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Research article

THE EFFECTS OF ANTIOXIDANTS PROVIDED WITH FEED ON CERTAIN QUALITY PARAMETERS OF BULL SEMEN UNDER HEAT STRESS CONDITIONS

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The aim of the current research was to assess the effects of the feed additive made of lyophilised melon juice (source of superoxide dismutase, SOD) and inactivated live *Saccharomyces cerevisiae* (strain R397) cells added to the feed via the product containing high levels of organically bound selenium (source of selenium-dependant glutathione peroxidase, Se-GPx) on the semen quality of bulls in heat stress conditions. The 15 bulls chosen for the experiment were assigned to three equal groups (control –group C; treated group M, given the source of SOD; and group A, treated with the source of Se-GPx). The research was conducted in summer. The activities of SOD and Se-GPx in seminal plasma were determined spectrophotometrically. Computer-assisted semen analysis was done to determine the sperm counts, motility and velocity. The temperature and humidity were recorded with a digital data logger.

The average SOD activity in the control bulls was significantly lower than in M ($p<0.001$) and A ($p<0.001$), whilst the average activities in the treated groups did not differ significantly ($p=0.784$). Higher average SOD activity compared to the control in the treated groups showed that both feed additives increased the antioxidative capacity of the seminal fluid. The average GPx activity in the control was significantly lower than in groups A ($p=0.001$) and M ($p=0.005$), whilst the two treatments did not lead to significantly different results ($p=0.701$). The analysis of relations between the activity of each enzyme and sperm motility and progressive motility in each of the bulls failed to detect a significant correlation. The analysis of the relation between THI (temperature-humidity index) and the activity of the antioxidative enzymes revealed that the increase in THI coincided with the decrease in the SOD activity in the control group, but with its increase in the treated groups ($p>0.05$). In all of the three groups with the increase in THI there was an increase in GPx activity ($p>0.05$). It can be concluded that in all of the three groups of bulls there was an increase in the activity of both enzymes in

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the seminal plasma, but the increase was significantly lower in the control. Thus, the antioxidative capacity of the seminal plasma of untreated bulls was proven to be lower in comparison with those of the treated animals.

Key words: antioxidative enzymes, bull, motility, sperm

INTRODUCTION

One of the most important factors which contributes to lower bull semen quality is oxidative stress (OS) [1,2]. OS is a condition followed by cell damage done by oxygen and oxidants produced from oxygen, which are commonly known as reactive oxygen species - ROS [3]. Breeding animals are exposed to numerous biological and environmental factors, such as variations in diet, climate changes, transport, regrouping, preventive and therapeutic measures, various stressogenic factors etc. [4-6]. The capacity of sires to overcome these factors is important to maintain their health and fertility [7]. Any disturbance in homeostasis leads to increased ROS production, even beyond the detoxifying capacity of local tissues or the organism [8]. Uncontrolled ROS production, beyond the antioxidative capacities of seminal plasma, results in OS, which is detrimental to the sperm [9]. All cellular components, including lipids, proteins, nucleic acids and carbohydrates are possible targets for OS damages [10]. Bull spermatozoa are extremely sensitive to oxidative damage caused by high concentrations of oxygen radicals due to the oxidation of lipids in the membranes [11-13], proteins and DNA damage [14,15], which results in disturbances in the function of their membrane, metabolism, morphology and motility [8]. In summer, ROS production significantly increases in sperm, leading to lipid peroxidation (LPO) and major sperm defects [16]. In a previous study from Rahman et al. [17] artificial scrotal insulation was performed to detect at which period sperm is mostly affected by heat. Significantly higher abnormal morphology and a decrease in motility and viability of sperm were detected in the semen ejaculated between 14 and 42 days after scrotal insulation. Spermatogenesis requires approximately 61 days in total to be completed in the bull: 21 days of spermatocytogenesis, 23 days of meiosis and 17 days of spermiogenesis. Increased testicular temperature affected mostly those sperm cells that were at the meiotic and the beginning of spermiogenic stages of development at the time of scrotal insulation.

Spermatozoa are protected from oxidative damage by various antioxidants, which are present in the sperm plasma and the cells themselves [18]. Antioxidants are substances which break the chain of oxidative reactions and, by acting so, decrease oxidative stress [19,20]. They play a role in the neutralisation and suppression of ROS synthesis or prevent their activity. Enzyme antioxidants are known as natural antioxidants, which neutralise the superfluous ROS and prevent damage to cell structures. This group of compounds comprises, among others, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). The role of GPx in the protection of sperm cells and the tissues of the male genital tract from

peroxidative damage is very important due to the presence of CAT in mammalian sperm in minimal concentrations. Thus, due to the absence or extremely low activities of CAT, GPx is the most important protector of sperm cells and testis tissues from the negative impact of hydrogen peroxide. In contrast to CAT, which reacts only with H_2O_2 when its concentrations are very high ($>10^{-6}$ M), GPx enables the fine regulation of H_2O_2 concentrations in physiological conditions. In addition to this, GPx enables the reparation of complex molecules (e.g. cell membrane lipids) [21]. The specific structure of sperm cell plasma membrane, which is rich in unsaturated fatty acids, is targeted by free radicals, which points to the importance of GPx in the protection from damage caused by peroxides. In the mammalian epididymis, mature spermatozoa are highly protected by the activity of GPx. Its presence has been confirmed in all stages of sperm development [22,23]. GPx has been detected in sperm plasma and in the subacrosomal region of spermatozoa [24,25], but there is evidence that it can be found in the sperm cell nuclei, which is also true for GPx4 [26]. In addition, GPx5 is present in the cytoplasm of the epithelium cells in the *caput epididymi*s [25], as well as in the apical cells of the epididymis [27]. The presence of GPx5 has been confirmed in the free epididymal fluid, which is considered to be connected to the vesicles rich in lipids, known as *epididymosomes* [27,28]. Considerable quantities of plasma GPx and GPx3 in the epithelium of the *cauda epididymis* enable effective protection from damage by ROS [29]. Increased environmental temperature in the summer may increase the temperature in the testes, quicken the metabolism and the demands for oxygen [30]. If the increased metabolism is not followed by increased blood flow, hypoxia develops in the testicular tissue, which leads to increased production of ROS and LPO, oxidative stress and lower sperm motility [16,30]. In young bulls more intense oxidative processes in the semen plasma and cells were detected in the summer, which was in positive correlation with the temperature-humidity index - THI [5]. At the same time, the Se-GPx activity in sperm cells increased, but was insufficient to eliminate the influence of oxidative processes and resulted in decreased progressive motility of the sperm cells. Selenium-dependent enzymes play an important role in sperm protection from oxidative damage, which is why the deficiency in GPx can lead to morphopathological changes in the sperm cell mitochondria [31,32].

The assessment of OS and treatment with antioxidants are not widely used in routine veterinary practice. Accordingly, the aim of the current research was to assess the effects of two natural antioxidants added to the feed intended for mature sires on the quality of their semen in heat stress conditions. Moreover, this research may contribute to the evaluation of the justification for the introduction of ROS and OS assessment in every-day analysis of the bull semen quality.

MATERIAL AND METHODS

There were three groups of Simmental bulls in the experiment, each consisting of 5 animals, equalized for age (bulls were between 3 and 6 years of age), semen production results and exterior characteristics. All procedures on animals were approved by the Ethical Committee of the Faculty of veterinary medicine University of Belgrade, Serbia, Ministry of Agriculture and Water management and Forestry Republic of Serbia (No 323-07-5369/2018-05/1, dated 04.06.2018), according to the Serbian Animal Welfare Protection Law, and Directive 2010/63/ EU.

Before the start of the experiment, concentrated feed mixtures were prepared with the addition of the tested feed supplement. The actual concentration of the added antioxidant in the feed was checked. The animals were fed with such feed for 120 days (from 1 June to 30 September).

Group M was given *Melofeed*® (Lallemand Animal Nutrition, France), a supplement made from dried melon juice, rich in SOD, which is gradually released from the coated particles over time in the digestive system a daily dose of 50 μg/animal, as recommended by the manufacturer.

Group A was given the feed with the addition of *Alkosel*®, (Lallemand Animal Nutrition, France), which contains organically bound selenium as L(+) selenomethionine, originating from *Saccharomyces cerevisiae* strain *R397* cells, in a daily dose of 3 mg selenomethionine/animal, in compliance with the recommendations of the manufacturer.

The third group, C, was the control one and was not given any antioxidants in the feed.

Thirty days from the beginning of the experiment the ejaculate sampling of the ejaculate began in all of the animals. The ejaculate was sampled twice a month per each bull until the end of the experiment (seven times from each bull, i.e. 105 samples in total) using the artificial vagina. The consecutively taken samples were labelled with numbers (S1 to S7) and those taken at the same time point were named a set of samples. Each ejaculate was inspected macroscopically and under the microscope, diluted and frozen.

To determine the activities of SOD and GPx seminal plasma was separated by centrifugation of the samples of deep-frozen semen after thawing.

SOD activity in seminal plasma was determined spectrophotometrically [33]. The method by Günzler et al. [34] was used to determine GPx activity in the presence of hydrogen peroxide (H_2O_2) as a substrate and reduced glutathione (GSH), on a spectrophotometer BK-36 S390 (BIOBASE, Osgood Common Fremont, CA, USA).

The quality of the semen was determined based on the concentration of spermatozoa, their motility and velocity, using the computer-assisted semen analysis (CASA) system (Sperm Class Analyzer, CASA System, Microptic, Barcelona, Spain).

The temperature and the air humidity in the stables were recorded with the AMT-116 temperature and humidity data logger (Amtast USA Inc., Lakeland, FL, USA). The

temperature and the relative humidity in stable were recorded 24 times per day. The daily temperature-humidity index (THI) values were calculated using the equation by Kibler: THI=1.8Ta-(1-RH)(Ta-14.3)+32 where: Ta-measured ambient temperature in °C, RH –relative humidity as a fraction of the unit.

The significance of the differences in the average activities of SOD and GPx between the three groups of bulls and in all of the seven sets of samples (taken at the same time point) was determined using the two-way ANOVA, between the three groups in the same sample set with one-way ANOVA, and within each group in the seven consecutively taken samples- repeated-measures ANOVA. The pairs of averages were compared by means of Tukey test, and to detect the correlation between the variables Pearson's correlation coefficient was determined. Given the variability of the data on spermatozoa motility and progressive motility, to decide on the relation between these and SOD and GPx, Spearman's rank correlation coefficient was calculated.

RESULTS

The two-way ANOVA detected significant differences between the three groups of bulls in the experiment in the average SOD activities ($F=33.491$, $p<0.001$): the average activity in the control group was significantly lower ($p<0.001$) than those in group A ($p<0.001$) and M. However, the activities in both treatment groups, A and M, did not differ significantly ($p=0.784$).

The semen samples originating from the same bull group significantly differed ($F=6.749$, $p<0.001$) regarding SOD activity. In S1 the activity was significantly lower than in S4 ($p=0.002$), in S5 ($p<0.001$) and S6 ($p=0.036$). SOD activity in S5 was significantly higher by comparison with S2 ($p=0.001$) and S7 ($p=0.021$). The interaction of group and sampling order factors was not statistically significant ($F=0.613$, $p=0.824$).

The use of ANOVA for repeated measurements within each of the bull groups revealed statistically significant differences in average SOD activities in the seven consecutively taken samples in the control group ($F=7.165$, $p<0.001$), but these differences proved to be insignificant within group A ($F=1.435$, $p=0.243$) and M ($F=2.362$, $p=0.062$). In C group the average SOD activity in S1 was significantly lower than that in S4 ($p=0.0102$) and those which were taken afterwards ($p_{1/5}<0.001$, $p_{1/6}=0.003$ and $p_{1/7}=0.006$). In addition, the average SOD activity in S2 was significantly lower compared to the values S5 ($p=0.014$).

The average SOD activity differed significantly between the groups in the first five sampling time points ($F_1=5.356$, $p_1=0.022$; $F_2=4.586$, $p_2=0.033$; $F_3=4.843$, $p_3=0.029$; $F_4=4.742$, $p_4=0.030$ and $F_5=4.568$, $p_5=0.033$), but not in those which followed ($F_6=1.075$, $p_6=0.372$ and $F_7=0.825$, $p_7=0.462$) (Figure 1.). Pair wise comparison of the differences in the enzyme activities proved that in the first five sampling time points the average SOD activities in the control bulls were significantly lower than both those in group A ($p_1=0.035$, $p_2=0.046$, $p_3=0.0495$, $p_4=0.048$ and $p_5=0.023$), and in group M in the S1,S3 and S5 ($p_1=0.040$, $p_3=0.046$ a d $p_5=0.022$).

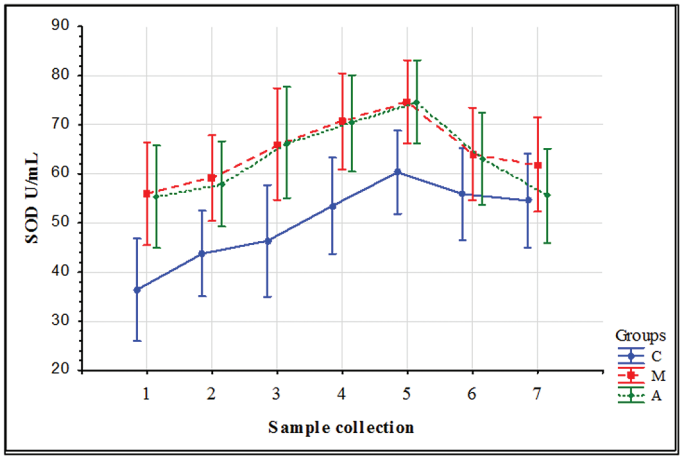

Figure 1. Mean SOD activity in semen plasma of bulls treated with various antioxidants (bars presenting 95% confidence intervals)

The average activities of GPx between the three groups of bulls in this experiment differed significantly (F=12.832, p=0.001, Figure 2). According to the results of Tukey test, the average GPx activity in group C was significantly lower than the averages in group A(p=0.001) and in group M (p=0.005), whilst se average activities did not differ significantly between the two treatment groups (p=0.701).

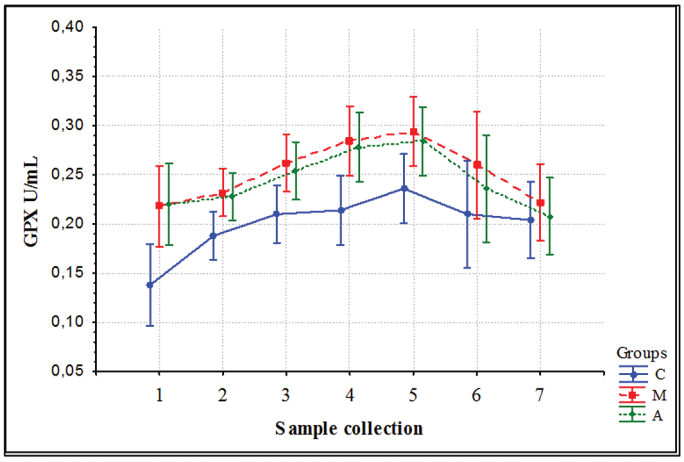

Figure 2. Mean GPx activity in semen plasma of bulls treated with various antioxidants (bars presenting 95% confidence intervals)

The comparison of average GPx activities between the semen samples taken at the same time point in all of the three groups revealed significant differences in all of the seven time points (F=8.252, p<0.001). At the beginning, in the set of samples taken in the first time point, the average values were significantly lower than in S3

(p=0.009), S4 (p<0.001), S5 (p<0.001) and in S6 (p=0.036). In S2 and S7 GPx activities were significantly lower than in S4 ($p_{2/4}$=0.041 i$p_{7/4}$=0.016) and S 5 ($p_{2/5}$=0.003 and $p_{7/5}$=0.001).

The interaction of group and time point of sampling did not affect significantly the activities of GPx (F=0.647, p=0.795).

Between the average activities of GPx in semen samples taken at different time points (samples of the same sampling order) within each of the groups certain differences were detected: significant in group C (F=3.361, p=0.015) and in group A (F=4.920, p=0.002), but not in group M (F=2.213, p=0.077). In group C significantly lower average GPx activity was in S1 in comparison with S5 (p=0.008). In group A the average GPx activity in S4 was significantly higher in comparison to that in S1 (p=0.030), and S7 (p=0.047), and in S5 in comparison to S1 (p=0.009), S2 (p=0.047) and S7 (p=0.014).

On comparison of the average activities of GPx in semen samples taken at the same time points detected (Figure 2) between the groups, significant differences were noticed in the first four samples (F_1=6.089, p_1=0.015; F_2=4.774, p_2=0.015; F_3=4.356, p_3=0.038 and F_4=5.746, p_4=0.018), but not in the others (F_5=3.707, p_5=0.056; F_6=1.000, p_6=0.397 and F_7=0.278, p_7=0.762). The pair comparison of bull groups revealed that the average activities of GPx were lower in the control than both those in the first four samples in group A (p_1=0.029, p_2=0.040, p_3=0.044 and p_4=0.025), and in S1 and S4 in group M (p_1=0.025 and p_4=0.040).

Analysis of the correlation between SOD activity, GPx activity and sperm cell velocity

The results of the analysis of the relationship between the tested characteristics of each of the bulls in the 7 samples (Table 1) indicated the presence of a strong negative relationship between the activity of SOD and the percentage of progressively motile sperm cells (ρ=-0.857, p=0.014) in one bull (ID=1) from A group only, which means that with the increase in SOD activity the percentage of progressively motile spermatozoa declined significantly. In addition to this, in only one bull (ID=2) from group M there was a strong negative relationship between GPx activity and the progressive sperm motility (ρ=-0.775, p=0.041), i.e., the increase in GPx activity correlated with the significant decline in the percentage of progressively motile sperm cells.

In the control bulls, in S6, a negative deterministic (functional) relationship between the paired data was revealed: the activities of GPx and sperm motility (ρ≈-1.000, p≈0.000), and between the same enzyme activity and the progressive sperm motility (ρ≈-1.000, p≈0.000, Table 2). In group M the correlation between SOD activity and progressive sperm velocity was negative and very strong in S2 (ρ=-0.975, p=0.005) and very strong but positive in S5 (ρ=0.975, p=0.037). In this group, a very strong

positive correlation between SOD activity and sperm velocity (ρ=0.975, p=0.037) is characteristic of the S5. In addition, the relationship of GPx activity and sperm velocity was functional in S3 (ρ≈-1.000, p≈0.000) and very strong in S5 (ρ=-0.975, t=-7.550, p=0.005). In group A no significant correlation was detected between the tested parameters when sperm samples were compared at each time point.

Table 1. Parameters of statistical relationship between the variables (enzyme activities vs. percentages of motile or progressively motile sperm) in all bulls and seven consecutively taken samples

Bull ID	Enzyme	Sperm characteristics	Control		Group M		Group A	
			ρ	p	ρ	p	ρ	p
1	SOD	Motility	-0.214	0.645	0.036	0.939	-0.464	0.294
		Progressive motility	-0.321	0.482	0.214	0.645	**-0.857**	**0.014**
	GPx	Motility	0.162	0.728	-0.216	0.641	0.214	0.645
		Progressive motility	0.216	0.641	-0.180	0.699	0.214	0.645
2	SOD	Motility	-0.685	0.090	-0.179	0.702	-0.324	0.478
		Progressive motility	-0.739	0.058	-0.429	0.337	-0.324	0.478
	GPx	Motility	-0.595	0.159	-0.685	0.090	-0.306	0.504
		Progressive motility	-0.721	0.068	**-0.775**	**0.041**	-0.306	0.504
3	SOD	Motility	-0.429	0.337	0.450	0.310	-0.414	0.355
		Progressive motility	-0.179	0.702	0.631	0.129	-0.180	0.699
	GPx	Motility	-0.291	0.527	0.418	0.350	-0.382	0.398
		Progressive motility	0.091	0.846	0.364	0.423	-0.509	0.243
4	SOD	Motility	<0.001	≈1.000	-0.107	0.819	-0.429	0.337
		Progressive motility	-0.036	0.939	-0.321	0.482	-0.321	0.482
	GPx	Motility	-0.536	0.215	-0.111	0.812	-0.107	0.819
		Progressive motility	-0.571	0.180	-0.259	0.574	0.179	0.702
5	SOD	Motility	-0.500	0.253	-0.036	0.939	-0.643	0.119
		Progressive motility	-0.286	0.535	-0.126	0.788	-0.643	0.119
	GPx	Motility	-0.145	0.756	-0.429	0.337	-0.180	0.699
		Progressive motility	-0.018	0.969	-0.286	0.535	-0.180	0.699

ρ-Spearman's rank coefficient correlation

If all the consecutively taken samples are considered together (Table 2), it can be seen that the only significant is the correlation between SOD activity and sperm mobility in the control group (ρ=-0.383, p=0.023).

Table 2. Indicators of statistical relationships between the activities of SOD or GPx, and sperm motility or progressive motility in groups and in samples taken at the same time points

Sample order no.	Enzyme	Characteristics	Control		Group M		Group A	
			ρ	p	ρ	p	ρ	p
1	SOD	Motility	-0.400	0.505	0.300	0.624	-0.700	0.188
		Progressive motility	-0.400	0.505	-0.100	0.873	-0.300	0.624
	GPx	Motility	-0.600	0.285	-0.154	0.805	-0.667	0.219
		Progressive motility	-0.600	0.285	-0.359	0.553	-0.718	0.172
2	SOD	Motility	0.800	0.104	0.872	0.054	0.600	0.285
		Progressive motility	0.800	0.104	**0.975**	**0.005**	0.300	0.624
	GPx	Motility	0.400	0.505	0.205	0.741	0.671	0.215
		Progressive motility	0.400	0.505	0.359	0.553	0.671	0.215
3	SOD	Motility	-0.667	0.219	-0.300	0.624	0.100	0.873
		Progressive motility	-0.308	0.614	<0.001	≈1.000	0.100	0.873
	GPx	Motility	0.300	0.624	**≈1.000**	**<0.000**	0.100	0.873
		Progressive motility	0.600	0.285	0.700	0.188	0.100	0.873
4	SOD	Motility	0.600	0.285	0.205	0.741	-0.600	0.285
		Progressive motility	0.300	0.624	0.051	0.935	-0.700	0.188
	GPx	Motility	-0.100	0.873	0.800	0.104	0.600	0.285
		Progressive motility	-0.300	0.624	0.600	0.285	0.700	0.188
5	SOD	Motility	0.300	0.624	**0.900**	**0.037**	0.600	0.285
		Progressive motility	0.700	0.188	**0.900**	**0.037**	0.600	0.285
	GPx	Motility	0.051	0.935	**0.975**	**0.005**	0.200	0.747
		Progressive motility	-0.410	0.493	0.872	0.054	0.200	0.747
6	SOD	Motility	0.300	0.624	-0.500	0.391	-0.100	0.873
		Progressive motility	0.300	0.624	-0.600	0.285	-0.100	0.873
	GPx	Motility	**-1.000**	**<0.001**	-0.100	0.873	0.103	0.870
		Progressive motility	**-1.000**	**<0.001**	<0.000	≈1.000	0.103	0.870
7	SOD	Motility	-0.872	0.054	0.400	0.505	-0.600	0.285
		Progressive motility	-0.872	0.054	0.500	0.391	-0.600	0.285
	GPx	Motility	0.051	0.935	0.718	0.172	0.308	0.614
		Progressive motility	-0.205	0.741	0.154	0.805	0.308	0.614
In total	SOD	Motility	**-0.383**	**0.023**	-0.023	0.894	-0.259	0.133
		Progressive motility	-0.332	0.052	-0.088	0.615	-0.301	0.079
	GPx	Motility	-0.277	0.108	-0.047	0.789	-0.063	0.721
		Progressive motility	-0.265	0.123	-0.129	0.459	-0.021	0.902

ρ-Spearman's rank coefficient correlation

Analysis of the relation between sperm motility and THI

One of the goals in this work was to assess the influence of THI on bull sperm cell motility. The effect of THI on sperm quality was monitored based on 14-day averages of temperature and relative humidity calculated before each sampling. Given the heterogeneity of the data, for the quantification of the strength of the relationship between THI and the percentage of motile sperm cells, and the relationship between THI and percentages of progressively motile cells, Spearman's rank coefficient was calculated (Table 3). The negative coefficients indicated that with the increase in THI both the sperm motility and progressive motility decreased. However, it was revealed that between the three variables (motility, progressive motility and THI) the relationship was not significant ($p>0.05$), i.e. there almost was no relationship between these variables.

Table 3. Results of correlation analysis between sperm cell motility, progressive motility and THI in analyzed bull groups

Parameter pairs	Group	ρ	p
Sperm motility/THI	C	-0.006	0.977
	M	-0.001	0.995
	A	-0.017	0.929
Sperm progressive motility/THI	C	-0.030	0.876
	M	-0.039	0.836
	A	-0.136	0.472

Analysis of the relationship between the enzyme activities and THI

Data on SOD and GPx activities in the semen plasma, and THI in all of the three groups of bulls were homogeneous (cv<30%), which is why the strength of the relationship between these variables was assessed according to Pearson's correlation coefficient (Table 4). The only negative relationship was proven in the control group between SOD and THI: the increase in THI coincided with the decline in SOD activity. However, the relationship between these variables was not significant. In groups M and A the increase in THI was parallel with the increase in SOD activity, but without a significant relationship. In all of the three groups THI increased with the increase in GPx activity, but the relationship between these variables was not strong.

Table 4. Results of correlation analysis between the activities of SOD and GPx, and THI in bull groups

Parameter pairs	Group	r	p
SOD / THI	C	-0.015	0.517
	M	0.185	0.328
	A	0.347	0.060
GPx / THI	C	0.108	0.575
	M	0.320	0.084
	A	0.356	0.053

DISCUSSION

Oxidative stress results from increased production of free radicals and/or decreased antioxidative capacity of the organism [35]. Williams et al. [36] state that the process of oxidation is necessary to almost all cells to provide them with energy needed for all vital functions.

Mammalian sperm cells have a developed defence mechanism against OS owing to the enzyme antioxidative system. The best-known enzymes of this system are SOD, CAT and GPx. Their activity in seminal plasma and sperm cells varies between animal species [37-39]. According to the research by Bilodeau et al. [40], the activity of CAT in the bull semen is low, which was confirmed by Kadirve et al. [41], in buffalo semen. The average SOD activity in the control group was significantly lower than in groups M ($p<0.001$) and A ($p<0.001$), whilst the means in the treated groups did not differ significantly ($p=0.784$). Analysis of seven consecutively taken bull semen samples detected that the average activities of SOD differed significantly within the groups ($p<0.001$) and that in all groups they were on the increase until S5 (when they reached a peak), and decreased afterwards and were lowest in S7. The average activities of SOD significantly differed between groups in S1-5, but not afterwards, which can be explained by high SOD consumption in the neutralisation of free radicals resulting from long-lasting heat stress. This finding is in compliance with the results published by Vince et al. [42], who detected high activities of SOD in Simmental bulls in summer. Nichi et al.[16] proved in bulls of the same breed a higher degree of lipid peroxidation in the semen in the summer period, which means that the organism by means of its antioxidative enzyme system tends to resist to the process and thus prevent the production of semen of lower quality. In their experiment on Karan Fries bulls Soren et al. [43] detected increased SOD activity in the semen during the summer period. Nair et al. [44] detected a negative correlation between the activity of SOD and lipid peroxidation. These findings are in compliance with the results obtained in our research, with higher average SOD activities in the treated groups in comparison with the control, which means that both feed supplements increased the antioxidative capacity. However, Sariözakan et al. [45] found lower SOD activity in the semen of HF bulls diluted with diluters containing antioxidants, in comparison with those diluted without the addition of antioxidants.

The average GPx activity in the control was significantly lower than that in group M ($p=0.001$) and group A ($p=0.005$), but the averages did not differ significantly between the treated groups ($p=0.701$). The average GPx activities in the seven semen samples within the same group varied significantly ($F=8.252$, $p<0.001$). They tended to rise until the time when S5 samples were taken, peaked, and were on decrease afterwards, which can be explained by GPx consumption in the antioxidative processes [46]. Analysis of the semen samples taken in a particular time point revealed that the groups differed in the mean GPx activity in the first four samples taken, but not in the period which followed, and that the averages in S1-4 were less in the control bulls

than those in group M (p_1=0.029, p_2=0.040, p_3=0.044 and p_4=0.025) and group A in S1 and S4 (p_1=0.025 and p_4=0.040). This implies that due to heat stress there is a compensatory increase in the activity of GPx in the semen of all tested groups. This is in agreement with the research conducted by Nichi et al. [16], who also recorded high activity of this enzyme in Simmental bull semen, significantly higher than in that of Nelore bulls. In the semen of young Simmental bulls [5] and in the semen of Karan Fries bulls [43] increased activities of GPx were detected in the summer. Given that we detected a significantly higher average GPx activities in S1-4 in both treated groups in comparison with the control, the feed supplements increased the antioxidative capacity of the semen.

The analysis of the relationship between the activities of SOD or GPx and the percentages of motile and progressively motile sperm cells within bull groups and between the samples taken at the same time points indicated that in the control, in S6 there was a negative, entirely deterministic relationship between GPx and the percentage of motile sperm cells ($\rho \approx -1.000$, $p \approx 0.000$), as well as the percentage of progressive motile sperm cells ($\rho \approx -1.000$, $p \approx 0.000$). In group M of bulls a negative, very strong relationship between SOD activity and the percentage of progressive motile sperm cells (ρ=-0.975, p=0.005) was detected in S2, in which a slight increase in SOD activity in the semen in comparison with S1 was detected, which was not high enough to considerably soften the negative influence of the heat stress. In S5 a very strong positive relationship (ρ=0.975, p=0.037) was revealed between SOD activity and the percentage of progressive motile sperm cells, as well as between the enzyme activity and the proportion of motile spermatozoa. Lindemann et al. [47] and O'Flaherty et al. [48, 49] described the positive influence of SOD on the motility of buffalo's frozen/thawed semen. Losano et al. [50] wrote about the positive influence of SOD on the motility of the bulls' spermatozoa. In group The relationship between the activities of SOD or GPx and the proportions of motile and progressive motile sperm cells was not significant.

Given all what was said, it can be concluded that the increase in the activities of both enzymes in the tested groups of bulls was not sufficient (with the exception of group M, in which it was partially sufficient) to completely mitigate the negative influence of heat stress on the semen quality. Rajoriya et al. [51] failed to confirm a relationship between SOD activity and progressive sperm motility of thawed Tharparkar bull's semen. Lone et al. [52] did not find any significant correlation between SOD activity and progressive sperm motility of buffalo semen. Vince et al. [42] detected higher SOD activity in the semen of young Simmental bulls than in older animals of the same breed in summer but this was not enough to entirely cushion the effects of oxidative stress in summer, which eventually led to lower sperm motility. By contrast, Kadirve et al. [41] detected a positive relationship between the activities of SOD, GPx and GSH, and the motility of the buffalo spermatozoa, similar to the results obtained by Nair et al. [44], who found a positive relationship between SOD, GPx and G6PD activities, and the motility of the sperm cells in bulls and buffalos. Permual [53] claimed that the

addition of SOD to the diluter correlated positively with sperm motility in Mithun bulls. Olfati Karaji et al. [54] concluded that the addition of reduced glutathione and SOD to the diluter results in increased motility and progressive motility of HF bull's sperm. Majic Balic et al. [5] recorded increased GPx activities in young Simmental bull's semen in summer, but they were insufficient to mitigate the intensive oxidative processes, which could have contributed to the decrease in the progressive motility of semen. Based on the results obtained in our experiment, it can be concluded that the antioxidants added to bull's feed had positive influence on the sperm motility, which was more pronounced in group M.

In the experiment conducted by Majić Balić et al. [5] on Simmental bulls it was proven that THI correlated negatively with progressive sperm motility i.e. that progressive motility was the least in summer, when THI was the highest, which is in compliance withour results. By contrast, in research by Luceno et al. [55], who investigated the influence of heat stress on HF bulls in the Netherlands, a conclusion was reached that increased THI index did not influence strongly neither the morphology nor the progressive motility of sperm cells. This is in line with the results published by Mathevom et al. [56], who claimed that sperm cell concentration and motility in the ejaculates of HF bulls are higher in winter (lower THI) than in summer. The variations in the semen quality may result from the breed, age, diet, general condition and the adaptive capacity of the animals, temperature and relative humidity in the course of the experiment, as well as the duration of heat stress.

The analysis of the relationship between the THI and the activity of antioxidative enzymes in our research revealed that the increase in THI in the control group led to decrease in the SOD activity, whilst in both treated groups the activity increased ($p>0.05$). The increase in GPx activity ($p>0.05$) with the rise in THI was characteristic of in all three groups of bulls.

Based on the facts which are stated, it can be concluded that in all of the three groups of bulls the activities of SOD and GPx in the semen were on the increase, but the increase was significantly lower than in the control. In the control group the activity of SOD decreased, whilst in the treated groups it increased. In all of the three groups the activity of GPx was also on the increase, but not significantly. Thus, a lower antioxidative capacity of the semen from the control group was proven, that is an increased one in the semen of bulls treated with the tested supplements (Alkosel and Melofeed). Moreover, the increase in THI was followed by the decrease in the motile and progressive motile sperm proportions in all of the groups. The addition of the tested feed supplements to bull feed in order to prevent the negative impact of heat stress on the semen can be considered a good solution. The results obtained render further research necessary to detect whether the use of the feed additives tested (or some others of similar composition) before heat stress could contribute to the decrease in its negative influence on the bull sperm quality.

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Authors' contributions

PS and MM carried out the experiment, made substantial contribution to acquisition, analysis and interpretation of data and participated in manuscript writing. LN performed the statistical analysis and made substantial contributions to interpretation of data. AN and MJ coordinated experiment performance and has been involved in manuscript writing. RM made substantial contributions to interpretation of data and writing the manuscript. SZ conceived and designed the study, made substantial contributions to the writing of manuscript, critically revised the manuscript and approved its submission.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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EFEKAT DODADAVANJA ANTIOKSIDANASA U HRANI NA ODREĐENE PARAMETRE KVALITETA SEMENA BIKOVA U USLOVIMA TOPLOTNOG STRESA

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Cilj ovog istraživanja bio je ispitivanje efekta liofilizovanog preparata voćne pulpe dinje (izvor superoksid dismutase – SOD) i inaktivisanih živih ćelija kvasca *Sacharomycess cerevisae* (soj R397) dodatih u hranu kroz preparat koji sadrži visok nivo organski vezanog selena (izvor selen zavisne glutation-peroksidaze – Se-GPx) na kvalitet semena bikova u uslovima toplotnog stresa. U ogledje bilo uključeno 15 bikova podeljenih u tri grupe (kontrolna – C grupa, ogledna grupa-M kojoj je dodavan izvor SOD – i ogledna grupa – A tretirana izvorom Se-GPx). Ogled je sproveden u letnjem periodu. Aktivnosti SOD i Se-GPx u seminalnoj plazmi određivane su spektofotometrijski. Za utvrđivanje koncentracija spermatozoida, pokretljivosti i brzinskih parametara korišćena je CASA *(computer-assisted semen analysis*), a za merenje temeperature i vlažnosti koristili smo digitalni data loger.

Prosečna aktivnost SOD u kontrolnoj grupi bikova bila je značajno niža od one u M grupi ($p<0,001$) i A grupi ($p<0,001$), dok se prosečne vrednosti u tretiranim grupama nisu međusobno značajno razlikovale ($p=0,784$). Više prosečne vrednosti SOD su u tretiranim grupama u odnosu na kontrolnu ukazuju da su oba dodatka hrani povećala antioksidativni kapacitet semene plazme. Prosečna aktivnost GPx za kontrolnu grupu je bila značajno niža nego u A ($p=0,001$) i M grupi bikova ($p=0.005$), dok se međusobno M i A grupa nisu značajno razlikovale ($p=0,701$). Analizom korelacije između aktivnosti svakog od enzima i procenta pokretljivih i progresivno pokretljivih spermatozoida kod svakog bika ponaosob nije ustanovljena značajna povezanost. Analizom veze između THI (temperature-humidity index) i aktivnosti antioksidativnih enzima uočeno je da je sa porastom THI u kontrolnoj grupi došlo do smanjenja aktivnosti SOD, dok je u tretiranim grupama nastalo povećanje aktivnosti SOD ($p>0.05$). U sve tri grupe bikova povećanjem THI povećavala se i aktivnost GPx ($p>0.05$). Može se zaključiti da je kod sve tri grupe bikova došlo do povećanja aktivnosti oba enzima u semenoj plazmi, ali je povećanje značajno niže u kontrolnoj grupi nego u tretiranim grupama. Time je dokazan manji antioksidativni kapacitet semene plazme netretiranih bikova u odnosu na tretirane.