone is produced. Three technical repetitions were made for each sample. The total protein content in all SP samples was calculated as 1.2 mg/mL. 500 µL of 10mM DNPH in 2M HCl was added to 500 µL of each sample. The samples were then vortexed every 10-15 minutes and incubated for 1 h at room temperature. A control sample was prepared for each sample using 2M HCl instead of DNPH. The proteins were precipitated by adding 500 µL of 20% trichloroacetic acid (TCA) (w/v). The solution was then centrifuged (11000 × g, 5 min, 18°C), and the supernatant was removed. The precipitate was resuspended with 1 mL of ethanol-ethyl acetate (1:1 v/v) to remove free reagent, and after a 10 min incubation at room temperature, it was centrifugated at 11000 × g for 3 min at 18°C. The supernatant was discarded. This process was repeated twice. In order to redissolve the precipitated protein, the following reagents were used: V1 – 1.2 mL of 6M Guanidine with 20 mM potassium phosphate (pH 2.3); V2 – 1.2 mL of 0.1M NaOH. After the addition of the reagents, the samples were incubated at 37°C for 15 min and then centrifuged (13000 × g, 5 min, 4°C) to remove any insoluble material. The supernatant was then transferred to a new Eppendorf tube, and absorbance at 366 nm was measured spectrophotometrically (DU 800 SPECTROPHOTOMETER, Beckman Coulter). The content of protein carbonyl groups was calculated according to the Lambert-Beer law based on the above-mentioned measurement results, using the molar absorbance coefficient adopted as 22.000 $M^{-1}cm^{-1}$ (Swinehart 1962, Augustyniak et al. 2015). The obtained results show the content of carbonyl groups in nmol/mg of protein.

Statistical analysis

The results were analyzed statistically using Statistica 13.3 (Stat Soft Incorporation, Tulsa, OK, USA). The results are shown as means with standard deviation (mean±SD). The comparison of the results of individual variants (V1, V2) was carried out using Student's t-test for independent samples. Linear regression plots were also made. Pearson's correlation coefficient was used to determine the relationship between individual samples.

Results

Carbonyl group content in canine seminal plasma proteins

This study presents the average total protein content and the carbonyl group concentration in the canine SP, depending on the reagent variant applied (Table 1). In the canine SP samples with numbers 1, 6, 7, and 8,

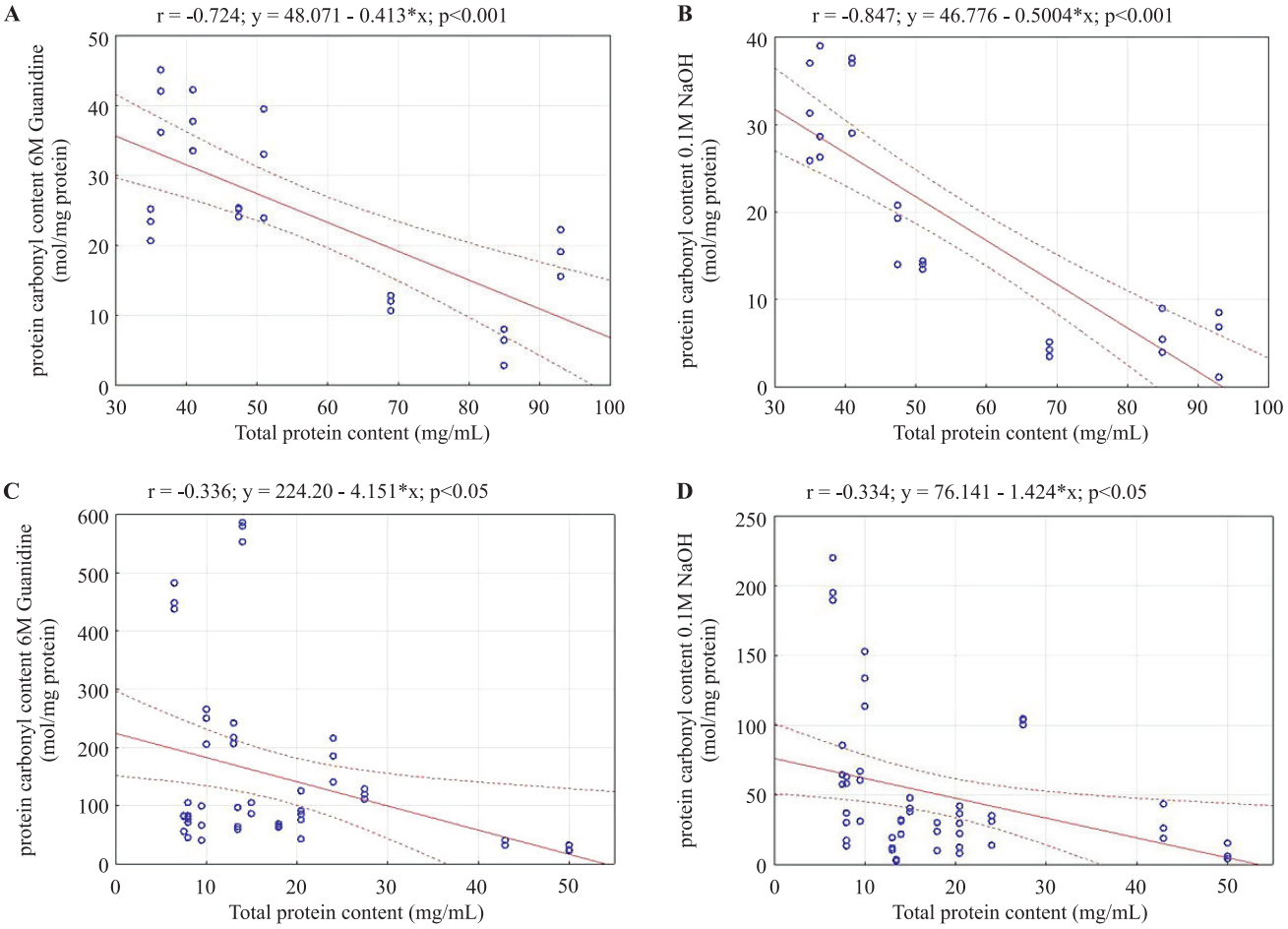

Fig. 1. Dependence of the protein carbonyl group content on the total protein content using different variants of reagents (V1 – 6M Guanidine; V2 – 0.1M NaOH) in dog seminal plasma (**A** – V1; **B** – V2) and in stallion seminal plasma (**C** –V1; **D** – V2).

statistically significantly ($p \leq 0.05$) lower SP protein carbonyl group contents using 0.1M NaOH (V2) were observed, as compared to the values of measurements using 6M Guanidine (V1) (Table 1). As regards the canine SP samples with numbers 2, 3, 4, and 5, no statistically significant ($p \geq 0.05$) differences were noted between variants V1 and V2.

When investigating the relationship between total protein content and the number of carbonyl groups in canine SP proteins, the statistical analyses revealed a negative, highly significant (and very strong) correlation in both V1 ($r = -0.724$; $p \leq 0.001$; Fig. 1A) and V2 ($r = -0.847$; $p \leq 0.001$; Fig. 1B).

Carbonyl group content in stallion seminal plasma proteins

This study presents the average total protein contents and the carbonyl group concentration in stallion SP, depending on the reagent variant applied (Table 2). In the samples under analysis (1 to 6 and 8 to 15), statistically significantly ($p \leq 0.05$) lower SP protein carbonyl group contents using 0.1M NaOH (V2) were observed, as compared to the values of measurements using 6M Guanidine (V1) (Table 2). As regards the remaining samples (7, 16, 17), no statistically significant ($p \geq 0.05$) differences were noted between the individual sample variants. Statistical analysis of the results demonstrated a negative significant (moderately strong) correlation between the total protein content and the carbonyl group content in seminal stallion plasma. The relationship was demonstrated in both V1 ($r = -0.336$; $p \leq 0.05$; Fig. 1C) and V2 ($r = -0.334$; $p \leq 0.05$; Fig. 1D).

The statistical analysis of the results demonstrated a highly statistically significant ($p \leq 0.01$) difference in the average carbonyl protein content in seminal stallion plasma between the ejaculates collected during the breeding season and the samples acquired during the non-breeding season. A higher level of stallion SP protein oxidation was observed outside the breeding season (Fig. 2).

A detailed analysis of the dates on which the semen was acquired demonstrated a positive, highly statistically significant (moderately strong) correlation between the carbonyl group content and the month

Table 2. Content of total protein and protein carbonyl groups in stallion seminal plasma during the breeding and non-breeding season depending on the variant of reagents used for its measurement: V1 – 6M Guanidine, V2 – 0.1M NaOH. Different letters (a, b) indicate statistically significant differences between different variants (V1, V2) of samples ($p \leq 0.05$).

Sample	Total protein content (mg/mL)	Protein carbonyl group content [nmol/mg protein]		p value
		V1 6M Guanidine	V2 0.1M NaOH	
Breeding season				
1	50	26.06 ± 4.86[a]	8.48 ± 5.81[b]	0.0150
2	15	92.12 ± 11.02[a]	42.02 ± 5.22[b]	0.0021
3	8	73.30 ± 30.50[a]	22.35 ± 12.79[b]	0.0459
4	20.5	100.22 ± 21.47[a]	33.41 ± 10.09[b]	0.0082
5	20.5	53.73 ± 19.33[a]	16.70 ± 11.48[b]	0.0462
6	14	572.84 ± 17.24[a]	28.14 ± 5.85[b]	0.0001
7	43	34.71 ± 5.19	29.32 ± 12.67	0.5326
Non-breeding season				
8	24	180.76 ± 38.17[a]	26.52 ± 11.35[b]	0.0026
9	8	79.80 ± 2.94[a]	50.38 ± 17.74[b]	0.0471
10	10	240.00 ± 31.22[a]	133.33 ± 19.55[b]	0.0074
11	27.5	119.61 ± 8.43[a]	102.75 ± 2.44[b]	0.0292
12	18	65.15 ± 21.47[a]	21.13 ± 10.06[b]	0.0019
13	13	221.56 ± 18.38[a]	13.87 ± 4.70[b]	0.0001
14	13.5	73.18 ± 20.70[a]	3.14 ± 0.70[b]	0.0042
15	6.5	455.71 ± 23.80[a]	201.63 ± 16.39[b]	0.0001
16	7.5	73.33 ± 15.22	69.09 ± 14.56	0.7448
17	9.5	68.58 ± 29.00	52.79 ± 19.08	0.4749
Av.	18.15 ± 12.10	148.86 ± 149.45	50.30 ± 51.64	

in which the ejaculate was collected. This correlation was confirmed for both V1 ($r = 0.478$; $p \leq 0.001$; Fig. 3A) and V2 ($r = 0.428$; $p \leq 0.01$; Fig. 3B).

Discussion

A number of different methods for determining carbonyl protein levels are known worldwide. The selection of a spectrophotometric method, i.e., the reaction with DNPH, is justified by its cost-effectiveness, resulting from the absence of the need to purchase specific antibodies required for Western blot analysis or expensive commercial ELISA tests (Soglia et al. 2016). The available literature, however, contains little information on the specific methods or parameters required for investigation of the carbonyl group content in the SP of individual animal species (Weber et al. 2015).

In a study describing the methodology for measuring carbonyl groups in oxidatively modified proteins the 6M Guanidine and 0.1M NaOH were considered to be alternatively used to dissolve the protein precipitate (Levine et al. 1990). The results in the canine and stallion SP showed differences in measurements between the above-mentioned reagents in both species tested. Lower values were found when 0.1M NaOH was used for protein dissolving in both species. The results indicate that both reagents might be successfully applied to oxidative process measurements in the canine and stallion SP proteins. However, it should be noted that these results are influenced by the substance applied; therefore, it is of particular significance when comparing the samples.

Differences achieved between protein carbonyl group content measured using 6M Guanidine and 0.1M NaOH are difficult to explain but may be due to the specificity of the proteins being constituents of individual ejaculates. These proteins may possess a different number of chemical groups which undergo various reactions with 6M Guanidine and 0.1M NaOH. Suscep-

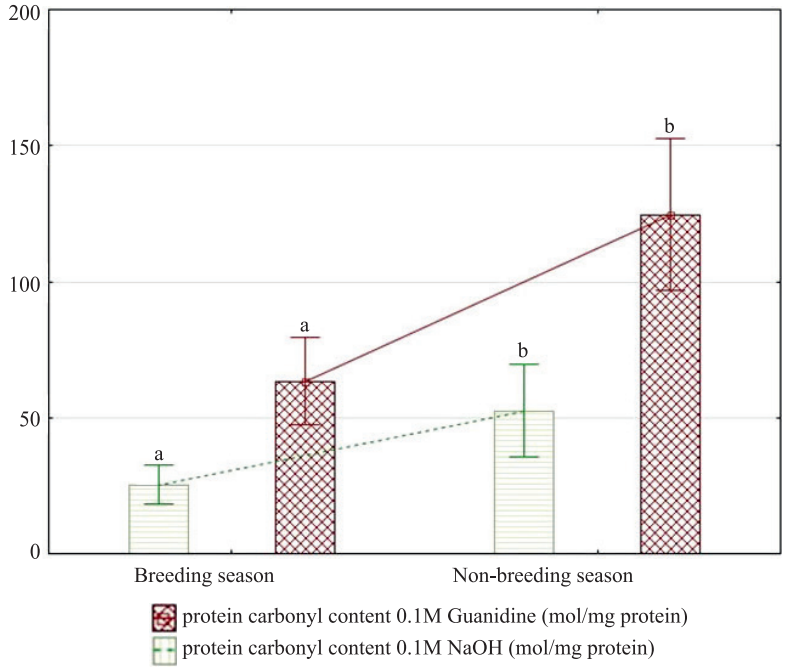

Fig. 2. Average content of protein carbonyl groups in the stallion seminal plasma proteins during the breeding season (n = 7) and non-breeding season (n = 10) measured using different variants of reagents: red graph bar – 6M Guanidine (V1); green graph bar – 0.1M NaOH (V2). Different letters (a, b) indicate statistically significant differences between seasons ($p \leq 0.01$).

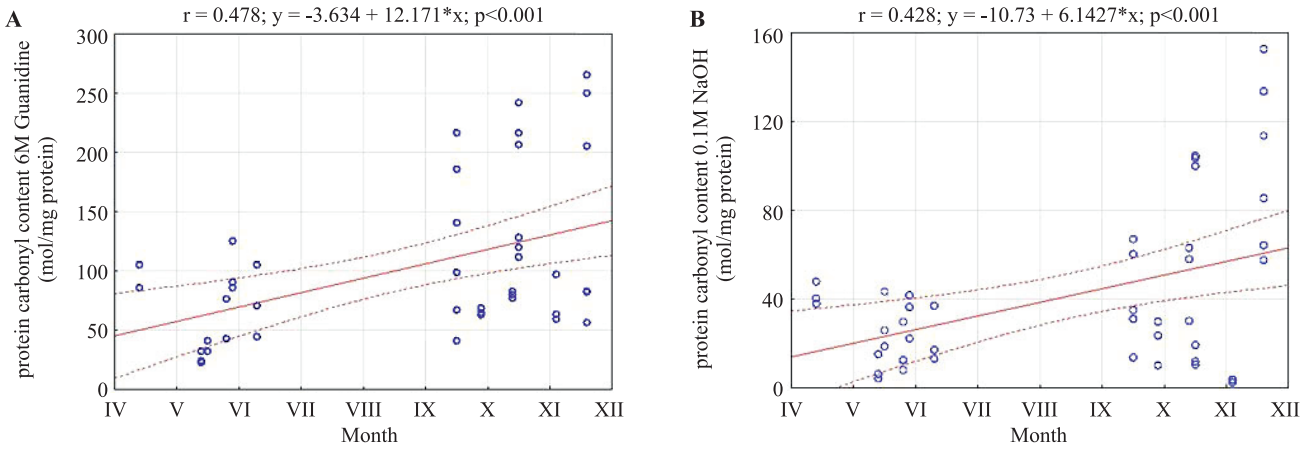

Fig. 3. Protein carbonyl group content in the stallion seminal plasma dependence of the month in which the ejaculate was collected, measured using different variants of reagents: **A** – 6M Guanidine (V1); **B** – 0.1M NaOH (V2).

tibility of individual proteins to dissolving may be different.

The current study demonstrated a negative correlation between the total protein content and the number of carbonyl groups in both canine and stallion SP proteins. This phenomenon might be explained by the higher content of antioxidant enzymes that protect reactive groups of proteins susceptible to oxidation processes. Sharma et al. (2016) demonstrated, in a study of bull SP, a positive correlation between the total protein content and the concentration of superoxide dismutase, i.e., an enzyme involved in antioxidative processes. However, the present thesis needs to be further studied to obtain convincing evidence.

The average protein content in the SP of the English Springer Spaniel dogs was higher when compared to non-breed dogs in which the results of measurements were obtained using the same method (Cheema et al. 2011). The differences in the results might be related to breed determinants, although this issue requires further research.

It should be emphasized that in the present study, the content of the carbonyl groups in the canine SP was demonstrated for the first time, and the lack of such results in the literature precludes a direct discussion of the results obtained in the current study. In the experiment the average content of carbonyl groups in canine SP was 24 nmol/mg protein. The only available litera-

ture on the subject presents results obtained in human (Mohanty et al. 2016) and stallion SP (Morte et al. 2008). Mohanty et al. (2016) have demonstrated a relationship between the increased carbonyl group content in human SP proteins and the increased percentage of damaged sperm and the degree of lipid peroxidation. In the above-mentioned study, the carbonyl protein content, measured using the reaction with DNPH, was 1.7 nmol/mg protein in healthy males and 4.3 nmol/mg protein in males with reduced fertility (Mohanty et al. 2016).

Our data obtained in the stallion SP revealed that the average content of carbonyl groups was 79 nmol/mg protein. Morte et al. (2008) showed the average content of carbonyl groups in stallion SP as 50 nmol/mg protein. Susceptibility of individual proteins to carbonylation and oxidative modifications may be due to the presence of a metal-binding site in their structure or higher amounts of amino acid residues (Cai and Yan 2013).

Morte et al. (2008) found differences in the carbonyl group content in the stallion SP between samples collected during the breeding and non-breeding season, which is congruent with our results, in which we observed a higher level of stallion SP protein oxidation in the non-breading season. This relationship may result from the shift to a winter diet regime which is poor in antioxidants such as vitamin E and selenium (Muirhead et al. 2010). It can be suggested that during the breeding season, access to pasture and a significant proportion of green forage in the feed improves the oxidative balance in stallion SP, which may consequently result in a reduction of oxidized proteins. Moreover, stallions during the breeding season usually exhibit greater copulatory activity, which is associated with the increased secretory activity of the reproductive system accessory glands, whose secretions contain numerous substances that protect sperm against oxidative stress.

This study has confirmed the usefulness of both studied reagents in the reaction with DNPH as an efficient spectrophotometric method for determining the carbonyl group content in the canine and stallion SP proteins. However, we must be aware that using the DNPH method to measure oxidative damage of SP proteins in different animal species might give different results, which was confirmed in our study.

Conclusions

This study showed the applicability of 6M Guanidine and 0.1M NaOH in the reaction with DNPH for spectrophotometric determination of the carbonyl group content in the stallion and canine SP proteins. The study also confirmed the occurrence of seasonal variations in the carbonyl group content in stallion SP proteins. The applied method, due to its simplicity and cost-effectiveness, appears to be suitable for large-scale measurements of the carbonyl group content in canine and stallion SP proteins. Additionally, it was showed that for protein precipitate dissolving, both 6M Guanidine and 0.1M NaOH may be used.

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References

Alomari E, Bruno S, Ronda L, Paredi G, Bettati S, Mozzarelli A **(2018)** Protein carbonylation detection methods: A comparison. Data Brief 19: 2215-2220.

Aquino-Cortez A, Machado da Silva LD, de Araujo AA, da Silva Bezerra de Menezes E, Moura AA **(2016)** Proteins of the canine seminal plasma. Ciencia Rural 46: 901-908.

Augustyniak E, Adam A, Wojdyla K, Rogowska-Wrzesinska A, Willetts R, Korkmaz A, Atalay M, Weber D, Grune T, Borsa C, Gradinaru D, Bollineni RC, Fedorova M, Griffiths HR **(2015)** Validation of protein carbonyl measurement: a multi-centre study. Redox Biol 4: 149-157.

Cai Z, Yan LJ **(2013)** Protein oxidative modifications: Beneficial roles in disease and health. J Biochem Pharmacol Res 1: 15-26.

Cheema RS, Bhakri G, Gandotra VK, Dhanju CK **(2011)** Characterization of Mongrel dog seminal plasma proteins and their correlation with semen characteristics. J Reprod Stem Cell Biotech 2: 55-63.

Dalle-Donne I, Scaloni A, Giustarini D, Cavarra E, Tell G, Lungarella G, Colombo R, Rossi R, Milzani A **(2005)** Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. Mass Spectrom Rev 24: 55-99.

Fagan JM, Sleczka BG, Sohar I **(1999)** Quantitation of oxidative damage to tissue proteins. Int J Biochem Cell Biol 31: 751-757.

Ferramosca A, Provenzano SP, Montagna DD, Coppola L, Zara V **(2013)** Oxidative Stress Negatively Affects Human Sperm Mitochondrial Respiration. Urology 82: 78-83.

Figueroa E, Lee-Estevez M, Valdebenito I, Watanabe I, Oliveira RPS, Romero J, Castillo RL, Farias JG **(2019)** Effects of cryopreservation on mitochondrial function and sperm quality in fish. Aquaculture 511: 634190.

Huang C, Cao X, Pang D, Li C, Luo Q, Zou Y, Feng B, Li L, Cheng A, Chen Z **(2018)** Is male infertility associated with increased oxidative stress in seminal plasma? A-meta analysis. Oncotarget 9: 24494-24513.

Kehm R, Baldensperger T, Raupbach J, Hohn A **(2021)** Protein oxidation – formation mechanisms, detection and relevance as biomarkers in human diseases. Redox Biol 42: 101901.

Klaiwattana P, Srisook K, Srisook E, Vuthiphandchai V, Neumvonk J **(2016)** Effect of cryopreservation on lipid composition and antioxidant enzyme activity of seabass (Lates calcarifer) sperm. Iran J Fish Sci 15: 157-169.

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz A-G, Ahn B-W, Shaltiel S, Stadtman ER **(1990)** Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 186: 464-478.

Li P, Li ZH, Dzyuba B, Hulak M, Rodina M, Linhart O **(2010)** Evaluating the impacts of osmotic and oxidative stress on common carp (Cyprinus carpio, L.) sperm caused by cryop-reservation techniques. Biol Reprod 83: 852-858.

Majzoub A, Arafa M, Mahdi M, Agarwal A, Al Said S, Al-Emadi I, El Ansari W, Alattar A, Al Rumaihi K, Elbardisi H **(2018)** Oxidation-reduction potential and sperm DNA fragmentation, and their associations with sperm morphological anomalies amongst fertile and infertile men. Arab J Urol 16: 87-95.

Mogielnicka-Brzozowska M, Fraser L, Dziekońska A, Gackowska K, Sobiewska M, Kuzborska A, Majewska AM, Filipowicz K, Kordan W **(2019)** Identification of proteoforms of albumin and kallikrein in stallion seminal plasma and their correlations with sperm motility. Pol J Vet Sci 22: 227-235.

Mohanty G, Swain N, Goswami C, Kar S, Samanta L **(2016)** Histone retention, protein carbonylation, and lipid peroxidation in spermatozoa: Possible role in recurrent pregnancy loss. Syst Biol Reprod Med 62: 201-212.

Moraes CR, Meyers S **(2018)** The sperm mitochondrion: organelle of many functions. Anim Reprod Sci 194: 71-80.

Morte MI, Rodrigues AM, Soares D, Rodrigues AS, Gamboa S, Ramalho-Santos J **(2008)** The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: Seasonal distinctions and correlations with DNA strand breaks, classical seminal parameters and stallion fertility. Anim Reprod Sci 106: 36-47.

Mostek A, Dietrich MA, Słowińska M, Ciereszko A **(2017)** Cryopreservation of bull semen is associated with carbonylation of sperm proteins. Theriogenology 92: 95-102.

Mostek A, Słowińska M, Judycka S, Karol H, Ciereszko A, Dietrich MA **(2018)** Identification of oxidatively modified proteins due to cryopreservation of carp semen. J Anim Sci 96: 1453-1465.

Muirhead TL, Wichtel JJ, Stryhn H, McClure JT **(2010)** The selenium and vitamin E status of horses in Prince Edward Island. Can Vet J 51: 979-985.

Niżański W, Partyka A, Prochowska S **(2016)** Evaluation of spermatozoal function – useful tools or just science. Reprod Domest Anim 51S: 37-45.

Ponglowhapan S, Essen-Gustavsson B, Forsberg CL **(2004)** Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. Theriogenology 62: 1498-1517.

Shacter E **(2000)** Quantification and significance of protein oxidation in biological samples. Drug Metab Rev 32: 307-326.

Sharma L, Pandey V, Nigam R, Saxena A, Sawin DK, Yadav B **(2016)** Association of oxidative status and semen characteristics with seminal plasma proteins of buffalo semen. Iran J Vet Res 17: 226-230.

Soglia F, Petracci M, Ertbjerg P **(2016)** Novel DNPH-based method for determination of protein carbonylation in muscle and meat. Food Chem 197: 670-675.

Swinehart DF **(1962)** The Beer-Lamber Law. J Chem Educ 39: 333-335.

Weber D, Davies MJ, Grune T **(2015)** Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: Focus on sample preparation and derivatization conditions. Redox Biol 5: 367-380.