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Cholinesterase Activities and Oxidative Stress in Cattle Experimentally Exposed to Nitrate/Nitrite in Cultivated Pasture with Different Fertilization Schemes

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ABSTRACT

***Background*:** Nitrate and nitrite poisoning is associated with pasture intake that has high nitrate levels and leads to acute methemoglobinemia. Pasture may accumulate nitrate under certain conditions, such as excessively fertilized soil or environmental conditions that enhance the N absorption (rain preceded by a period of drought). After ingestion of plants, this substrate reaches the rumen and, in physiological conditions, is reduced to nitrite and afterward to ammonia. The aim of this study was to evaluate changes in cholinesterase activities and oxidative stress caused by subclinical poisoning for nitrate and nitrite in cattle fed with *Pennisetum glaucum* in three different fertilization schemes.

***Materials, Methods & Results*:** In order to perform the experimental poisoning, the pasture was cultivated in three different paddocks: with nitrogen topdressing (urea; group 1), organic fertilizer (group 2) or without fertilizer (group 3; control). Nitrate accumulation in forage was evaluated by the diphenylamine test. After food fasting of 12 h, nine bovine were randomly allocated to one of the experimental groups and fed with fresh forage (*ad libitum*) from respective paddock. In different time points from beginning of pasture intake (0, 2, 4, 6 and 9 h) heart rate and respiratory frequency were assessed, as well as mucous membrane color and behavioral changes. Blood samples from jugular vein into vials with and without anticoagulant were collected. From blood samples, serum nitrite levels, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme activity were evaluated, as well as oxidative stress through the following parameters: levels of nitrate/nitrite (NO_x), thiobarbituric acid reactive substances (TBARS) and reactive oxygen species (ROS), beyond the antioxidant system by enzyme activity measurement of catalase (CAT) and superoxide dismutase (SOD). The diphenylamine test was positive to group 1 and 2, so that the pasture presented 3.16 mg/kg, 2.98 mg/kg and 1.67 mg/kg of nitrate for group 1, 2 and 3, respectively. In addition, cows from group 1 demonstrated increased ($P < 0.05$) nitrite levels in serum, compared to other groups, and greater heart rate after 9 h ($P < 0.05$). The AChE and BChE activity in group 1 showed significant increase ($P < 0.05$) at 4 and 6 h (AChE), and 4 and 9 h (BChE) compared to group 3. Also, NOx levels were lower at 6 and 9 h ($P < 0.05$) and at 9 h ($P < 0.05$) for animals of group 1 and 2, respectively, when compared to group 3. Furthermore, in the group 1 levels of ROS and TBARS were significantly higher ($P < 0.05$) after 2 and 4 h, and 6 and 9 h compared to other groups, respectively. The CAT activity increased significantly ($P < 0.05$) with 2 and 4 h of the experiment, but on the other hand, decreased at 6 and 9 h in group 1. Nevertheless, the animals from group 2 presented only a significant reduction in this enzyme activity at 9 h. Furthermore, SOD activity was reduced in animals of groups 1 ($P < 0.05$) at 4, 6 and 9 h, compared to other groups.

***Discussion*:** It was concluded that the nitrate and nitrite poisoning by pasture intake cultivated and fertilized with urea leads to increased levels of serum nitrite, as well as the cholinesterase activity and causes oxidative stress in cattle. It is conjectured that the cholinesterase activity and oxidative stress may assist in understanding the pathophysiology of changes caused by poisoning.

Keywords: plant toxicology, poisoning, methemoglobin, cholinergic system, oxidative stress.

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INTRODUCTION

Nitrate/nitrite poisoning is usually associated with mortality of cattle introduced in pastures with high nitrate levels [33]. Under normal conditions, pasture absorbs nitrogen in the soil and converts it into plant material, however, under favorable conditions such as excessively fertilized soil or environmental conditions that enhance the N absorption (rain preceded by a period of drought), nitrate (NO_3) accumulation in the pasture with toxic potential when ingested in sufficient quantities could occur [17,22,33]. After ingestion of plants with high NO_3 levels, this substrate reaches the rumen and, in physiological conditions, is reduced to nitrite (NO_2) and afterward to ammonia (NH_3). When NO_3 is ingested in large quantities, its reduction into NH_3 is not efficient and NO_2 accumulates in the rumen, which is absorbed into the circulatory system [19]. In the bloodstream, NO_2 oxidizes the iron ion of hemoglobin from ferrous (Fe^{+2}) state to ferric (Fe^{+3}), reducing the hemoglobin to methemoglobin that is unable to carry oxygen to the tissue [33]. Therefore, these changes may affect other system as the cholinergic, involved in several physiological functions of the organism.

Oxidative stress was already described in clinical poisoning by plants with high levels of nitrate and nitrite [2] and by *Senecio* sp. [4]. However, in most cases the poisoning is subclinical, not identified by farmers, which may cause production losses. Therefore, the aims of this study were to evaluate the cholinesterase activities and the status of oxidative stress in cattle fed with *Pennisetum glaucum* cultivated in three different fertilizer schemes, targeting the subclinical poisoning by nitrate/nitrite.

MATERIALS AND METHODS

Experimental design

The cultivation of *Pennisetum glaucum* (pearl millet) was performed in approximately 600 m² area, with 50 kg of seeds per hectare (ha), divided into three paddocks of equal proportions and with individualized fertilization. In the paddock 1 nitrogen topdressing (urea 45%) was performed at ratio of 150 kg/ha, fifteen days before de experiment; the paddock 2 received organic fertilizer (swine and cattle manure) at dose of 5 t/ha, thirty and fifteen days before de experiment; and the paddock 3, used as control, received no fertilizer of any kind. When de pasture reached the cutoff point the diphenylamine test to evaluate nitrate accumulation was conducted. Briefly, 0.5 g of diphenylamine were diluted in 20 mL of distilled water plus sulfuric acid until 100 mL, to form the diphenylamine reagent. The test consists of obtaining a few drops of vegetable extract placed on glass slide with addition of 2-3 drops of diphenylamine[1] reagent. The reaction is considered positive when in less than 10 seconds form an intense blue staining [14].

Nine male bovine, Holstein breed, aged about 12 months, body weight medium of 200 kg, were used in the experiment. After food fasting of 12 h (*ad libitum* access to water), animals were randomly allocated in three groups (n = 3 bovine/group). For each group fresh pasture from respective paddock in individual feeders (Table 1) was provided once, *ad libitum*, for 3 h. During the experiment (under resting state) heart rate and respiratory frequency (auscultation), mucous membranes color (visual evaluation) and possible behavioral changes, besides the quantity of pasture intake were evaluated. All evaluations were performed at hour zero, two, four, six and nine after beginning pasture intake.

Table 1. Pasture intake and nitrate levels in different groups.

	Grup 1	Grup 2	Grup 3
Fertilizer	Urea	Manure	Control
Average Body weigth (kg)	244	254	172
Pasture intake/animal (kg)	9.33	11.33	3.9
Pasture intake/ body weigth (%)	3.71	4.70	2.36
Dyphenylamine	+++	+	-
Pasture nitrate (mg/kg)	3.16	2.98	1.67

*Dyphenylamine test: (+++) Strong positive reaction; (+) Positive reaction; (-) Negative Reaction.

At the same time points after beginning pasture intake (0, 2, 4, 6 and 9 h) blood samples were collected from jugular vein into three vials containing EDTA, sodium citrate or without anticoagulant. The blood from vials without anticoagulant were kept at room temperature after collection until blood clotting and centrifuged at 16000 *g* for 10 min to obtain the serum, which was properly stored at -20°C for analysis of nitrite, ROS, TBARS, BChE and NOx. The blood in sodium citrate was stored at -20°C until analysis of CAT and SOD. Likewise, the blood with EDTA was homogenized in hemolytic buffer (1:50) and frozen (-20°C) for AChE analysis.

Biochemical analysis

Nitrite was measured by spectrophotometric method using diazotization of sulfanilic acid with nitrite ion coupled with (naphthyl)ethylenediamine[1], generating a measurable pink metabolite with absorbance measured at 540 nm [11]. For this, 300 uL of serum was used and the results expressed in mmol/mL.

For determination of NO_x, a serum aliquot (200 uL) was homogenized in 200 mM Zn_2SO_4 and acetonitrile[1]. Then, the solution was centrifuged at 16.000 *g* for 30 min at 4°C and the supernatant was collected for analysis of the NO_x levels as previously described [24]. NO_x was determined by 570 nm absorbance technique and the results expressed in µmol/mg protein.

Oxidative profile was determined using two biochemical techniques, which aims to measure levels of TBARS and ROS. TBARS was used to determine lipid peroxidation in serum samples, as described [16]. The methodology used spectrophotometry (535 nm) and results were expressed as nmol of malondialdehyde[1] (MDA) per mL. To determinate ROS levels, serum samples were prepared as described [1], and thereafter, the serum was diluted (1:10) with 10 nM Tris (pH 7.4) and 5 uL of 2',7'-dichlorofluorescein diacetate1 (DCFH-DA) was added in methanol at 37°C in 5% CO_2 for 15 min, enough time to esterase form a non-fluorescent compound (2',7'dichlorofluorescein - (DCFH(2)) [3]. The ROS formation was quantitated from the standard curve of DCF in methanol (0.05 - 1.0 mM), and the results were expressed as U DCF/mg protein.

The antioxidant status was assessed by analysis of CAT and SOD enzyme activities using spectrophotometry. The determination of CAT activity was performed according to the modified method [29], using 0.02 mL of blood aliquot (diluted 1:10 with saline solution) and homogenized in 0.910 mL potassium phosphate buffer[1] at 50 mM and pH 7.0. Whereas the measurement of SOD activity was based on inhibition of superoxide radical reaction with epinephrine, as described [21]. The CAT and SOD results were expressed in nmol CAD per mg protein and IU of SOD per mg protein, respectively.

The cholinesterase was measured in whole blood (AChE) and serum (BChE). Enzymatic analysis of AChE in whole blood was determined [36], which aims to determine the specific activity of AChE in whole blood from the quotient between the AChE activity and hemoglobin content. The determination of BChE activity in serum followed the method [9] using the substrate butirilcolina[1]. The results of AChE and BChE were presented in mU/L molHb and µmoles of BcSCh/h per mg protein, respectively.

Statistical analysis

Relative data for the evaluated parameters of AChE, BChE, NO_x, ROS, TBARS, CAT and SOD were initially analyzed using descriptive statistics for contingency of information and to obtain new hypotheses, being presented as mean and standard deviation. Data were tested for normality of variance through the Shapiro-Wilk test, for asymmetry, and homogeneity by the Levene test, prior the analysis. One-way ANOVA was used to analyze all the above mentioned parameters and compared the average between groups in each time point (0, 2, 4, 6, and 9 h of the experiment), followed by Tukey test. Values were considered different significantly at $P < 0.05$. All the statistical process was carried out by R-language, v.2.15.[2] (R Development Core Team)[2].

The statistical analysis of heart rate and respiratory frequency was performed using SPSS 25.0 software for Macintosh OSX (IBM)[3] and the Kolmogorov-Smirnov test was used to determine whether the distributions were parametric. The results between groups were compared using the Student *t*-test when the distributions were parametric and the Mann-Whitney U test for nonparametric distributions.

RESULTS

The diphenylamine test performed in the pasture paddocks was strongly positive for group 1, moderately positive for group 2 and negative for group 3 (Figure 1). The pasture intake by animals was not enough to cause clinical poisoning, being the intake about 3.71% of live weight in group 1, 4.70% in group 2 and 2.36% in group 3. Nitrate analysis on pasture showed values of 3.16 mg/kg in group 1, 2.98 mg/kg for group 2 and 1.67 mg/kg for group 3 (Table 1).

Regarding clinical parameters measured, respiratory rate did not differ between groups throughout the experiment, as well as the mucous membranes color. The heart rate was significantly different ($P < 0.05$) in the group 1 when compared with group 3 at the hour 9 of the experiment; in other words, there was an increase in heart rate of animals in group 1. Nitrite serum dosage was significantly higher ($P < 0.05$) for group 1 when compared to group 3, an increase of 43.56%, 73.33% and 78.78% at 4, 6 and 9 h, respectively. On the other hand, serum nitrite level was not significantly different ($P > 0.05$; Figure 2) in group 2 compared to the group 3 (Table 2).

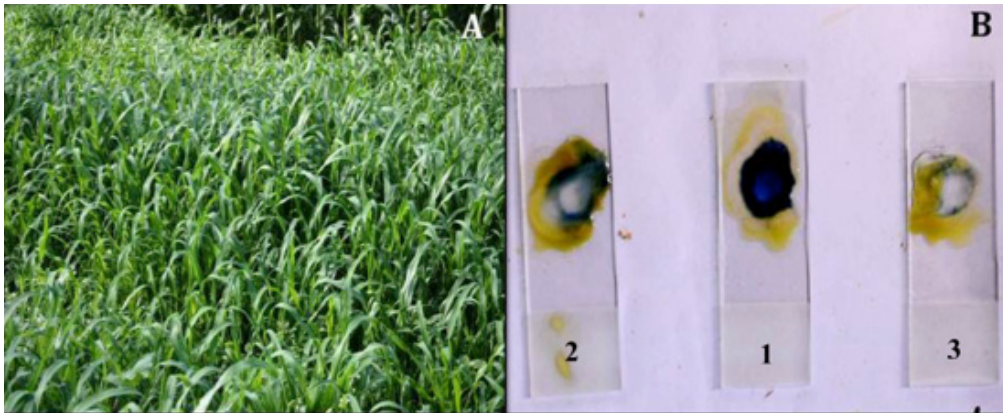

Figure 1. A- *Pennisetum glaucum* cultivation used in the experiment (group 1). B- diphenylamine test with strong positive reaction in the group 1 (+++), positive in group 2 (+) and negative in group 3 (-) for the grazing of pickets A, B and C, respectively. Reaction considered positive when displayed intense blue staining seconds after mixing of the reagent.

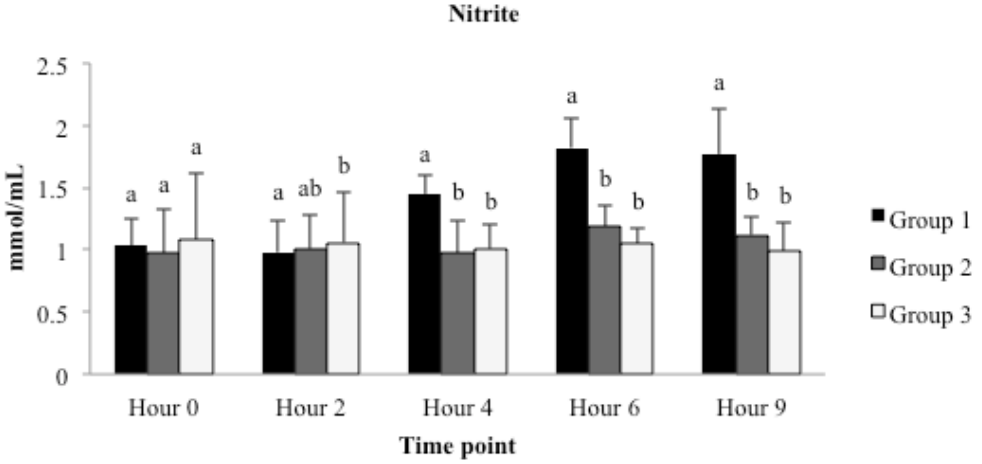

Figure 2. Mean and standard deviation of nitrite measured in cattle fed pasture fertilized with urea (group 1), organic fertilizer (group 2) and control without fertilization (group 3). Same letters in the same series show not statistical difference ($P > 0.05$).

Table 2. Respiratory and cardiac rate in different groups.

Parameter	Hours	Mean		
		Group 1	Group 2	Group 3
Heart rate	0	92.0 ± 28.0[a]	100.7 ± 4.2[a]	98.7 ± 2.3[a]
(bpm)	2	72.7 ± 13.3[a]	89.3 ± 10.1[a]	89.3 ± 9.2[a]
	4	76.0 ± 4.0[a]	93.3 ± 18.9[a]	81.3 ± 9.2[a]
	6	81.3 ± 10.1[a]	90.7 ± 10.1[a]	80.7 ± 22.5[a]
	9	100 ± 4.3[a]	90.7 ± 2.2[b]	77.3 ± 2.3[c]
Respiratory rate	0	42.7 ± 16.8[a]	67.3 ± 27.3[a]	53.3 ± 7.6[a]
(mpm)	2	40 ± 17.4[a]	45.3 ± 4.6[a]	36 ± 8.0[a]
	4	44.0 ± 10.6[a]	40.7 ± 9.4[a]	44 ± 10.6[a]
	6	36.0 ± 6.9[a]	42.0 ± 7.2[a]	42.0 ± 5.3[a]
	9	52.7 ± 11.7[a]	52.0 ± 10.6[a]	46.7 ±11.0[a]

Different letters in the same series show statistical difference ($P < 0.05$).

The AChE activity was different ($P < 0.05$) in the group 1, 100% higher at 4 h and 144% at 6 h after beginning the experiment, when compared to group 3 (Figure 3). The BChE activity was also increased at 4 and 9 h in group 1 ($P < 0.05$), being this increase of 22.3% and 50.3% when compared to group 3. AChE and BChE activity in the group 2 did not present significant difference to group 3 ($P > 0.05$).

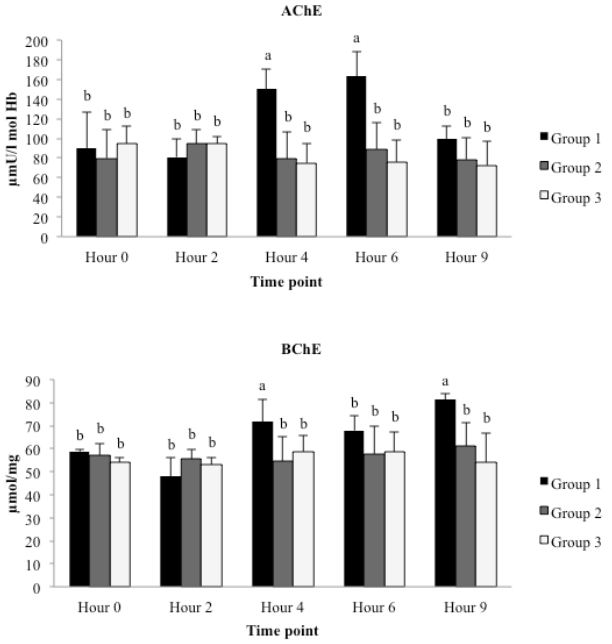

Figure 3. Mean and standard deviation of AChE and BChE activities measured in cattle fed pasture fertilized with urea (group 1), organic fertilizer (group 2) and control without fertilization (group 3). Same letters in the same series show not statistical difference ($P > 0.05$).

The NO_x values were significantly higher (48.2%) in animals from group 1 compared to group 3 at 2 h of the experiment. However, with 6 and 9 h there was a reduction in serum NO_x levels that reached 39.41% and 77.66% when compared to group 3, respectively (Figure 4). At hour 9, a significant difference between groups 1 and 2, also as compared to the control group, was observed; with difference of 64.17% between groups 1 and 2, and 37.66% higher for group 3 than the group 2 (Figure 4). The ROS levels increased significantly in animals from group 1 compared to group 3 in 2 and 4 h, that is, 52.20% and 40.61% higher in bovine fed with nitrogen topdressing pasture; however, with 9 h of experiment the ROS levels of group 1 reduced about 29.54% compared to group 3 (Figure 4). Lipid peroxidation also occurred in group 1, due an increase in TBARS levels of 31.01%, 66.03% and 56.88% at hours 2, 6 and 9 of the experiment, respectively, when compared to group 3 (Figure 4).

Animals from group 1 showed a significant increase in CAT activity at 2 and 4 h of the experiment, and this difference was 55.74% and 32.29% higher, respectively, compared to group 3. However, with 6 and 9 h a decreased CAT activity in animals from group 1 compared to group 3 (35.74% and 42.30%, respectively) were observed. Furthermore, at hour 9 of the experiment animals from group 2 also showed lower CAT activity compared to group 3 (a decrease of 45.20%; Figure 5). The SOD enzyme activity had a significant reduction in bovines from group 1 compared to group 3, and this decrease occurred at 4, 6 and 9 h, being 27.00%, 39.70% and 39.90%, respectively (Figure 5).

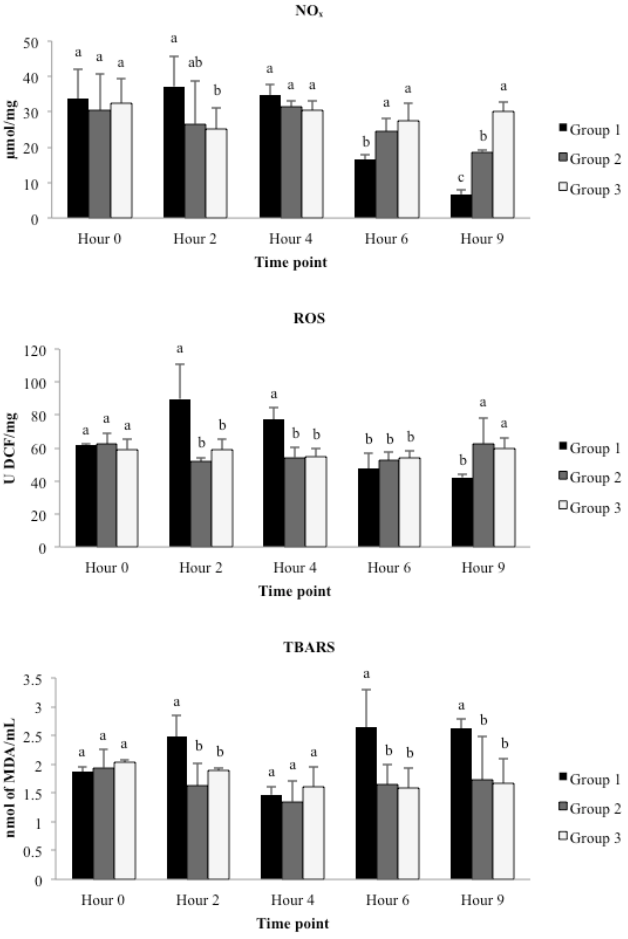

Figure 4. Mean and standard deviation of NO*x*, ROS and TBARS levels measured in cattle fed pasture fertilized with urea (group 1), organic fertilizer (group 2) and control without fertilization (group 3). Same letters in the same series show not statistical difference ($P > 0.05$).

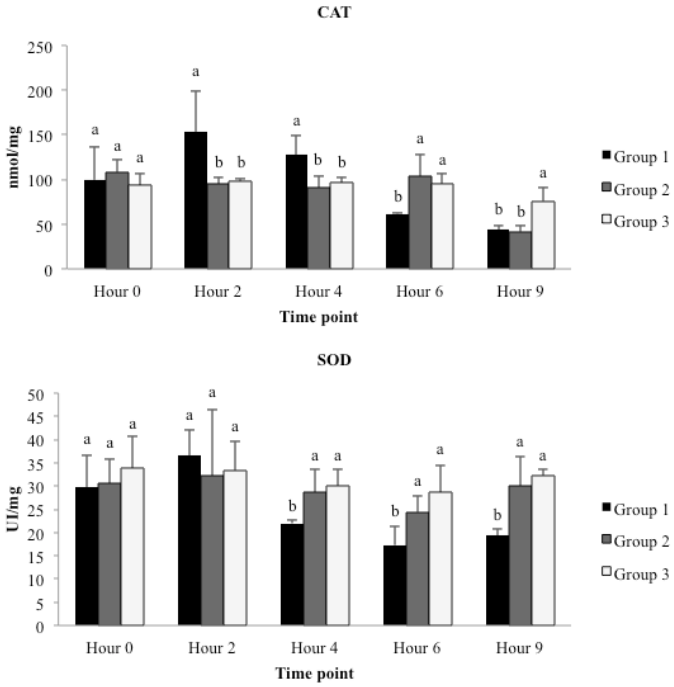

Figure 5. Mean and standard deviation of CAT and SOD levels measured in cattle fed pasture fertilized with urea (group 1), organic fertilizer (group 2) and control without fertilization (group 3). Same letters in the same series show not statistical difference ($P > 0.05$).

DISCUSSION

During the experiment none of animals presented clinical signs of poisoning by nitrate and nitrite with progressive dyspnea, cyanosis, ataxia and convulsions, according with described in literature [30,32]. However, animals of group 1 showed an increase in heart rate after 9 h of the experiment. Tachycardia is an important compensatory mechanism in any alteration of oxygen transport, when there is a deficient oxygenation, such as methemoglobinemia that attempts to raise the cardiac output, increasing blood pressure, heart rate and force of contraction [18,34]. Thereby, showing that even in subclinical poisoning the organism is altered to compensate the poisoning.

Regarding pasture consumption although group 3 ingested much less pasture per animal (3.9 kg), the pasture intake per body weight were quite similar between groups, ranging between 2.36 and 3.71, not interfering among groups. Furthermore, group 2 (manure) ingested even more pasture than group 1 (urea).

The pasture fertilization is directly related with nitrate accumulation in plants. The excessive fertilization is reported in several outbreaks of nitrate and nitrite poisoning [17,22], being described after use of nitrogen fertilizer [17] and also organic fertilizer, mainly from pigs and poultry [17,22]. The intense soil use, jointly with the fact that agricultural activity is linked to animal production, favors the use of organic fertilizer in the pasture. In addition, some climatic factors trigger greater absorption into the plant. Intensive rain preceded by a drought period is cited in the literature as one of the main factors to nitrate accumulation in plant [26,27]. It was mentioned [33] that pastures with nitrate levels above 1.5%, or between 0.5 to 4% [26] would be potentially toxic to cattle. Also, researchers [17] evaluated several outbreaks of nitrate/nitrite poisoning and found nitrate values in the pasture ranging from 0.3 to 3.36%, which agrees with levels found in this study, since a small amount of accumulated nitrate is able to induce subclinical poisoning.

One of the major consequences of nitrate/nitrite poisoning is abortion that occurs due the hypoxia generated by methemoglobinemia, inducing injuries to the fetus. Some studies have reported the occurrence of abortions in poisoned animals, treated or with spontaneous regression after few days of the poisoning [6,17]. The occurrence of abortions in animals that fed pasture with high nitrate levels was reported [28], but did not developed characteristic clinical symptoms

of poisoning. This indicates that even a mild nitrate/nitrite poisoning may lead to some fetal damage [26].

Studies with AChE and BChE enzymes showed the participation of this system in regulatory mechanisms of the organism by hypoxia, regulating the activity of the cholinergic system [5]. In nitrate and nitrite poisoning intense hydrolysis of acetylcholine due to increase of AChE and BChE activity in the synaptic cleft occurs. This mechanism appears in response to hypoxia generated by methemoglobinemia, where oxygen transport is poor. The AChE and BChE activity have parasympatholytic action, inhibiting cholinergic neurotransmission, an effect that leads the increase of vital signs in an attempt to compensate the oxygenation disorders [15,20,23]. The increase observed in cholinesterase activities may explain the pathophysiology of cardiac and respiratory changes, since intense degradation of acetylcholine with modulator cardiorespiratory effect occurs. This is due to inhibition of parasympathetic neurons and activation of the sympathetic pathway, mechanism activated in response to hypoxia in an effort to increase the oxygen transport to tissues.

The increase in nitrite serum levels indicates that the pasture intake in group 1 induced the ruminal conversion of nitrate to nitrite and, therefore, absorption into the bloodstream [33]. Oxidation of hemoglobin to methemoglobin occurs when nitrite reaches the bloodstream and oxidizes the iron ion of ferrous state (Fe^{+2}) to ferric (Fe^{+3}), which leads to hypoxia signs observed in group 1, characterized by increased heart rate [17,22,34]. Furthermore, nitrite in the bloodstream participates of the reaction catalyzed by peroxidase, especially in the presence of H_2O_2 that might lead to damage in many organic molecules, favoring cell injury as a result of the nitric oxide (NO) production [35]. It was described that high nitrite absorption, derived from plants with high NO_3 levels conducts to tissue damage due to NO production [7]. This observation corroborates the results found in this study, increased levels of nitrite and NO.

The NO has selective reactivity and is able to react with other paramagnetic molecules such as iron of the proteins with heme group (Fe^{+2} and Fe^{+3}) [35]. The NO in the bloodstream reacts primarily with the oxyhemoglobin (HbO_2), giving rise to methemoglobin ($MetHb^+$) and nitrite. In addition, this reaction can mediate the peroxynitrite formation, highly reactive radical. After nitrite formation, a stable subproduct of NO, is converted to nitrate and then removed from the bloodstream [29]. This dynamic may explain the reduction in NOx levels during the experiment. Once NO is formed it reacts with proteins of the erythrocytes, generating the $MetHb^+$ that is metabolized quickly and removed from bloodstream [18,29]. The formation of $MetHb^+$, in this case may support the pathogenesis of the disease, since this form is unable to deliver oxygen to the organism [17].

The ROS are extremely unstable and reactive molecules able to penetrate through the cell membranes [13] and lead to several changes in proteins, lipids and DNA [10]. One of the main consequences of ROS production is lipid peroxidation, which is due to conformational change in the double and triple bonds, modifying the lipid structure [8]. These lipids are the major components of cell membranes and one of the first site for free radical attack, whereas the oxidation can cause serious injury to the cellular permeability [10]. Associated to this, the formation of iron ions in the form Fe^{+3} [17] may potentiate the lipid peroxidation, increasing cell damage by releasing Fe^{+2}. The peroxides can be degraded when in contact with iron-containing molecules (hemoglobin and myoglobin) to radicals able of extracting hydrogen, such as peroxyl radical, forming the malondialdehyde as the final product of lipid peroxidation [12]. Serum TBARS levels showed an increase throughout the experiment in group 1, after 4 h. This may have occurred as a consequence of free radicals production, inducing deleterious reactions in organic molecules [31]. In a similar study [2], evaluating nitrate/nitrite poisoning, found values of TBARS 47% higher in the poisoned group, but animals were exposed to nitrate for a long time, tending to chronicity. Taken together, our results and those reported previously indicate that the nitrate/nitrite poisoning leads to lipid peroxidation in acute form, due to free radicals production, and this process is perpetuated until the nitrate exposure remains.

The enzymatic antioxidant system evaluated in this study by CAT and SOD enzymes plays an important role in the neutralization of free radicals, preventing cell injury. SOD is specific in the removal of superoxide radical, catalyzing the reaction that will form the H_2O_2. CAT performs the H_2O_2 degradation to H_2O and O_2. In the presence of iron ions or in H_2O_2 excess, with antioxidant activity saturation, the hydroxyl radical is formed by Fenton reaction [8]. It is believed

that depletion of the antioxidant activity expressed by reduction of SOD and CAT activity after four and six hours, respectively, occur as a consequence of oxidative stress triggered at the beginning of the experiment, leading to resolution of this condition and reduction of antioxidant activity. Reduction of 33% on antioxidant activity in cattle poisoned by nitrate/nitrite was described [2]. Equivalent results of the present study with a significant reduction of antioxidant activity after six hours of the experiment were observed.

CONCLUSION

Based on these results, we conclude that the nitrate and nitrite poisoning by pasture intake cultivated and fertilized with urea leads to increased levels of serum nitrite, as well as the cholinesterase activity and causes oxidative stress in cattle. It is conjectured that the cholinesterase activity and oxidative stress may assist in understanding the pathophysiology of changes caused by poisoning.

MANUFACTURERS

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***Ethical approval*.** Research project approved by Ethical Commission on the use of Animals of the Instituto Federal Catarinense Campus Concórdia, project 16/2015.

***Declaration of interest*.** The authors declare no conflict of interest with respect to the publication of this paper.

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