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Evaluation of the glutathione concentration in whole blood of dairy Holstein cows

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Abstract: The objective of this study was to evaluate the concentrations of the total (T-GSH), oxidised (GSSG) and reduced (GSH) glutathione in the blood of dairy cows, assess the relationships of the phase and number of lactation, milk production, body condition score (BCS) and selected biochemical parameters on its concentrations. We analysed 79 samples of whole blood from dairy Holstein cows. The concentration of glutathione was assessed by the spectrophotometric enzymatic method. The whole dataset shows the mean concentration of T-GSH, GSSG and GSH of 803 ± 22 μmol/l, 23 ± 2.5 μmol/l and 757 ± 27 μmol/l, respectively. The GSH/GSSG ratio was 95 ± 17. The phase of the lactation had a significant impact on the levels of T-GSH and GSH, but the number of lactation and BCS had no effect. Dry cows had higher levels of T-GSH (938 ± 44 μmol/l) than the fresh (713 ± 46 μmol/l) and peak lactation (785 ± 45 μmol/l) cows. The fresh cows had significantly lower concentrations of GSH (618 ± 44 μmol/l) than the peak lactation (719 ± 46 μmol/l) and dry cows (827 ± 43 μmol/l). On the basis of a regression analysis, blood glutathione was affected mainly by the liver function and energy metabolism. Glutathione as the marker of oxidation stress seems to be a promising tool in monitoring the health and welfare of the herd, yet intensive research in this field remains necessary.

Keywords: antioxidant defence; lactation period; liver metabolism; spectrophotometric method

Glutathione is the most abundant thiol-scavenging molecule that occurs in every eukaryotic cell. This peptide serves as a major and key antioxidant and it also modulates many biochemical processes among the cell. Numerous pathological conditions and metabolic disorders are conditioned by dysregulation in the glutathione synthesis (Giustarini et al. 2009).

Apart from its scavenging abilities, glutathione maintains several essential functions: it protects cells from oxidative stress and the impact of xenobiotics, serves as a storage form for cysteine and as an enzyme cofactor (Meister and Anderson 1983), it helps in recycling of vitamin E and C (Meister 1988) and in the transport of several amino acids, it participates in the synthesis and reparation of nucleic acids, proteins and prostaglandins, it protects thiol groups and many others. Glutathione can serve as a neuromodulator and contributes in cell differentiation and proliferation (Janaky et al. 1999).

Upon the common state and homeostasis of the cell, glutathione predominantly forms into a thiol

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reactant – reduced glutathione (GSH). Among the increase of oxidative demands on the cell, glutathione can be utilised and oxidised to its disulfide form – oxidised glutathione (GSSG).

Preferentially, GSH is found inside the cell in the range of 1–10 mmol/l (Meister 1988) and it accounts for > 98% of the T-GSH (Forman et al. 2009). GSH occurs in the cytoplasm in excess of 85%, 10–15% can be found in the mitochondria and a few percent account for the endoplasmic reticulum. Michelet et al. (1995) mentioned a plasma concentration of only about 0.4% of T-GSH. The liver serves as the only organ that releases GSH outside of the cell into the plasma and bile (Lautenburg et al. 1984).

The concentration of GSSG depends on the impact of the stress conditions, but upon the physiological conditions and redox homeostasis, it ranges one or two magnitude lower. The glutathione ratio (GSH/GSSG) serves as a marker of the stress conditions and can be used to monitor the oxidative status of the cell. Iwasaki et al. (2009) mentioned that the glutathione ratio can decrease from 10–100 : 1 up to 1 : 1 upon enhanced demands and stress burdens.

Monitoring the glutathione concentrations has gained attention mainly in human medicine. Several authors proved the relationships between glutathione and the onset of neurodegenerative diseases (Spina and Cohen 1989), diabetes mellitus (Yoshida et al. 1995), rheumatoid arthritis (Hassan et al. 2001) and early ageing (Christon et al. 1995). Glutathione also seems a promising therapeutic tool in the fight against neoplastic processes (Kigawa et al. 1998).

In dairy cattle, information about the glutathione concentrations is scarce and it is evaluated on an experimental basis only. It is suggested that, in dairy cows, high levels of oxidative stress can lead to the dysregulation of the GSH synthesis, which consequently manifests in a milk yield diminution, reproductive disorders and these conditions go hand in hand with subclinical changes which can easily end in clinically observed changes, if prolonged. Sharma et al. (2011) evaluated the GSH concentrations in the blood of dairy cows around parturition and found lower values in the close-up period in comparison with the first weeks of lactation. Aitken et al. (2009) mentioned that dairy cows are prone to increased oxidative stress during the transition period and if they experience stress conditions, particularly in the parturition, this can be the main cause of immune and inflammatory dysfunction in dairy cattle. Moreover, the increased incidence of disease during the periparturient period is directly related to numerous genetic, physiological and environmental factors that can compromise the cow's immunological defences (Sordillo 2005). Necasova et al. (2019) found a significant effect of the age together with the type of feeding of calves on the concentration of GSSG and the GSH/GSSG ratio. Calves fed colostrum, transition or native milk showed an increase in the GSSG levels compared to calves receiving milk replacer and calves after weaning.

Nevertheless, comparing the available data on glutathione concentrations is difficult. Some of the glutathione metabolic processes are still unclear and not promptly elucidated, moreover, the availability of glutathione to react with multiple compounds makes it difficult to measure. Several authors neglected to evaluate all the individual forms of glutathione and no standard method has been set both in the human and veterinary fields.

Several methods to determine the glutathione levels have been developed. Many of them have been abandoned due to the many pitfalls that have to be considered in its evaluation. Only a few methods can be used for the measurement nowadays and, still, all of them also have disadvantages among their benefits. Mostly, spectrophotometric- and high-performance liquid chromatography (HPLC) based methods are used. Isotachophoresis (ITP) has proven to be a quick and cheap method for the detection of both GSH and GSSG (Bodor et al. 2018). No standard method exists nowadays, therefore, comparing the results was difficult due to the different types of samples, the techniques used in the sample collection, preparation and measurements. The authors use different units and some of them found GSSG levels to be negligible which can then distort the levels of GSH and T-GSH.

The objective of our study was to determine the concentrations of the GSH, GSSG and total glutathione (T-GSH) in the whole blood of clinically healthy dairy cows and to assess the effect of the phase and number of the lactation period, the milk production and body condition of dairy cows on its concentrations. We also assessed the impact and relationships between the individual forms of glutathione and selected biochemical parameters in dairy cows.

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MATERIAL AND METHODS

Animals

Our study comprised a total of 79 Holstein dairy cows. The cows were divided into three groups according the phase of lactation. Group F – fresh cows (n = 22) consisted of cows from 8 to 38 days in lactation, Group L – top of lactation (n = 30) consisted of cows from 54 to 155 days in lactation and Group D – dry cows (n = 27) involved cows in the close-up period (up to 2 weeks before parturition). All the animals involved in the study were clinically examined by checking their respiratory rate, heart rate and temperature, hydration status and the colour of the mucosa. Only healthy individuals were included in the study.

The study was performed on an intensive dairy farm in South Moravia. The animals were stalled in a cow-shed with a free-box stabling system and a robotic milking system (Lely Astronaut A4 and A5; Lely Industries N.V., Maassluis, The Netherlands). The blood withdrawal took benefits as a part of the routine preventive diagnostic activity.

The cows were fed with a total mixed ration (TMR) twice a day. The composition of the daily ration is shown in Table 1. The lactating cows received a production mixture (Table 1) according to the phase of the lactation and the milk production in the amounts of 0.5–8 kg per day.

Table 1. The nutrient content in the total mixed ration for the milking cows, dry cows and nutrient content in production mixture

Analytical components in kg of dry matter	TMR (milking cows)	TMR (dry cows)	Production mixture
Crude protein	161.46 g	136.03 g	202.88 g
NEL	6.83 MJ	5.15 MJ	7.10 MJ
Crude fibre	163.71 g	285.26 g	54.34 g
Starch	246.61 g	44.17 g	413.39 g
Fat	45.67 g	19.63 g	48.07 g
Calcium	9.84 g	12.39 g	14.60 g
Phosphorus	4.77 g	3.32 g	6.35 g
Magnesium	3.26 g	3.68 g	3.87 g
Vitamin A	12 290 IU	9 880 IU	20 060 IU
Vitamin D3	2 230 IU	2 960 IU	3 600 IU
Vitamin E	32 mg	98.78 mg	83.66 mg
Copper	19.22 mg	26.65 mg	26.63 mg
Zinc	81.10 mg	99.53 mg	135.91 mg
Iodine	1.16 mg	5.65 mg	1.96 mg
Selenium	0.39 mg	0.49 mg	0.48 mg

NEL = net energy for lactation; TMR = total mixed ration

The milk yield ranged between 25–58 l and 22–62 l with an average yield of 38.6 and 39.2 per day in the group of fresh and lactation cows, respectively.

We evaluated the body condition score (BCS) using a five-point scale (1–5) with 0.25-point intervals according to Edmondson's system (Edmondson et al. 1989). For the purpose of the body condition evaluation, we divided the cows in four groups: Group 1 (BCS 1.50–2.75), Group 2 (BCS 3.00–3.25), Group 3 (BCS 3.50–3.75) and Group 4 (BCS over 4.00).

Sampling procedure

The blood was collected by venepuncture of the *vena coccigea* into two Hemos tubes (Gama Group a. s., České Budějovice, Czech Republic) after the morning feeding.

One of the tubes was left unused to obtain the serum for the biochemical analysis. Just after coagulation, the serum was gained by centrifugation at 2 500 *g* for 15 min (Jouan B4i centrifuge; Trigon Plus s.r.o, Čestlice, Czech Republic) and stored at –20 °C until use. The second tube was promptly transferred into a vacutainer tube with the addition of ethylenediaminetetraacetic acid tripotassium salt (K_3EDTA) as an anticoagulant (Greiner Bio-One, Kremsmünster, Austria). A small amount of whole blood was conserved with sodium fluoride for the glucose assessment. Consequently, 50 and 100 µl of the K_3EDTA-treated blood was placed into Eppendorf tubes; one with a 10 µl addition of the scavenger 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP) for the determination of the GSSG. The second tube with 50 µl of blood was used for the measurement of the T-GSH. The samples were immediately placed on ice and, after transportation, stored at –70 °C until the day of the analysis.

Analytical procedure

All the laboratory analyses were performed at the Department of Animal Protection, Welfare and Behaviour, University of Veterinary Sciences Brno.

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The concentration of T-GSH and GSH glutathione was determined in the whole blood. The other biochemical indicators were measured in the blood serum with the exception of the glucose (Glu) which was measured in fluoride plasma. We monitored the following biochemical indicators in the serum: the total protein (TP), albumin (Alb), total bilirubin (Tbil), urea (U), creatinine (Crea), cholesterol (Chol), beta-hydroxybutyrate (BHB), triacylglycerols (TAG), non-esterified fatty acids (NEFA), aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), calcium (Ca), inorganic phosphorus (P) and chlorides (Cl).

The concentration of glutathione was assessed by a commercial photometric assay, Bioxytech GSH/GSSG-412, purchased from Oxis International, Inc. (Portland, USA) with the use of a Varioskan Flash spectrophotometric analyser (Thermo Fisher Scientific, Waltham, USA). The principle of the procedure involves Ellman's reagent (5, 5'-dithiobis-2-benzoic acid, DNTB) that reacts with GSH and forms a spectrophotometrically detectable product at 412 nm (Tietze 1969). The change in the colour development is proportional to the T-GSH and GSSG concentrations. To avoid autooxidation during the blood collection and whole preanalytical phase, GSH is scavenged by 1-methyl-2-vinyl pyridinium trifluoromethane sulfonate (M2VP), which can prevent the reaction of the thiol group of GSH with other reagents. The GSSG is then, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), reduced by glutathione reductase to GSH and afterwards evaluated by the same reaction as mentioned above. This is a key factor preventing the overestimation of GSSG, because even small amounts of GSH undergoing oxidation simply lead to a great increase in the GSSG levels afterwards. The concentration of GSH and the GSH/GSSG ratio was assessed by calculation.

The biochemical parameters were assessed by an Indiko – Clinical Chemistry Analyser, type 863 automatic analyser (Thermo Fisher Scientific, Waltham, USA). All the parameters were measured by standardised laboratory methods. We used the following sets for the measurement of the individual parameters provided by Biovendor – Laboratory Medicine a. s. (Brno, Czech Republic). TP [LTotal Protein (biuret) Cat. No. 12 751], Alb (LAlbumin, Cat. No. 10 001), Tbil (LTotal Bilirubin, Cat. No. 10 552), U (LUrea, Cat. No. 12 702), Crea (LCreatinine Jaffé, Cat. No. 10 917), Chol (LCholesterol, Cat. No. 10 851), BHB (LBeta-Hydroxybutyrate, Cat. No. 10 835), Glu (LGlucose GOD-POD, Cat. No. 11 601), TAG (LTriacylglycerols Mono, Cat. No. 12 805), NEFA (LNon-esterified Fatty Acids, Cat. No. 13 002), AST (LAST, Cat. No. 10 352), ALP (LALP, Cat. No. 10 252), ALT (LALT, Cat. No. 10 452), GGT (LGGT, Cat. No. 11 502), Ca (LCalcium Arzenazo III, Cat. No. 12 101), P (LInorganic Phosphorus, Cat. No. 11 354), Cl (LChlorides, Cat. No. 11 951).

Statistical evaluation

All the data were statistically evaluated using UNISTAT v6.0 (Unistat Ltd, London, United Kingdom). The basic statistical parameters for all the variables were calculated and are shown in the tables. The normality of the data distribution was assessed using the Kolmogorov-Smirnov test. As the data on the glutathione concentrations and most of the other evaluated biochemical variables were not normally distributed, non-parametric tests were used for the statistical evaluation. The difference between the groups according to the phase, rank of lactation and BCS was tested by the Kruskal-Wallis test and the Dunn post hoc test. The results are shown as a mean ± standard error of the mean (SEM).

The relationships between the glutathione and the milk yield or biochemical parameters were evaluated by regression analysis and Spearman's correlation coefficient is shown. The statistical significant level was set at $P \leq 0.05$.

RESULTS

The concentration of glutathione in blood of cows

The basic statistical characteristics for the particular forms of glutathione and the glutathione ratio are shown in Table 2. We found a relatively high variability in the concentration of the glutathione in the whole blood of the dairy cows. The coefficient of variability was 30.5% for T-GSH, 95.2% for GSSG, 31.7% for GSH and 159.7% for the GSH/GSSG ratio. The distribution of the data is shown in Figure 1A–C for the T-GSH, GSSG and GSH, respectively.

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Table 2. The concentration of the total glutathione (T-GSH), oxidised glutathione (GSSG), reduced glutathione (GSH) and the glutathione ratio (GSH/GSSG) in the whole blood of the dairy cows (n = 79)

Glutathione type	Mean	Median	SEM	Minimum	Maximum
T-GSH (μmol/l)	802.8	798.4	27.6	99.6	1 301.0
GSSG (μmol/l)	23.0	16.5	2.5	0.8	95.3
GSH (μmol/l)	756.8	714.4	27.0	24.4	1 286.7
GSH/GSSG	95.1	42.6	17.1	0.6	867.7

SEM = standard error of the mean

The relationship between the glutathione and the phase of lactation, number of lactation, BCS and milk yield

Three different phases of lactation were compared – fresh cows, top-of-lactation cows and dry cows. We only found a significant impact on the levels of the T-GSH. The cows in the close-up period had higher levels of T-GSH in comparison with the fresh cows. The results are shown in Table 3.

The number of lactation did not show any significant effect on the concentrations of the individual forms of glutathione. The same was observed in the relationship with the BCS, although an increase in the energetic reserves went hand in hand with an increase in the GSH, T-GSH and also the GSSG, but with a decrease in the GSH/GSSG ratio. The results are shown in Table 3.

The relationship between the glutathione and the milk yield (n = 52) showed a statistically significant correlation only for the GSSG (the correlation coefficient was −0.309; P = 0.013). The correlations for T-GSH and GSH were −0.105 (P = 0.229) and −0.053 (P = 0.355), respectively.

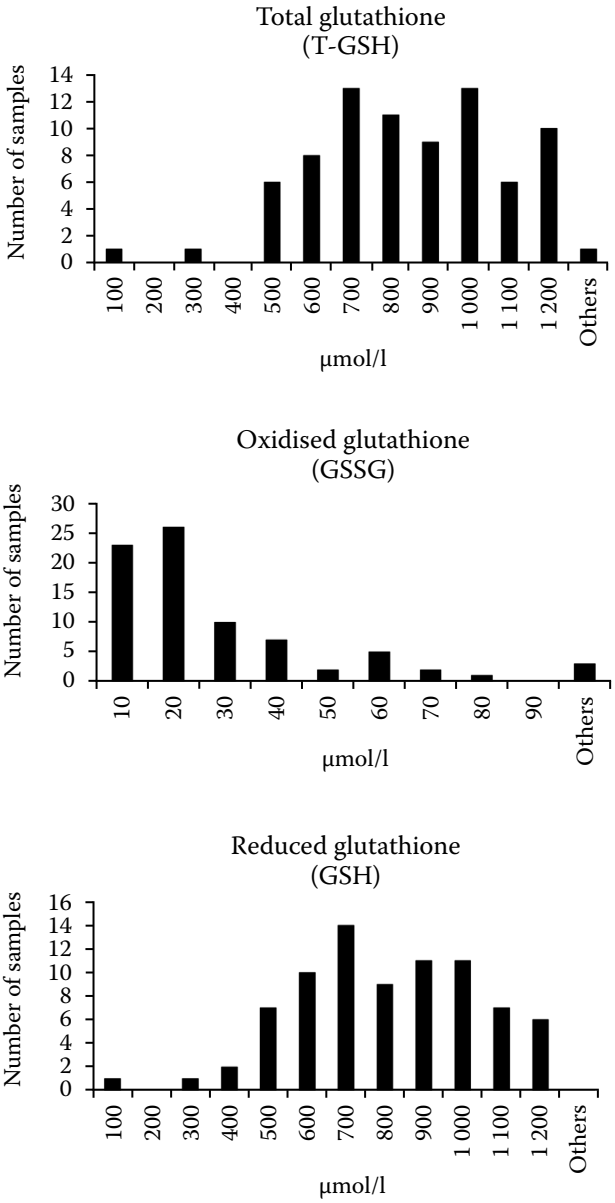

Figure 1. Frequency distributions of the concentrations of the individual forms of glutathione in the whole blood of the dairy cows

(A) Total glutathione (T-GSH). (B) Oxidised glutathione (GSSG). (C) Reduced glutathione (GSH)

The relationship between the glutathione and the biochemical parameters

The basic statistical characteristics of the selected biochemical parameters of the complete data set (n = 79) along with Spearman's correlation coefficient with the particular forms of glutathione are given in Table 4. The concentration of T-GSH and GSH had significant positive correlations with Alb, Crea, Tbil, TAG, ALP and Ca, and negative with Glu, NEFA and ALT. T-GSH shows a negative correlation with the GGT.

Significant negative correlations were found in the GSSG in relationship to Chol, AST and GGT. GSSG positively correlated only with BHB. The GSH/GSSG ratio had a significant positive correlation with Urea, even stronger with Chol, AST, GGT and Cl. The GSH/GSSG ratio negatively correlated with BHB.

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Table 3. The concentration (mean ± SEM) of the total glutathione (T-GSH), oxidised glutathione (GSSG), reduced glutathione (GSH) and the GSH/GSSG ratio in relation to the lactation phase, number of lactation and body condition score (BCS)

Categories	No. of animals	T-GSH (μmol/l)	GSSG (μmol/l)	GSH (μmol/l)	GSH/GSSG
Lactation phase					
Fresh cows	22	720.8 ± 46.2*	20.1 ± 3.8	680.6 ± 44.0	104.3 ± 44.0
Top of lactation	30	765.6 ± 45.7	18.5 ± 3.0	728.6 ± 46.4	93.8 ± 22.6
Dry cows	27	910.8 ± 44.3*	30.4 ± 5.4	850.1 ± 43.8	89.1 ± 25.5
P-value	–	0.020	0.260	0.058	0.523
Number of lactation					
1	22	752.1 ± 51.2	21.9 ± 4.1	708.2 ± 48.7	93.5 ± 39.1
2	27	818.2 ± 51.1	19.1 ± 3.1	780.1 ± 52.0	117.5 ± 32.0
3	18	855.7 ± 53.6	21.2 ± 5.6	813.3 ± 53.0	103.7 ± 31.8
≥ 4	12	786.9 ± 76.4	36.8 ± 9.8	713.2 ± 66.8	36.2 ± 7.9
P-value	–	0.341	0.233	0.227	0.185
Body condition score					
≤ 2.75	16	668.7 ± 74.6	12.6 ± 2.3	643.5 ± 76.5	105.9 ± 35.9
3.00–3.25	27	786.1 ± 40.7	22.0 ± 3.7	742.2 ± 41.1	85.5 ± 24.2
3.50–3.75	19	860.7 ± 52.2	27.3 ± 5.9	806.1 ± 48.7	67.6 ± 20.0
≥ 4.00	17	890.6 ± 53.1	29.6 ± 6.7	831.4 ± 52.4	131.0 ± 57.6
P-value	–	0.110	0.138	0.226	0.627

SEM = standard error of the mean
*$P \leq 0.05$

Table 4. The selected biochemical parameters and their correlation with the total glutathione (T-GSH), reduced glutathione (GSH), oxidized glutathione (GSSG) and the GSH/GSSG ratio in the complete dataset (n = 79)

Parameter	Mean	Median	SEM	Spearman's correlation coefficient			
				T-GSH	GSSG	GSH	GSH/GSSG
Albumin (g/l)	41.40	40.90	0.55	0.354**	0.029	0.335**	0.078
Total protein (g/l)	86.50	86.20	0.95	0.019	–0.022	0.025	0.019
Glucose (mmol/l)	3.26	3.30	0.04	–0.232*	0.091	–0.227*	–0.097
Urea (mmol/l)	4.04	3.80	0.10	0.099	–0.175	0.101	0.196*
Creatinine (μmol/l)	92.10	87.00	2.16	0.279**	0.022	0.262**	0.053
Total bilirubin (μmol/l)	2.43	2.00	0.16	0.405**	–0.023	0.413**	0.168
Cholesterol (mmol/l)	6.06	6.30	0.19	0.028	–0.446**	0.102	0.447**
Beta-hydroxybutyrate (mmol/l)	0.49	0.46	0.02	0.038	0.198**	–0.015	–0.195*
Triacylglycerols (mmol/l)	0.11	0.10	0.01	0.585**	0.146	0.548**	0.035
NEFA (mmol/l)	0.27	0.19	0.03	–0.211*	0.045	–0.201*	–0.074
AST (μkat/l)	2.20	1.94	0.10	0.064	–0.485*	0.136	0.503**
ALT (μkat/l)	0.60	0.60	0.04	–0.455**	0.058	–0.452**	–0.183
GGT (μkat/l)	0.72	0.59	0.05	–0.191*	–0.330**	–0.130	0.297**
ALP (μkat/l)	1.21	1.01	0.09	0.444**	–0.002	0.428**	0.124
Calcium (mmol/l)	2.55	2.54	0.03	0.458**	–0.027	0.478**	0.180
Inorganic phosphorus (mmol/l)	1.94	1.92	0.05	–0.063	0.114	–0.088	–0.132
Chloride (mmol/l)	95.79	95.40	0.75	0.106	–0.074	0.100	0.360**

ALP = alkaline phosphatase; ALT = alanine amino transaminase; AST = aspartate transaminase; GGT = gamma glutamyl transaminase; NEFA = non-esterified fatty acids; SEM = standard error of the mean
*$P \leq 0.05$, **$P \leq 0.01$

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DISCUSSION

The comparison of the concentration of the particular forms of glutathione determined in our study with other authors appears to be quite difficult, because a standard method for the evaluation of glutathione does not exist. Various authors have used different methods and various samples, some experiments are missing valid data about the sample preparation and some papers even consider GSSG concentrations to be negligible. Sharma et al. (2011) found low mean concentrations of GSH (94 ± 21 µmol/l for dry cows and 234 ± 37 µmol/l for fresh cows), but, in another paper (Bozukluhan et al. 2017), much higher values of T-GSH (76.30 ± 3.61 mg/dl; recalculated 2 482 ± 118 µmol/l) in the full blood of healthy cows were found. Kaneko (1997) mentioned levels of GSH in cattle 2 890 ± 460 µmol/l, nevertheless there is no information concerning the method that was used. In a case study, Talukder et al. (2014) examined the plasmatic GSH by the spectrophotometric method in dairy cow diagnosed with follicular cystic ovarian degeneration. The concentrations ranged from 2.5 µmol/l to 3 µmol/l. Uzlu et al. (2016) measured in the whole blood of bulls with a mean GSH of 63.43 ± 2.92 mg/dl (recalculated 2 064 ± 95 µmol/l). They used the method of Beutler (1963) which is based on the spectrophotometric evaluation of the colour complex (after the reaction of the GSH with Ellman's reagent) at 412 nm. However, this method does not consider the GSSG concentrations, not to mention its tendency to autooxidation caused by any time delay between the sample collection and laboratory evaluation. Furthermore, no stabilising agent was added after the withdrawal. This may be the cause of the higher values in comparison to ours. Bodor et al. (2018) used two different methods for the evaluation of glutathione in the whole blood of dairy cows. The enzymatic spectrophotometric method and isotachophoresis were applied to compare the concentrations of GSH and GSSG. The levels of GSH ranged between 206 µmol/l to 1 406 µmol/l and GSSG from 13 µmol/l to 59 µmol/l. Isotachophoresis is basically a new method and, contrary to other methods, it allows for the direct measurement of both GSH and GSSG during one analysis. Autooxidation is blocked by the reaction of GSH with an iodoacetic acid supplement at the time of the blood collection. This method seems promising in evaluating the glutathione in production animals as it is cheap and applicable for field conditions. In this study, a high variability in the GSH and GSSG levels among the individuals were documented. Similar values were evaluated by both methods.

Compared with the other experimental data, our results correspond with the GSH concentrations detected in human blood. Michelet et al. (1995) measured the mean T-GSH in whole human blood as 872 ± 157 µmol/l and GSH as 849 ± 171 µmol/l, the plasmatic level of GSH was 3.39 ± 1.04 µmol/l. Plasmatic concentrations of GSH are of a micromolar range, because GSH is mainly stored in a cytosolic environment. Serru et al. (2001) mentioned levels of GSH in whole human blood at 486 ± 85 µmol/l, GSSG at 68 ± 26 µmol/l and T-GSH at 553 ± 90 µmol/l. Giustarini et al. (2003) measured the mean T-GSH and GSSG levels in whole blood as 1 378 ± 96 µmol/l and 3.21 ± 0.87 µmol/l, respectively. Michelet et al. (1995) reported, in their study, an average T-GSH of 941 ± 155 µmol/l and GSH of 849 ± 63 µmol/l in whole blood.

Our results show the effect of the lactation phase on the concentration of the T-GSH in the blood. The concentration of T-GSH was higher in the dry cows in comparison with the lactating cows. We suppose that it can be due to the lower metabolic burden of the liver, the lower occurrence of subclinical metabolic disorders and a lower stress extent in general. Sharma et al. (2011) also evaluated the effect of the lactation phase. Similarly, they found lower mean concentrations of GSH in cows 4 weeks in the lactation period (94 ± 21 µmol/l) than 4 weeks before calving (234 ± 37 µmol/l). They used Beutler's method (Beutler et al. 1963) for the evaluation of the GSH. Still, these authors again did not evaluate the individual forms of glutathione and the metabolic state of the examined herd could have been different. The rising glutathione levels were explained as the consequence of lipid peroxidation. They found a negative correlation with the glutathione peroxidase activity. The theory of the changes is explained by the growth of the oxidative stress during the periparturient period. This theory could be supported by our finding of the positive significant correlation of T-GSH and GSH with the TAG, which shows that the synthesis of glutathione by the liver is in connection to the lipid metabolism in the liver. A decrease in the concentration of the TAG occurs during the

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development of liver steatosis (Pechova et al. 1997). Thus, we can suppose that the occurrence of liver steatosis has a negative effect on the glutathione concentrations in blood. GSSG significantly negatively correlated with ALT which is also a marker of liver steatosis (Pechova et al. 1997). This could be explained by a mild insult to the hepatocytes, which can still react by usage of the GSH and the formation of GSSG. Severe cell damage or quick hepatocyte exhaustion leads to the improper synthesis of glutathione, the disability of the scavenge function and, therefore, lower levels of GSSG.

The liver serves as the only organ capable of supplying the circulating blood with GSH. Most of the *de novo* synthesised GSH is being transported into the blood stream and promptly utilised by the cells. The lack of GSH in the liver for their own metabolic demands, despite its needs, can end in a GSH liver deficiency followed by the depression of GSH in the blood. This also supports negative correlations between the GSH and liver enzymes (ALT, GGT). Positive correlations were only found between the Tbil and GSH which is not clear in connection to the liver function.

For a better understanding of the relationships between glutathione and the liver function, it is necessary to provide more experiments mainly in animals with more severe energy deficiencies and liver diseases.

In high yielding dairy cows, the milking period is the cause of a negative energy balance. The food intake is insufficient to cover the metabolic needs of the body. In order to maintain the yield, the animals exploit their body fat reserves (Celeska et al. 2010). Mobilising the tissue lipid and protein in order to sustain the productive function shows interesting results on the glutathione concentrations. Increasing concentrations of glucose and NEFA go hand in hand with a decrease in the T-GSH and GSH.

Nevertheless, an increase in these two parameters shows a different energy situation. An increase in the NEFA is a sign of lipid mobilisation and energy deficiency, but an increasing glucose concentration shows a lower degree of energy deficiency. The primary glucose demand for lactogenesis cannot be sufficient and increased levels are followed by aggravation of the NEFA. This status is considered a physiological condition in fresh cows and the T-GSH and GSSG tend to decrease. With a deepened negative energy balance and higher daily milk yield, the quick energy supply is exhausted and ketone bodies are formed. The levels of BHB increase and this positively correlates with the GSSG. This promotes the antioxidant defence system to react to such conditions.

It is supposed that the oxidative stress in the periparturient period can lead to periparturient disorders (Waller 2000). Kirbas et al. (2014) documented the decrease of glutathione concentration in Akkaraman sheep during rumen acidosis. They detected GSH in healthy sheep at 560 ± 122 µmol/l and in affected animals at 339 ± 160 µmol/l. The relationships between glutathione and some infectious diseases have been documented over the last several years. Bozukluhan et al. (2017) detected diminished levels of glutathione in cattle suffering with brucellosis. Uzlu et al. (2016) mentioned a decrease in glutathione in bulls with foot and mouth disease (mentioned above). In cows with lumpy skin disease compared to healthy individuals, the serum GSH levels were 130 ± 0.16 µmol/l and 358 ± 0.29 µmol/l, respectively (El-Mandrawy and Alam 2018). In general, the GSH levels tends to reduce upon stress conditions, while GSSG levels increase. Proven in various experiments, during the course of a disease GSH decreases, yet many of the authors did not evaluate the concentration of GSSG in the affected animals. The results show that animals experiencing a disease can restore the GSH levels while getting over the disease (Uzlu et al. 2016; Bozukluhan et al. 2017; El-Mandrawy and Alam 2018).

Some steps in the glutathione metabolism and its pool are still hidden and unclear. Proven in this and various studies, its levels are mainly closely related to the liver metabolism. The liver plays a central role in the interorgan homeostasis of GSH and the dysregulation of the hepatic GSH synthesis has a systematic impact on the GSH homeostasis (Shelly 2012).

Various factors that can affect glutathione levels have to be considered. Even in healthy individuals, upon metabolic demands, we can observe relationships between the energy metabolism and the liver functions.

The glutathione issue is a complex task and further investigation in this field would bring valuable data, mainly in diseased animals. We proved the importance of the measurement of all forms of glutathione while considering the impact of stress on the organism.

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In cattle, glutathione seems to be promising marker in the evaluation of the oxidation stress and welfare conditions of the herd. However, there is still a lack of data and intensive research on this topic is necessary for the better understanding of glutathione metabolism in cattle.

Conflict of interest

The authors declare no conflict of interest.

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