



Glutathione redox state, glutathione peroxidase activity and selenium concentration in periparturient dairy cows, and their relation with negative energy balance

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ABSTRACT. The aim of the study was to evaluate glutathione redox state, glutathione peroxidase (GPx) activity, and selenium (Se), non-esterified fatty acids (NEFA) and β -hydroxybutyrate (BHB) concentrations in 15 Holstein periparturient dairy cows and to monitor the effect of negative energy balance (NEB) on the level of oxidative processes ongoing in dairy cows in postpartum period. The body condition score (BCS) was recorded and blood samples were collected 4 times during periparturient period. A significantly increased NEFA concentration was recorded on calving day ($P < 0.05$) and 7 days *post partum* (p.p.; $P < 0.01$) compared to 7 days *ante partum* (a.p.). The reduced glutathione (GSH) concentration was significantly decreased on calving day and 7 days p.p. ($P < 0.05$) as compared to 7 days a.p. The oxidized glutathione (GSSG) concentration was significantly higher 7 days p.p. as compared to calving day ($P < 0.01$) and 14 days p.p. ($P < 0.05$). Between the GSSG concentration and the GSH/GSSG ratio was found a significantly negative ($r = -0.84$; $P < 0.001$) correlation. The significant decrease in GPx activity was found 14 days p.p. as compared to 7 days a.p. ($P < 0.05$). The BCS value was significantly positively correlated ($r = 0.44$; $P < 0.05$) with GSSG concentration. The results of the study indicate significant changes of antioxidant/oxidant markers and also confirm that in the postpartum period oxidative stress occurs in dairy cows. It also seems that BCS correlates with these indicators and may influence the level of oxidative processes in cows during the periparturient period.

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Introduction

Negative energy balance (NEB) occurs in dairy cows during the periparturient period and is induced by increased energy and nutrients requirements. This higher energy and nutrient demand is important for fetal development and for postpartum milk production. There is also a significant decrease in intake of dry matter (Sordillo and Aitken, 2009; Esposito et al., 2014). NEB is associated with metabolic disorders, increased

inflammation, immunosuppression and oxidative stress (Mayasari et al., 2016). Metabolic changes in the transition period are accompanied by increased production of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) followed by lipid peroxidation and cellular damage to tissues. Carbohydrate insufficiency in dairy cows with NEB leads to lipid mobilization and increased production of non-esterified fatty acids (NEFA) and ketone bodies in the liver (Sordillo, 2005; Spears and Weiss, 2008; Esposito et al., 2014).

Glutathione is a very important component of the antioxidant defense system because of its ability to protect cells from oxidative stress. One method of protecting cells is by using glutathione-dependent enzymes such as glutathione peroxidase (GPx) (Meister, 1983; Enkvetchakul et al., 1995). During reduction of H_2O_2 to H_2O , which is catalyzed by GPx, reduced glutathione (GSH) is transformed into oxidized glutathione (GSSG) and serves as an electron donor. GSSG is converted back to GSH by glutathione reductase using reduced nicotinamide adenine dinucleotide phosphate (NADPH). Under physiological conditions, the reduced form is present in a higher concentration. Oxidative stress can be the reason of the increase in GSSG concentration, or of the decrease in the GSH/GSSG ratio (Meister and Anderson, 1983; Cao et al., 2013). Thus, significant changes in the intracellular GSH and GSSG concentrations usually indicate oxidative stress and the GSH/GSSG ratio is considered to be one of the best indicators of oxidative stress (Tarin, 1996; Tarin et al., 1998; Avanzo et al., 2001; Kaneko et al., 2001).

Along with the concentration of reduced and oxidized form of glutathione and the ratio of GSH/GSSG, determining GPx activity is considered to be one of the main indicators of oxidative stress (Pilarczyk et al., 2012; Cao et al., 2013). GPx, as a selenium-dependent antioxidant enzyme, is also used in indirect determination of selenium (Se) status and indicates long-term Se supplementation (Pavlata et al., 2000). Se status in organisms, especially in the periparturient period, is very important for health, immunity and growth. A deficiency of this antioxidant can contribute to increased incidence of oxidative stress and the associated increased incidence of mastitis (Gong and Xiao, 2016).

Based on the above findings, we decided to monitor the effect of NEB on the level of oxidative stress in dairy cows. The NEB that occurs in dairy cows after parturition was monitored using BCS value and the determination of the NEFA concentration as an indicator of the energy balance in dairy cows (Omur et al., 2016). Cows with a higher body condition score (BCS) value before parturition are predisposed to lose more body condition in the periparturient period and are characterized by a significantly higher NEFA concentration around parturition due to the already mentioned ongoing lipid mobilization (Omur et al., 2016). Along with these changes, we also expected an increase in oxidative stress indicators and a decrease in antioxidants in postpartum period in dairy cows. According to our hypothesis, the changes in BCS during periparturient period in dairy cows should be proportional to

the level of ongoing oxidative processes after parturition. The aim of the study was to evaluate glutathione redox state, GPx activity and Se concentration in dairy cows during periparturient period. NEFA and β -hydroxybutyrate (BHB) concentrations were also measured.

Material and methods

Experiments were approved by the Czech Animal Experiments Committee (21599/2014-MZE-17214; PP 11-2017).

Animals and diets

The study was carried out using 15 Holstein dairy cows at a farm located in the village of Uherčice (Břeclav, South Moravia, Czech Republic). Cows selected for testing had no complications of the diseases, the BCS was 3.50 and all of the cows were multiparous, specifically cows ranging from the second to the fourth lactation were included in the experiment. None of the cows were treated for diseases during the experimental period, and none of them had calving complications (only single pregnancy). There were no instances of death or culling. The mean milk production was 10 337 l per last lactation (it ranged from 6 287 to 12 722 l). The cows were fed a total mixed ration (TMR) according to the antepartum and postpartum period (formulated feed amounts and nutritional values are given in Table 1).

Study design

Blood samples were collected 4 times during the periparturient period according to different *ante partum* (a.p.) and *post partum* (p.p.) stages: 7 days a.p. (n = 15), calving day (n = 15), 7 days p.p. (n = 15) and 14 days p.p. (n = 15). Indicators of antioxidant status and metabolites concentrations were determined in a total of 60 blood samples. The BCS was recorded at every single blood collection of the cows. At 7 days a.p. mean values of BCS were 3.50 ± 0.25 , on calving day 3.50 ± 0.27 , at 7 days p.p. 3.25 ± 0.40 and at 14 days p.p. 3.00 ± 0.35 . The average milk yield was 49.7 l in the first month of lactation.

Sampling and analysis

NEFA and BHB concentrations. Blood samples were collected from the vena coccygea mediana into Hemos sampling tubes without anticoagulant. For serum samples, blood was allowed to coagulate at room temperature and centrifuged (3000 rpm) for 10 min. After blood collection, samples were immediately analyzed or stored at -70°C .

Table 1. Total mixed ration composition

Indices	<i>Ante partum</i>	<i>Post partum</i>
Ingredients, kg		
alfalfa hay	2	1
barley straw	2.2	0
concentrate (DOVP) ¹	0	6
concentrate (DOVP – a.p.) ²	2.8	0
post-extraction rapeseed meal	0.8	0
palmitate	0	0.15
MP iont ³	0.5	0
high moisture maize	0	3
brewers grains	0	4
alfalfa haylage	0	6
maize silage	15	19
total amount	23.30	39.15
DM ⁴ , kg	12.9	19.5
DM, %	55.4	49.8
Nutrient content		
crude protein, % of DM	15.0	16.8
crude fibre, % of DM	21.1	15.5
crude fat, % of DM	3.1	4.6
NEL ⁵ , MJ of DM	5.63	6.67
Ca, % of DM	0.88	0.69
P, % of DM	0.48	0.41
Na, % of DM	0.24	0.40
K, % of DM	1.33	1.21
Mg, % of DM	0.60	0.27
Cl, % of DM	1.12	0.38
S, % of DM	0.38	0.28
Zn, mg of DM	68	114
Mn, mg of DM	57	139
Cu, mg of DM	31	33
Co, mg of DM	0.32	0.73
I, mg of DM	1.14	0.82
Se, mg of DM	1.09	0.49
vitamin A, IU	216	255
vitamin D, IU	49	37
vitamin E, mg	17600	884

¹ DOVP – complementary feed for lactating dairy cows; ² DOVP –a.p. – complementary feed for dairy cows *ante partum*; ³ MP iont – mixture of anions (CaCl₂, MgSO₄), mineral supplements and protein concentrate (to prevent postpartum hypocalcaemia); ⁴ DM – dry matter; ⁵ NEL – net energy lactation

Serum NEFA and BHB concentrations were measured with standardized kits (Randox Laboratories Ltd., Crumlin, UK) using an automatic biochemical analyzer Konelab 20XT (Thermo Fisher Scientific, Vantaa, Finland).

Glutathione redox state. Blood samples were collected from the vena coccygea mediana into Hemos sampling tubes with heparin anticoagulant. For analysis of GSH concentration 50 µl of whole blood was frozen and stored at –70 °C before

determination, and for analysis of GSSG concentration 100 µl of whole blood was added to 10 µl of thiol-scavenging reagent 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP) and was then frozen and stored at –70 °C before determination. Reduced and oxidized glutathione concentrations were measured with a BIOXYTECH GSH/GSSG-412 kit (OxisResearch, Portland, OR, USA) using a colorimetric enzymatic method developed by Tietze (1969). This method is based on the change in colour development during the reaction. Reaction rate is proportional to the GSH and GSSG concentrations. 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reacts with GSH/GSSG to form a spectrophotometrically detectable product at 412 nm. An Evolution 160 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure GSH and GSSG concentrations.

GPx activity and Se concentration. Blood samples were collected from the vena coccygea mediana into Hemos sampling tubes with heparin. After blood collection, samples were immediately analyzed or stored at –70 °C. GPx activity in whole blood was measured by a RANSEL kit (Randox Laboratories Ltd., Crumlin, UK) using a UV method based on that of Paglia and Valentine (1967). This method is based on measuring the decrease in absorbance at 340 nm due to NADPH oxidation by the reaction with glutathione reductase (GR). For determination of GPx the automatic biochemical analyzer Konelab 20XT (Thermo Fisher Scientific, Vantaa, Finland) was used. The Se concentration in whole blood was analyzed using hydride generation atomic absorption spectrometry – HG AAS (SOLAAR, Thermo Fisher Scientific, Waltham, MA, USA). The samples were prepared by mineralization with HNO₃ and H₂O₂ using microwave digestion system ETHOS TOUCH CONTROL (Milestone, Sorisole, Italy) followed by evaporation.

Statistical analysis

The obtained results were tested for the homogeneity of variances (Hartley-Cochran-Bartlett test) and the normality of distribution (Shapiro-Wilk test). The data were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Fisher LSD post-hoc test. All results were expressed as mean value (x) and standard error of means (SEM). The relationship between the glutathione concentrations in whole blood and BCS values were evaluated by the correlation coefficient and the significance of correlation using linear regression analysis.

Results

Negative energy balance – BCS values, NEFA and BHB concentrations

A significantly increased NEFA concentration was recorded on calving day ($P < 0.05$) and 7 days p.p. ($P < 0.01$) compared to 7 days a.p. (Table 2). The highest mean BHB concentration was found 7 days p.p. The difference in the increase was not, however, significant ($P > 0.05$). Between the BCS value and NEFA concentration no significant correlation was found ($P > 0.05$). The differences in BCS value and NEFA concentration in individual groups are shown in Figure 1.

Table 2. Non-esterified fatty acids (NEFA) and β -hydroxybutyrate (BHB) concentrations in dairy cows from 7 days *ante partum* (a.p.) to 14 days *post partum* (p.p.)

Peripartum stages		NEFA, mmol/l	BHB, mmol/l
7 days a.p.	x	0.27 ^{aA}	0.57
	SEM	0.06	0.04
calving day	x	0.50 ^a	0.55
	SEM	0.10	0.05
7 days p.p.	x	0.56 ^A	0.64
	SEM	0.08	0.06
14 days p.p.	x	0.38	0.59
	SEM	0.06	0.05

x – mean value; SEM – standard error of means; significant differences between groups are indicated by using the same indices in a column: a – $P < 0.05$, A – $P < 0.01$

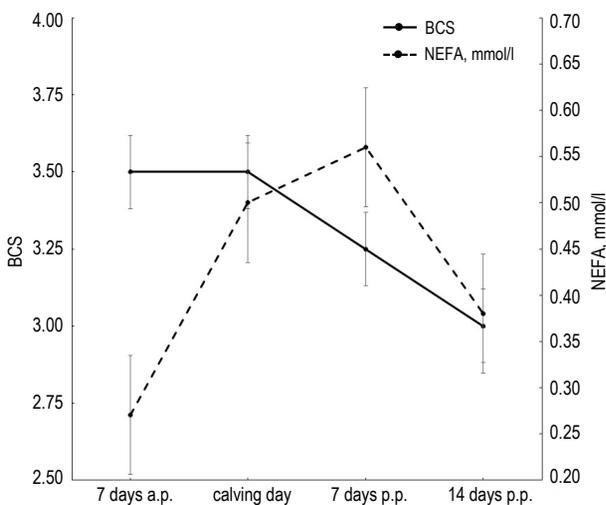


Figure 1. Mean body condition score (BCS) value and serum non-esterified fatty acid (NEFA) concentration in dairy cows during the negative energy balance (from 7 days *ante partum* (a.p.) to 14 days *post partum* (p.p.))

Glutathione redox state

The results of reduced and oxidized glutathione are presented in Table 3. The lowest mean GSH concentration was recorded on calving day and the

Table 3. Glutathione redox state in dairy cows from 7 days *ante partum* (a.p.) to 14 days *post partum* (p.p.)

Peripartum stages		GSH, $\mu\text{mol/l}$	GSSG, $\mu\text{mol/l}$	GSH/GSSG ratio
7 days a.p.	x	1005 ^{aB}	5.18	246
	SEM	37.7	0.58	39.5
calving day	x	867 ^a	4.25 ^A	248
	SEM	53.3	0.55	34.1
7 days p.p.	x	881 ^B	6.61 ^{aA}	171
	SEM	38.8	0.61	44.0
14 days p.p.	x	915	4.74 ^a	256
	SEM	58.7	0.79	43.8

x – mean value; SEM – standard error of means; significant differences between groups are indicated by using the same indices in a column: a, B – $P < 0.05$, A – $P < 0.01$; GSH – reduced glutathione; GSSG – oxidized glutathione; GSH/GSSG – glutathione ratio

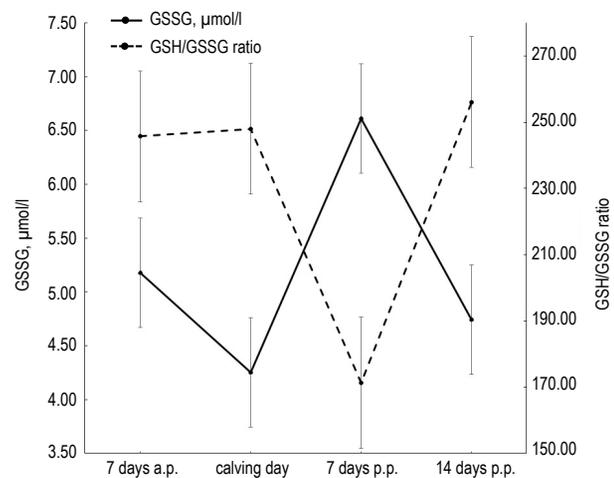


Figure 2. Mean whole blood oxidized glutathione (GSSG) concentration and GSH/GSSG ratio value in dairy cows during the negative energy balance (from 7 days *ante partum* (a.p.) to 14 days *post partum* (p.p.))

difference in the decreases was significant as compared to 7 days a.p. ($P < 0.05$). The GSH concentration was also significantly decreased 7 days p.p. as compared to 7 days a.p. ($P < 0.05$). In contrast to GSH concentration, the mean GSSG concentration in the study was significantly higher 7 days p.p. as compared to calving day ($P < 0.01$) and 14 days p.p. ($P < 0.05$). The differences in GSSG concentration and GSH/GSSG ratio in individual groups are shown in Figure 2. The differences in GSH/GSSG ratio were not, however, significant ($P > 0.05$). A significantly negative ($r = -0.84$; $P < 0.001$) correlation was found between the GSSG concentration and the GSH/GSSG ratio (Figure 3). The BCS value was significantly positively correlated ($r = 0.44$; $P < 0.05$) to GSSG concentration. Between the BCS value and GSH/GSSG ratio a negative correlation was found ($r = -0.30$) but was not, however, significant

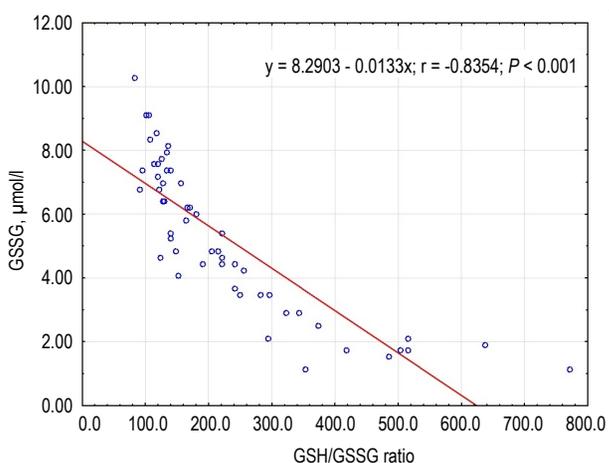


Figure 3. Correlation between whole blood oxidized glutathione (GSSG) concentration and GSH/GSSG ratio value in dairy cows during the negative energy balance

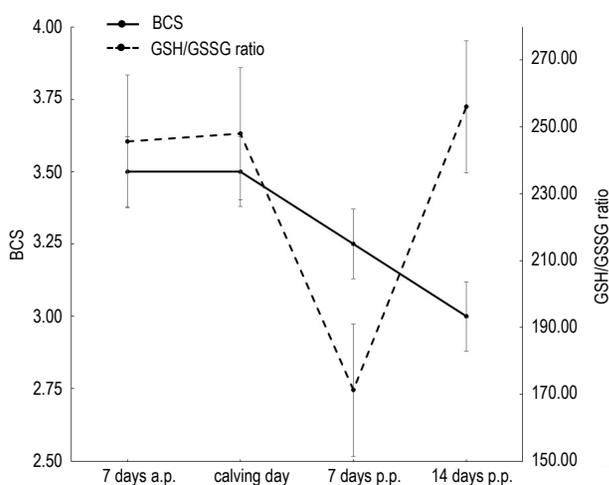


Figure 4. Mean body condition score (BCS) and whole blood GSH/GSSG ratio values in dairy cows during the negative energy balance (from 7 days ante partum (a.p.) to 14 days post partum (p.p.))

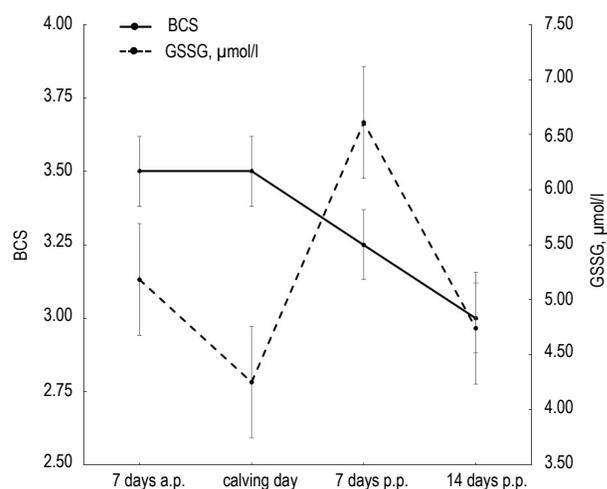


Figure 5. Mean body condition score (BCS) value and whole blood oxidized glutathione (GSSG) concentration in dairy cows during the negative energy balance (from 7 days ante partum (a.p.) to 14 days post partum (p.p.))

($P > 0.05$). The differences in BCS value and GSH/GSSG ratio and GSSG concentration in individual groups are shown in Figure 4 and 5, respectively.

GPx activity and selenium concentration

The results of GPx activity and Se concentration are presented in Table 4. The significant difference in GPx activity was found between 14 days p.p. and 7 days p.p. ($P < 0.05$). The lowest Se concentration was recorded at 7 days a.p., but it was not significant compared to the other blood samplings. No significant change ($P > 0.05$) in Se concentration between individual groups was recorded.

Table 4. Glutathione peroxidase activity (GPx) and selenium concentration (Se) in dairy cows from 7 days ante partum (a.p.) to 14 days post partum (p.p.)

Peripartum stages		GPx, $\mu\text{kat/l}$	Se, $\mu\text{g/l}$
7 days a.p.	x	1133 ^a	172
	SEM	53.1	9.00
calving day	x	1020	174
	SEM	53.3	8.22
7 days p.p.	x	1041	181
	SEM	52.8	10.29
14 days p.p.	x	954 ^a	179
	SEM	55.9	6.44

x – mean value; SEM – standard error of means; significant differences between groups are indicated by using the same indices in a column: a – $P < 0.05$

Discussion

Negative energy balance – BCS values, NEFA and BHB concentrations

Previous studies reported that higher BCS values (>3.0 , ≥ 3.5) relate to NEFA concentrations in dairy cows and also showed a relationship between high BCS values and metabolic changes leading to increased incidence of oxidative stress and diseases in the periparturient period (Bernabucci et al., 2005; O'Boyle et al., 2006). Insufficient energy intake in the periparturient period results in a decrease in glucose levels and also in an increase in NEFA levels as a response to lipomobilization (Omur et al., 2016). In the present study a significant increase in NEFA concentration around calving in cows with the higher BCS (3.50 at the beginning of the experiment) was found. The changes in BCS value, respectively the decrease in BCS after parturition were not, however, significant ($P > 0.05$). There was not thus a significant loss of body condition in dairy cows as expected. This assumption may result in a significantly elevated but non-pathological NEFA

concentration. A significant but non-pathological increase in NEFA concentration (0.56 ± 0.08 mmol/l) in this study, as well as in our previous study by Pišťková et al. (2018), may be the reason for BHB concentration stability during the periparturient period and dairy cows not being predisposed to an increased incidence of ketosis. A slight increase in BHB concentration was also observed in the present study.

Antioxidant status

In dairy cows periparturient period is a critical phase which is extremely important to health, productivity and fertility (Roche et al., 2009). It is characterized by the depletion of antioxidants and results in an imbalance between pro-oxidants and antioxidants. The amount of antioxidants is insufficient to cope with the production of ROS and so oxidative stress occurs in dairy cows (Castillo et al., 2005).

Glutathione redox state

A decrease in GSH/GSSG ratio indicates ongoing oxidation processes (Dobbelaar et al., 2010). The GSH concentration may start to decline in consequence of peroxide reduction free radical scavenging (O'Boyle et al., 2006) and ROS reduction, as their quantity continues to increase in the periparturient period (Sordillo et al., 2007), while GSSG concentration increases.

In the present study the reduction in GSH concentration was reported from calving day to 14 days p.p., when there was again an increase in concentration. Findings of the present study are similar to Sordillo et al. (2007), who found the decrease in whole blood GSH concentration from prepartum to postpartum period (488 ± 48 μ mol/l on calving day and 341 ± 19 μ mol/l at 21 days p.p.) and to a study by Elischer et al. (2015), in which GSH concentration declined after parturition and the lowest value was recorded at 7 days p.p. (226 ± 13 μ mol/l vs 288 ± 14 μ mol/l at 1 day p.p.). The significant increase in GSSG concentration after parturition in the present study was also in line with the findings of Sordillo et al. (2007), in which the highest GSSG concentration was observed on calving day (3.6 ± 0.7 μ mol/l). Elischer et al. (2015) reported the increased GSSG concentration after parturition in multiparous cows. Compared to GSH and GSSG concentration the differences in GSH/GSSG ratio were not, however, significant ($P > 0.05$). Data published in the previous studies (Sordillo et al., 2007; Elischer et al., 2015) are similar to findings

observed in the present study, which showed a decrease in GSH/GSSG ratio in the postpartum period, especially at 7 days p.p. Moreover, between the GSSG concentration and the GSH/GSSG ratio a significantly negative correlation was found, which is in line with a study by Sordillo et al. (2007), who reported that the negative correlation indicates a shift in the redox potential of whole blood ($r = -0.87$; $P < 0.001$).

In the study by O'Boyle et al. (2006), who recorded the change of GSH/GSSG ratio in relation to BCS in dairy cows, the GSH/GSSG ratio was 6.66 ± 5.1 in cows with normal BCS and 1.9 ± 1.0 in cows with high BCS. It seems that the cows with higher BCS are predisposed to a lower GSH/GSSG ratio compared to cows with normal BCS. On the basis of this finding, we predicted the lower values of GSH/GSSG ratio and also a higher level of oxidative processes in dairy cows (BCS – 3.50) in the present study. This could partially confirm the findings of the present study showing a significantly positive correlation between the BCS value and the GSSG concentration. This is also supported by the results of GSH and GSSG concentrations, and GSH/GSSG ratio in the present study, which are in line with previously reported data for multiparous cows using the same GSH/GSSG-412 kit by Oxis-Research (Sordillo et al., 2007; Elischer et al., 2015). Although the present study showed a decrease in the GSH/GSSG ratio, no significant correlation with BCS values was observed. The reason may be a not so significant decrease in BCS in the postpartum period, when the lowest BCS was recorded at 14 days p.p., which may indicate an attempt to cope with ROS and thus prevent a higher degree of oxidative processes.

GPx activity

In the case of GPx activity the depletion of antioxidant was recorded during the whole experiment. Compared to our previous study by Pišťková et al. (2018), in which the lowest GPx value was 793 ± 269 μ kat/l at 3 weeks p.p., a decrease in GPx activity in the present study was slower. Based on the findings, it could be associated with a lower BCS value before parturition (present study – 3.50 and previous study – 3.80) and confirm the fact mentioned above that cows with higher BCS are more susceptible to oxidative stress. As in the case of glutathione redox state, another possible explanation may be an insignificant change in BCS in the periparturient period in dairy cows and thus a lower level of ongoing oxidative processes. A decrease of antioxidant activity around calving could

result from depletion in the fight against ROS, which occur in higher concentrations in this period. The amount of antioxidants available is insufficient due to an ever increasing amount of ROS. O'Boyle et al. (2006) suggested that reduced antioxidant potential is more likely due to depleted antioxidant defense mechanism needed to reduce accumulated levels of ROS. As reported by Konvičná et al. (2015), decreased GPx activity is a reason for an increase in postpartal oxidative stress. The study by Cigliano et al. (2014), in which lower GPx and superoxide dismutase (SOD) activities were found in early lactating compared to mid-late lactating cows, reported that early lactation is related to a higher consumption of antioxidants because of the increased metabolic activity needed for milk production. Some researchers also suggested increased antioxidants activity as an effect of an increased risk of oxidative stress (Bernabucci et al., 2005; Dobbelaar et al., 2010).

Selenium concentration

Apart from determining GPx activity, selenium content was checked directly by checking selenium concentration in blood. Based on previous studies (Pilarczyk et al., 2012; Gong and Xiao, 2016) we expected a decreased Se concentration in the prepartum period due to the selenium transfer, necessary for the fetus, through the placental barrier. In the present study the lowest Se concentration was recorded at 7 days a.p., but was not significant compared to the other blood samplings. No significant changes in Se concentration were recorded from calving day until the last blood collection. A reason could be a sufficient concentration of selenium compared to reference values in cattle, so the concentration differences are not so prominent in dairy cows (Se whole blood concentration regarded as a reference value is 100–130 µg/l) (Pilarczyk et al., 2012; Pavlata et al., 2001). This could be also the reason of the slower decrease in GPx activity as a selenium-dependent enzyme. Se concentration should also be decreased in peak lactation due to Se transfer from the blood to the milk produced at peak lactation. This could not be recorded because of the small time range of blood collections. The insufficient time range of prepartal blood sampling in the experiment may be also the reason for non-significant Se concentration differences compared to other groups.

Conclusions

Since the decrease in body condition score (BCS) was not so significant in the present study, the correlation between lower BCS and the evaluated

non-esterified fatty acids (NEFA) concentration was also not significant. However, there is a presumption of correlation between BCS and NEFA concentration and its possible use as a suitable marker of energy balance. The results of the antioxidant/oxidant parameters in the present study indicate that cows did undergo oxidative stress in the postpartum period. Decreased reduced glutathione (GSH) concentration and GSH/GSSG ratio and increased oxidized glutathione (GSSG) concentration after parturition support the contention that the glutathione redox status is an indicator of oxidative stress. It also seems that BCS correlates with these indicators and that its value before parturition and also loss of body condition postpartum could influence the level of oxidative processes in cows during the periparturient period. However, further studies are needed to clarify this issue.

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