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Research article

SUPPLEMENTAL SELENIUM REDUCES THE LEVELS OF BIOMARKERS OF OXIDATIVE AND GENERAL STRESS IN PERIPARTUM DAIRY COWS

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The aim of this investigation was to determine the influence of oxidative stress upon general stress in dairy cows on parturition and whether the indicators of stress can be reduced by selenium (Se) supplementation.

A total of 36 animals were divided into 3 groups 21 days prepartum and treated with a single-term intra muscular supplement of sodium selenite: Control group - 0 mg; group Se10 - 10 mg; group Se20 - 20 mg.

Se supplementation significantly raised blood Se content and glutathione peroxidase (GPx) activity in groups Se10 and Se20, compared to Control, although there was no marked difference between supplemented groups. Plasma malondialdehyde (MDA) and cortisol concentrations were significantly reduced in supplemented groups Se10 and Se20, compared to Control. A negative correlation was detected between blood GPx activity and plasma MDA, while a positive correlation was determined between plasma MDA and cortisol concentrations.

These results indicate that prepartum Se supplementation can be utilized for a partial relief of stress in cows during labor by augmenting the antioxidative action of GPx.

Key words: Selenium, dairy cows, peripartum period, MDA, cortisol, stress

INTRODUCTION

The peripartum period in dairy cows (from 3 weeks prior, to 3 weeks after calving) is proven to be very stressful for the animal. While transitioning from pregnancy to lactation cows undergo substantial physiological and metabolic adaptations aimed to meet the increased demand of the organism for energy and other nutrients necessary for fetal growth, colostrum and milk production [1].

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Change in plasma cortisol concentration is commonly used for the assessment of stress intensity level in animals. In cow's plasma cortisol level increases during labor. Since the raise in cortisol exhibits immunosuppressive effects on phagocytosis and neutrophile migration, it may be involved in the development of major peripartum disorders such as retention of placenta and mastitis [2-4].

Taking into account various stress factors of commercial dairy production it is necessary to pay particular attention to the onset of oxidative stress, which is generally described as an imbalance between oxidant and antioxidant levels [5]. The considerable increase in oxygen requirements during times of increased metabolic demands results in an augmented production of reactive oxygen species (ROS). When the production of oxidants exceeds the capacity of the antioxidant defense, a state of oxidative stress is produced resulting in oxidative damage to macromolecules such as lipids, DNA and proteins [6]. Such damage can be a significant underlying factor to dysfunctional host immune and inflammatory responses that can increase the incidence of peripartum disorders during the transition period [7].

Malondialdehyde (MDA) is a low-molecular weight end-product of oxidative decomposition of polyunsaturated fatty acids that readily reacts with thiobarbituric acid producing a red pigment that can be measured spectrophotometrically [8]. Therefore, the accumulation of plasmatic MDA may be used as a quantitative indicator of the oxidative stress level.

Selenium (Se) incorporated in the form of selenocysteine is an essential component of a range of selenoproteins. They play an important role in the regulation of various physiological functions such as: antioxidative defense, thyroid hormones metabolism, immunity, reproduction and early postnatal viability [5] .

Antioxidative functions of Se are expressed through eight known isoforms of the selenoenzyme glutathione peroxidase (GPx) which eliminates hydrogen peroxide and various lipid hydroperoxides [9]. Thioredoxin reductase is another selenoenzyme that may function to prevent oxidative stress [10].

Numerous studies have indicated that prepartum Se supplementation of dairy cows fed diets low in Se can reduce the incidence of retained placenta following spontaneous [11] or induced labor [12]. Cows with retained fetal membranes had lower GPx activity in maternal and placental tissues compared to cows without retained placenta [13].

Whole blood Se concentration and GPx activity were negatively related to the prevalence of intramammary infection [14]. High serum Se concentrations were associated with reduced rates of mastitis and lower bulk-tank somatic cell counts [15].

Summarizing two decades of investigation of Se status indicators in plants and sheep, Valčić et al. [16] reported that the territory of Serbia can be generally categorized as Se deficient in hilly/mountainous central and southern parts, and marginally Se deficient in the northern part (Pannonian plain). According to investigations conducted by Jovanović et al. [17] in the region surrounding the experimental farm, Se content in

cereals and hay ranged from 40 to 62 μgSe/kg. These results are considered to be marginally deficient.

The beneficial effects of Se supplementation are thought to be due to the actions of certain antioxidant Se-dependent enzymes [18]. The recommended level of Se in dairy cows diets is 0.3 mg/kg dry matter (DM) but should be closely monitored to ensure that over supplementation does not occur [19].

The purpose of this article was to assertain whether oxidative stress should be considered as a significant component in overall stress in dairy cows in the transition period, and whether stress could be suppressed by selenium supplementation.

MATERIAL AND METHODS

Experimental groups and treatments

Thirty six (36) Holstein-Frisian cows included in this investigation were randomly divided into 3 groups of 12 animals each and supplemented with sodium selenite (NaSel) as follows:

- Group "Control" was not supplemented, serving as a negative control;
- Group "Se10" was supplemented 10 mg NaSel;
- Group "Se20" was supplemented 20 mg NaSel.

The supplement was administered by a single-term intramuscular (i/m) injection 21 days prepartum; parturition was induced using a single i/m injection of PGF-2α (2 mL, 500 μg of cloprostenol) not before day 275 of gestation; venous blood samples for analysis were taken from each individual animal 12 hours prepartum, at parturition and 12 hours postpartum.

All animals were clinically healthy, multiparous, single calve, with no record of previous peripartum disorders.

Blood Se concentration

Determination of selenium in whole blood samples of cows was carried out using atomic absorption spectrometry - hydride technique. Microwave digestion was used for sample preparation. Samples of whole blood (0.5g) were accurately weighted using analytical balance Denver Instrument, model TB-215D (Denver Instruments, USA), transferred into Teflon microwave vessels and digested with 8 mL of 69% nitric acid (Sigma-Aldrich, USA) and 2 mL of 30% hydrogen peroxide (Fluka Analytical, USA). Microwave oven (Milestone, Germany, model Touch control) was set to the following program: temperature ramp from ambient temperature at 180°C followed by 15 min holding time and 20 min of cooling time. Digested samples were transferred to volumetric flasks and diluted using 5M hydrochloric acid (Sigma-Aldrich, USA) to the final volume of 25 mL. Determination of selenium concentration was carried

out using SolAAr, Series 4 spectrometer equipped with VP70 hydride module and EC90 electrical furnace for precise temperature control of the analytical cuvette (Thermo Electron, UK). Measuring of absorption at 196 nm was performed after SeH_4 formation in the hydride system with 5% $NaBH_4$ (J.T.Baker, the Netherlands) and 0.6% NaOH (Merck, Germany). Stabilization and baseline delay times were 40 and 60 seconds respectively, reading time was 7 seconds in three replicates. Furnace was set to 900°C.

Quantification of Se content was performed using a five-point calibration curve (10-40 μg/kg, including zero) of reference standard solutions (Merck, Germany). Quality control was achieved using blank samples fortified at 20 μg/kg of Se and certified reference material (BCR 189). Good linearity was obtained from the calibration curve (r=0.998) and the measured concentration of the reference material was in the range of the reference value.

Blood glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was measured in whole blood samples using a coupled test [20]. All chemicals were obtained from Sigma Aldrich. Blood samples were hemolyzed using Drabkin's reagent (1.6 mM KCN, 1.2 mM K2Fe(CN)6 and 0.023 M $NaHCO_3$). The GPx present in the samples reduces tertiary butyl hydroperoxide (TBH). Glutathione (GSH) as the donor of hydrogen becomes oxidized to GS-SG. In the second phase of this coupled reaction GS-SG is reduced to GSH by NADPH and glutathione reductase (GR). Final concentrations of the used reagents were: 100 mM phosphate buffer (pH 7.4), 4 mM EDTA, 6 mM GSH, 0.375 IU/mL GR, 0.3 mM NADPH and 1.575 mM TBH. The low concentration of TBH (under 2.32 mM) as used in this method, determines only the activity of Se-dependent GPx. The reduction of NADPH was followed for 3 min at 366 nm using a Cecil Ce2021 spectrophotometer (UK) with a Peltier thermostat unit. Absorbance (A) values were taken at 30 seconds intervals and the results were expressed in microkatals per liter (μkat/L).

Serum MDA concentration

Serum MDA was measured using spectrophotometry [21]. All chemicals were obtained from Sigma Aldrich. Briefly, a 3 mL of 0.1% orthophosphoric acid, 1 mL of 0.6% thiobarbituric acid and 0.1 mL of 0.28% hydrated ferrous sulfate solution were added to 0.3 mL of serum. The reaction mixture was heated in boiling water for 60 minutes. The produced chromogen was extracted with n-butyl alcohol (4 mL). After centrifugation (2200 × g, 10 minutes), the butanol layer was separated for spectrophotometric measurement at 535 nm.

Serum cortisol concentration

Concentration of cortisol was measured in heparinized plasma samples using commercial standard RIA kits (INEP, Zemun). The assay is based on the competition

between unlabelled cortisol and a fixed quantity of ^{125}I labeled hormone for a limited number of binding sites on cortisol specific antibodies (bound to the tubes). Allowing a fixed amount of tracer and antibody to react with different amounts of unlabelled ligand the amount of tracer bound by the antibody will be inversely proportional to the concentration of the unlabelled ligand. Antigen-antibody complex is bound to the tubes and the supernatant is then decanted. Counting the radioactivity of the bound phase enables a standard curve to be constructed and samples to be quantified.

Statistical analysis

Data are presented as mean ± SD. Analysis was performed using *MS Excel 2007* and *Graph Pad Prism 5* statistical software packages. The differences between all experimental groups were analyzed using Student's t-test, except for cortisol where non-parametric Mann-Whitney test was used. In all cases the probability level $p<0.05$ was considered statistically significant.

RESULTS

Blood selenium concentration and GPx activity in both supplemented groups (Se10 or Se20) were significantly higher ($p<0.01$) compared to the control. However, there was no significant difference between groups Se10 and Se20 (Table 1).

Table 1. Blood Selenium concentration and GPx activity 12h pospartum, in cows given a single term supplementation with different amounts of Se as sodium selenite (0, 10 and 20 mg, i/m)

	Se (ng/mL)	GPx (μkat/L)
Control (n=12)	129.0 ± 18.0 ^{A,B}	90.6 ± 16.1 ^{C,D}
Se10 (n=12)	162.9 ± 30.4 ^{A}	178.2 ± 34.6 ^{C}
Se20 (n=12)	187.3 ± 32.6 ^{B}	185.0 ± 35.2 ^{D}

Student's t-test: ^{AA} $p<0,01$; $^{BB, CC, DD}$ = $p<0,001$

Plasmatic MDA concentrations were at the high level in all groups and did not markedly change from 12h prior, until 12h after parturition. However, MDA concentration was significantly ($p<0.001$) lower in both supplemented groups of animals (Se10 and Se20), compared to the Control, although there was no significant difference between groups Se10 and Se20 (Table 2).

Table 2. Blood plasma malondialdehyde (MDA) concentration 12h prepartum, at parturition and 12h postpartum, in cows given a single term supplementation with different amounts of Se as sodium selenite (0, 10 and 20 mg, i/m)

	MDA (μM)			
	Parturition - 12h	Parturition	Parturition + 12h	All Samples (n=36)
Control (n=12)	5.71 ± 0.94	5.76 ± 0.98	5.74 ± 0.85	5.74 ± 0.92^{A,B}
Se10 (n=12)	4.59 ± 1.20	4.44 ± 0.68	4.67 ± 0.80	4.57 ± 0.89^{A}
Se20 (n=12)	3.95 ± 0.88^{a}	4.95 ± 0.70^{a}	4.36 ± 0.72	4.42 ± 0.77^{B}

Student's t-test: $^{AA, BB}$ = $p<0.001$; ^{aa} = $p<0.05$

Cortisol concentration in the plasma of untreated cows from the control group rose steadily and significantly from 12h prior, until 12h after the parturition. This was not the case in the groups treated prepartaly with either 10 mg or 20 mg Se supplement. Additionally, cortisol concentration was significantly lower in animals treated 20 mg Se, compared to both Control and group Se10 (Table 3).

Table 3. Blood plasma cortisol concentration 12h prepartum, at parturition and 12h postpartum, in cows given a single term supplementation with different amounts of Se as sodium selenite (0, 10 and 20 mg, i/m)

	Cortisol (ng/mL)			
	Parturition - 12h	**Parturition**	**Parturition + 12h**	**All Samples (n=36)**
Control (n=12)	40.8 ± 17.1^{a}	54.2 ± 28.5^{b}	79.0 ± 23.3^{a,b}	58.0 ± 23.0^{A}
Se10 (n=12)	43.7 ± 19.1	40.2 ± 11.5	39.4 ± 19.9	41.1 ± 16.7^{B}
Se20 (n=12)	20.1 ± 11.8	22.7 ± 13.3	21.3 ± 12.9	21.4 ± 12.5^{A,B}

Mann-Whitney test: $^{AA, BB}$ = $p<0.01$; ^{aa} = $p<0.01$, ^{bb} = $p<0.05$

There was a significant negative correlation between individual blood GPx activities and plasma MDA concentrations 12 hours postpartum defined as:

$$y=-0.01x+6.3;\ r^2=0.237;\ p<0.01 \qquad \text{(Figure 1.a)}$$

and a significant positive correlation between individual plasmatic concentrations of MDA and cortisol in all animals throughout the observed peripartum period defined as:

$$y=30.9x-52.2;\ r^2=0.266;\ p<0.001 \qquad \text{(Figure 1.b)}$$

however, direct correlation between blood GPx and plasma cortisol could not be detected.

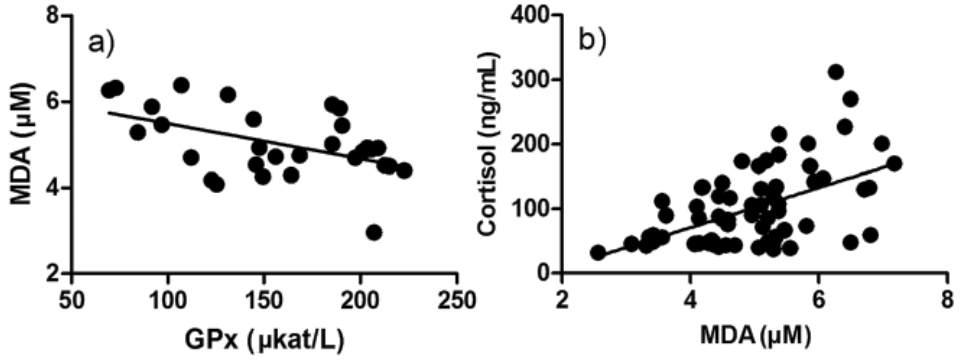

Figure 1. a) Correlation between individual plasmatic GPx activities and MDA concentrations of all experimental animals from 12h prepartum until 12h postpartum; **b)** correlation between individual plasmatic MDA and cortisol concentrations of all experimental animals from 12h prepartum until 12h postpartum. Correlation parameters given in the text.

DISCUSSION

Selenium concentration and GPx activity in whole blood do not vary over short periods, hence they are considered good indicators of the long-term selenium status. Single-term intramuscular injection of Se increases its blood content for a duration of 28 days, and GPx activity for 84 days [22]. Moreover, erythrocyte GPx activity accounts for 98-99 % of the total blood activity in ruminant species.

Blood Se concentrations in groups Se10 (162.9 ± 30.4 ng/mL) and Se20 (187.3 ± 32.6 ng/mL) were significantly higher than in the Control (129.0 ± 18.0 ng/mL) as a result of prepartum supplementation. However, Se dose of 20 mg did not significantly increase the Se content in group Se20, above that of group Se10 supplemented with 10 mg Se. Blood selenium concentration of 100 μg/L is usually considered physiologically adequate in cows [23] [24], although this estimate may be somewhat underestimated since Kommisrud et al. [25], using the functional approach, established that 100-150 ng/g represented the lower limit of blood Se content likely to protect most cows from peripartum disorders such as retained placenta.

In most mammalian species there is a strong positive correlation between blood Se concentration and selenoenzyme GPx activity, but only up to a certain point where GPx activity tends to plateau, independently of the Se content increase [26] [27]. The results of our experiment clearly reflect this dependency. Both supplemental doses of 10 or 20 mg Se significantly increased blood GPx activities in groups Se10 (178.2 ± 34.6 μkat/L) and Se20 (185.0 ± 35.2 μkat/L) above the control (90.6 ± 16.1 μkat/L), indicating that prepartum supplement dose of 10-15 mg Se should be sufficient to reach the plateau and satisfy the needs of the peripartum cows for this microelement. Similar results were reported by Wischral et al. [28].

Plasma MDA concentration readily changes according to the oxidative status of the animal. In our experiment plasmatic MDA content did not change significantly over the period from 12 h antepartum to 12 h postpartum. Similar results were observed by Erisir et al. [29]. Unified calculation for the whole period revealed that MDA content was significantly higher ($p<0.001$) in control (5.74 ± 0.92 μM), compared to supplemented groups Se10 (4.57 ± 0.89 μM) and Se20 (4.42 ± 0.77 μM). There was no significant difference between groups Se10 and Se20. In their experiment Erisir et al. [29] detected slightly lower MDA levels in cows without peripartum disorders (3.81 ± 0.21 μM). Kankofer et al. [13] measured the concentration of thiobarbituric acid reactive substances (TBARS), complementary to MDA, during the broader period around parturition, and found that they are on the rise from 2 weeks prepartum to 1 week postpartum irrespectively on the presence or absence of dystocia.

We detected a weak, but statistically significant ($p<0.01$) negative correlation between blood GPx activities and plasma MDA concentrations 12 hours postpartum (Figure 1a), indicating that Se supplementation can indeed be an efficient measure against oxidative stress through the raise of GPx antioxidative action. In partial contrast to

our finding Sharma et al. [30] reported the existence of a negative correlation of GPx with lipid peroxidation in cows in late pregnancy, but not in early lactation. However, plasma GPx in their experiment was considerably higher compared to ours, hence plateau (maximum) activity has been reached.

In the Control group of cows plasma cortisol concentration steadily rose from 12 h antepartum (40.8 ± 17.1 ng/mL) to 12 h postpartum (79.0 ± 23.3 ng/mL). In the selenium supplemented groups Se10 and Se20 cortisol levels did not change over the same period. Moreover, overall cortisol level in group Se20 (21.4 ± 12.5 ng/mL) was significantly lower ($p<0.01$) compared to group Se10 (41.1 ± 16.7 ng/mL). While normal sequence of events initiating parturition involves fetal cortisol induction of placental enzymes that direct steroid synthesis away from progesterone and toward estrogen [31], the rise of maternal cortisol is a sign of stress. Da Silva et al. [32] found the highest cortisol levels (22.05 ng/mL) in high-yield dairy cows 3 days postpartum, while Patel et al. [33] determined highest cortisol concentrations on parturition, declining to prepartum levels 3-5 days postpartum.

There was a statistically significant ($p<0.001$) positive correlation between plasmatic MDA and cortisol concentrations in our experiment, suggesting that the oxidative component is largely responsible for the overall stress level in cows during parturition. Similarly to our findings Gupta et al. [34] determined that prepartum treatment of cows with Se (and vitamin E) caused the reduction of plasma lipid peroxides, as well as a lower cortisol concentration at parturition.

Our results clearly suggest that single term i/m supplementation of dairy cows 21 days prepartum with 15 mg Se as sodium selenite can be instrumental in relieving animals from oxidative and general stress through the increase of selenoenzyme GPx antioxidant activity. This could be of particular significance in selenium marginal/deficient areas such as Serbia.

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DODATI SELEN SNIŽAVA NIVOE BIOMARKERA OKSIDATIVNOG I OPŠTEG STRESA KOD MLEČNIH KRAVA U PERIPARTALNOM PERIODU

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Cilj ispitivanja je bio da se odredi obim uticaja oksidativnog stresa na opšti stres kod mlečnih krava u toku 24 sata oko porođaja, kao i da li on može biti ublažen primenom selena.

Ukupno 36 krava je bilo podeljeno u tri grupe 21 dan pre porođaja i jednokratno tretirano natrijum selenitom aplikovanim intramuskularno u dozama: kontrolna grupa - 0 mg; grupa Se10 - 10 mg; grupa Se20 - 20mg.

Grupe Se10 i Se20 su imale značajno višu koncentraciju selena i aktivnost glutation peroksidaze (GPx) u krvi u odnosu na kontrolnu grupu, iako nije uočena razlika između suplementiranih grupa. Koncentracije malondialdehida (MDA) i kortizola su bile značajno niže u grupama Se10 i Se20 u odnosu na kontrolnu grupu. Uočena je negativna korelacija između aktivnosti GPx u krvi i koncentracije MDA u plazmi, a pozitivna korelacija između koncentracije MDA i kortizola u plazmi.

Rezultati ukazuju da prepartalna upotreba selena može biti korisna u smislu delimičnog smanjenja stresa u toku porođaja zbog povećanja antioksidativnog delovanja GPx.