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The oxidative status of milking goats after *per os* administration of N-acetylcysteine

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Investigated were changes in selected redox parameters – vitamin C, malondialdehyde (MDA) and glutathione (GSH) content of goat blood plasma – as markers of oxidative stress after *per os* administration the N-acetylcysteine (NAC). Used were 20 Polish White Improved goats, selected from the flock of 60 animals. Within the selected goats distinguished were four groups according to somatic cell counts (SCC) of milk: group I – below 1×10^6, group II – 1×10^6-2×10^6, group III – 2×10^6-4×10^6 and group IV – above 4×10^6/ml. Concentrations of GSH, MDA and vitamin C of blood plasma were assessed just at start of the experiment and then after 7 days of daily administration of 12 mg NAC per kg body weight to goats. After 7 days of administering NAC to goats the plasma concentration of both MDA and GSH dropped and that of vitamin C increased. It is concluded that NAC administered *per os* increases the anti-oxidant capacity and may reduce the content of lipid peroxidation products in blood plasma of milking goats.

KEY WORDS: glutathione / goat / lipid peroxidation / malondialdehyde / N-acetylcysteine / oxidative stress / vitamin C

When in physiological equilibrium, the organism possesses sufficient reserves of antioxidants, necessary for the neutralization of free radicals, systematically produced during metabolic processes [Castillo *et al*. 2001]. However, in pathologic situations,

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and among others in the case of subclinical inflammations of the mammary gland, the quantity of generated free radicals is much higher than that of antioxidants, what leads to condition described as oxidation stress [Castillo *et al*. 2003]. In such cases it is necessary to introduce exogenous antioxidants or their precursors into the diet, such as for instance N-acetylcysteine. Currently, several biomarkers are known which can be used as indicators of the oxidation status of the organism. GSH and vitamin C are worth special attention. In clinical practice a diverse test is also used to determine the extent of lipid peroxidation [Castillo *et al*. 2003]. NAC is a thiol antioxidant-free radical scavenger, that increases synthesis of intracellular glutathione [Bengtsson *et al*. 2001, Aitio 2005]. Reduced glutathione (GSH) is an important, naturally occurring antioxidant. Its function is to detoxify reactive oxygen metabolites of endogenous or exogenous origin [Corradi *et al*. 2004, Śliwa-Jóźwik *et al*. 2002, Świderska-Kołacz *et al*. 2007]. Malondialdehyde (MDA) is commonly used as a marker of both oxidative stress and the antioxidant status in various pathologies. An increase in free radicals causes overproduction of MDA [Grasso *et al*. 1990, Cook-Mills 2002]. Another redox parameter – vitamin C – is a water-soluble, cytosolic, chainbreaking antioxidant produced in the liver of ruminants [Weiss *et al*. 2004].

Due to the fact that the synthesis of GSH, the important antioxidant, depends among much else on the availability of sulphur-containing amino acids, the authors of the present report assumed, that supplementing the diet with NAC may lessen or even totally eliminate the mammary gland inflammations in milking animals. In the literature available no information about such investigations was found. Thus, the aim of this study was to determine the effect of offering N-acetylcysteine *per os* to milking goats with a differentiated somatic cell count (SCC) in milk, on changes in the content of GSH, MDA and vitamin C in their blood, as those are important biomarkers informing about the oxidation status of the organism.

Material and methods

Animals and sampling

Twenty goats-in-milk were selected from the flock of 60 Polish White Improved animals with the average live body weight of 53.0 kg (±3.5 kg) at the end of the third trimester of lactation 4th. The average milk yield per goat per lactation was 750 kg, containing 3.35% fat and 2.81% total protein. Four following groups were distinguished (n=5) based upon somatic cell count (SCC) of milk:

I – $<1\times10^6$ cells/ml of milk;
II – 1×10^6-2×10^6 cells/ml of milk;
III – 2×10^6-4×10^6 cells/ml of milk
IV – $>4\times10^6$ cells/ml of milk.

The goats were maintained in a loose barn and fed corn silage, wilted grass silage, concentrates and mineral-vitamin premix, according to the INRA guidelines, and had

free access to water. Goats were under veterinary care and showed no clinical signs of *mastitis*.

The experiment lasted 7 days. From each goat, jugular blood samples were collected twice: once before (at the morning feeding) and once after seven days of daily administering of N-acetylcysteine (NAC). Blood (9 ml) was withdrawn by an authorized veterinarian into Sarstedt Monovette tubes with heparine to assay MDA, or containing EDTA to assay GSH. To estimate the vitamin C, the blood serum, after collection into serum Sarstedt Monovette tubes, was used.

Simultaneously the milk samples were taken to estimate the somatic cell count using Fossomatic 90.

All procedures involving animals were performed in accordance with the Guiding Principles for the Care and Use of Research Animals and were approved by the III Local Ethics Commission for Experimentation on Animals (Warsaw Agricultural University; Permission No 48/2005).

NAC administration

Each goat was given *per os* 12 mg NAC / kg body weight in capsules (HEXAL®), once a day during evening milking (17.30 h), for 7 consecutive days. The dose of NAC was established based on Hexal's body weight-related references for humans (600 mg / animal per day) according to Marenzi *et al*. [2006].

Analytical

MDA in blood plasma was assessed spectrophotometrically using a kit provided by Bioxytech® (OxisResearch™, OXIS Health Products, Inc. USA). A 200 µl plasma sample was pipetted into an alcohol-rubbed, clean glass test tube followed by 650 µl of diluted R1 (N-methyl-2-phenylindole in acetonitrile) reagent. It was mixed gently by vortexing and 150 µl of concentrated (12 N) HCL was added. The mixture was incubated at 45°C for 60 min. followed by centrifugation of the turbid samples (15 000 x g for 10 min) to obtain a clear supernatant. The supernatant was pipetted into a cuvette and the absorbance determined at 586 nm (LambdaBio20 spectrophotometer, PERKIN ELMER, USA, 1999). The concentration of MDA was expressed in µmol/l of blood plasma.

Vitamin C concentrations were spectrophotometrically determined using a phosphotungstic acid method described by Omaya [1979]. The concentration of vitamin C was expressed in mg/dl of serum.

GSH content of blood plasma was determined spectrophotometrically using a kit provided by Bioxytech® (OxisResearch™, OXIS Health Products, Inc. USA). To a test tube of dark-light glass introduced were 200 µl of sample and 200 µl of buffer (potassium phosphate, Diethylenetriaminepentaacetic acid (DTPA), Lubrol, pH 7.8), 200 µl Reducing Agent (Tris(2-carboxyethyl) phosphine (TCEP) in HCl), 200 µl Chromogen and 200 µl Colour Developer to the reaction mixture and shocked. The mixture was subsequently incubated at room temperature in the dark for 30 min.

Measurement of the absorbance at 420 nm commenced. The concentration of GSH was expressed in μmol/l of whole blood.

Statistical

Statistical assessment was carried out using the GLM procedure of SAS Version 8e for Windows 2007 (Microsoft Inc., Silicon Valley, USA), SAS Institute, Cary, North Carolina) using the following model:

$$y_{ijk} = \mu + G_i + NAC_j + (G \times NAC)_{ijk} + e_{ijk,}$$

where:

y_{ijk} – observed mean of the trait;
μ – overall mean;
G_i – animal groups according to SCC level (I = I….IV);
NAC_j – time of sample collection (j = 1, 2);
$(G \times NAC)_{ij}$ – interaction of time of sample collection and number of animal subgroups;
e_{ijk} – error.

Results and discussion

The concentration of GSH and vitamin C in the blood plasma occurred related to both the SCC in milk and the administration of NAC (Tab. 1 and 2), while the MDA level changed only under the influence of NAC (Tab. 3). Before administering NAC, the GSH level increased significantly with the SCC increase of milk. A similar relation was observed earlier [Jóźwik *et al*. 2004]. The SCC in milk increase might be accompanied by subclinical inflammations of the mammary gland, occurring with

Table 1. Means and standard errors (SEM) for the level of GSH (μmol/l) in whole blood of goats before and after 7 days of the NAC daily administration

Sample collection	SCC group							
	I		II		III		IV	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
On day 0 of NAC administration	499$^{*\ AB}$	33.5	596$^{**\ Aa}$	36.7	626^{B}	33.3	704$^{**\ Aa}$	36.8
On day 7 of NAC administration	583$^{*\ a}$	36.8	484$^{**\ ab}$	33.6	581$^{\ b}$	34.6	572**	31.3

^{abAB}Within rows means bearing the same superscripts are significantly different: small letters – P≤0.05, capitals – P≤0.05.
*,**Means within columns are significantly different at P≤0.05, and P≤0.01, respectively.

Table 2. Means and standard errors (SEM) for the level of vitamin C (mg/dl) in serum of goats before and after 7 days of the NAC daily administration

Sample collection	SCC group							
	I		II		III		IV	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
On day 0 of NAC administration	0.83$^{**\,B}$	0.05	0.82$^{*\,A}$	0.06	0.74$^{**\,Bb}$	0.09	0.57^{**ABb}	0.08
On day 7 of NAC administration	1.10**	0.10	0.98$^{*\,A}$	0.10	1.09$^{**\,b}$	0.13	1.22$^{**\,Ab}$	0.07

^{abAB}Within rows means bearing the same superscripts are significantly different: small letters – P≤0.05, capitals – P≤0.05.
*,**Means within columns are significantly different at P≤0.05, and P≤0.01, respectively.

Table 3. Means and standard errors (SEM) for the level of MDA (μmol/l) of blood plasma in goats before and after 7 days of the NAC daily administration

Sample collection	SCC group							
	I		II		III		IV	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
On day 0 of NAC administration	9.01	0.23	8.94**	0.22	8.88**	0.22	9.37**	0.22
On day 7 of NAC administration	8.67	0.23	7.37**	0.21	7.32**	0.20	7.60**	0.21

**Means within columns are significantly different at P≤0.01.

different intensity and promoting the production of free radicals. Thus one may assume that an increased level of GSH in the animal's whole blood is a typical defensive response of the organism to the inflammation. This tri-peptide, due to the presence of the -SH groups, is considered the important substance, efficiently neutralizing free radicals. After administration of NAC, the level of GSH in the whole blood of goats (with the exception of group I, in which the GSH level was the lowest before introducing NAC) decreased significantly. After NAC administration the differences in the blood GSH level between goats from group IV producing milk with the highest SCC, and the remaining groups proved to be not significant. The decrease in GSH concentration after NAC administration was probably related to a decrease in the level of free radicals, what may be confirmed by the decreased SCC observed in earlier studies by Bagnicka *et al*. [2008] as well as in the present study (Fig. 1).

The decrease observed in the GSH concentration after the administration of NAC (with the exception of group I – Fig. 2) is difficult to interpret because, among much else, of a lack of reports from similar studies conducted on animals. From a biochemical point of view, the level of GSH in whole blood of animals after the administration of NAC

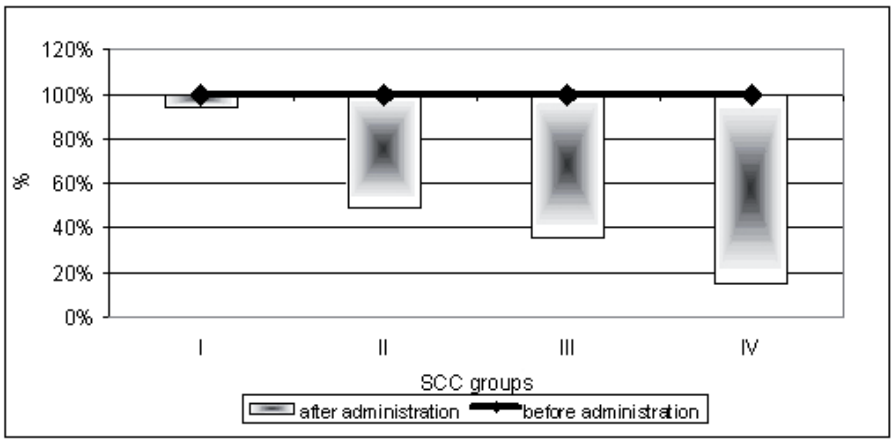

Fig. 1. The percentage changes of SCC level in milk after NAC supplementation in experimental groups

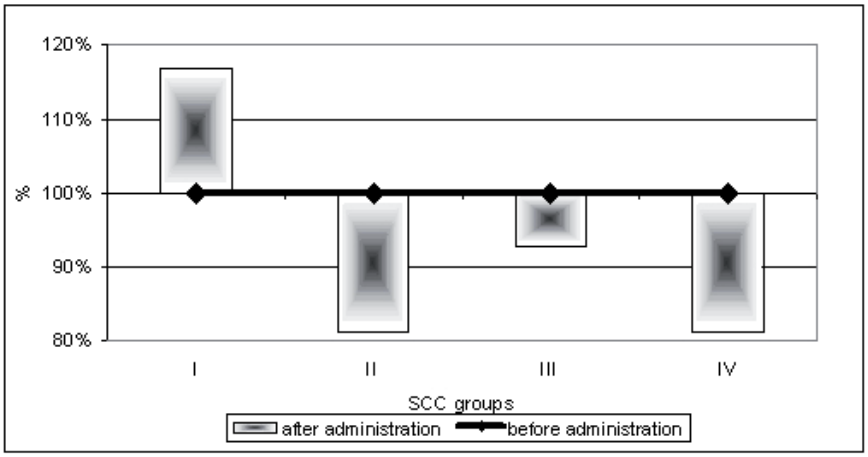

Fig. 2. The percentage changes of GSH in blood after NAC supplementation in experimental groups

should increase, as NAC acts as a cysteine prodrug and precursor of GSH [Zafarullah *et al*. 2003]. According to Atkuri *et al*. [2007] the decreased GSH content and its relatively uniform level in the goat blood after administering NAC might be caused by a its wide activity spectrum in the animals' organism. For some time the compound is already used in the treatment of various infections in humans (including the HIV virus), metabolic disturbances caused by various diseases and even certain genetic defects. NAC is also marked for its ability to reduce disulphide bonds in proteins [Harada *et al*. 2004], bind metals to form complexes [Koh *et al*. 2002] and scavenge free radicals [Aruoma *et al*. 1989]. The latter ability of NAC, *i.e*. a direct neutralization of free radicals, could result

in their lower plasma concentration. It leads to a decreased GSH synthesis, because the threat of the occurrence of an oxidation stress decreased. Moreover, the results of studies conducted on patients demonstrated that an oral administration of NAC, by a positive effect on the functions of neutrophils, lymphocytes and macrophages, may have a direct (or through metabolites) effect not only on the neutralization of ROS, but also on strengthening the immune resistance of the organism [Urban *et al*. 1997, Puerto *et al*. 2002]. The leucocytes mentioned are among the somatic cells present in milk. They strengthen the immune protection of the mammary gland against pathogenic infection, leading to an inflammation [Bradley 2002].

Contrary to GSH, the concentration of vitamin C in the blood serum before administering NAC, decreased significantly with increasing SCC of milk (Tab. 2). The level of vitamin C in group III was significantly lower only when compared to group I, while in group IV the content of vitamin C was significantly lower than in groups I, II and III. The increased GSH level, with a simultaneous drop in concentration of vitamin C in the blood serum, could indicate the subclinical udder inflammation. A higher intensity of inflammation was observed in the group of goats producing milk with the highest SCC. These observations are in accordance with results obtained on dairy cows in which sub-clinical and clinical *mastitis* was induced experimentally [Kizil *et al*. 2007]. The NAC caused increase of vitamin C level in serum of goats (Fig. 3). This growth can be related to increasing of antioxidative potential of blood after administering of NAC.

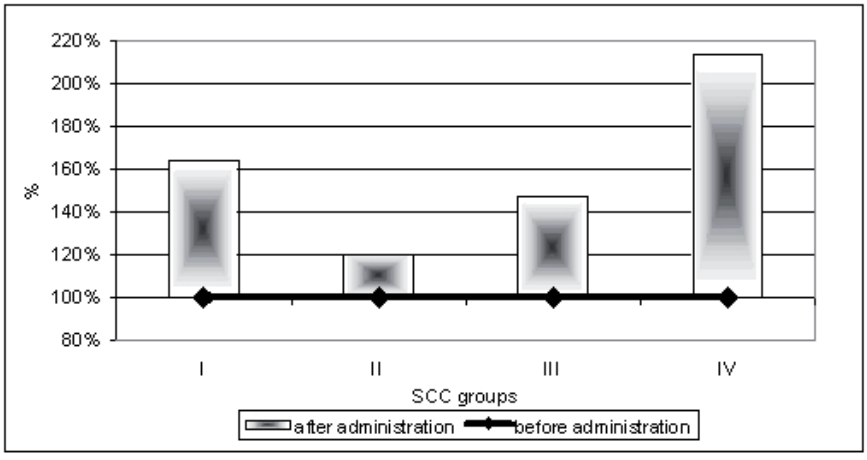

Fig. 3. The percentage changes of vitamin C in serum after NAC supplementation in experimental groups

The MDA concentration of blood plasma of goats before the administration of NAC was not significantly affected by SCC group (Tab. 3). After administering NAC the MDA plasma level (with the exception of group I) decreased significantly (Fig. 4). The MDA concentration of plasma in animals is positively correlated with the CMT

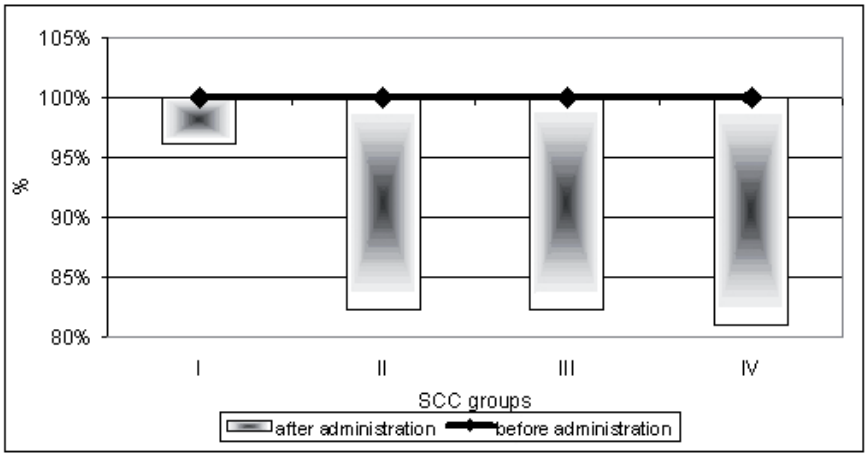

Fig. 4. The per cent changes of MDA in blood plasma after NAC administration in SCC groups

(California Mastitis Test) parameters. However, when significant changes occur in the GSH concentration even with no significant changes in the MDA level, a milk oxidation stress may appear [Kizil *et al*. 2007].

The significant attenuation of biochemical markers (MDA, vit. C and GSH) in all groups of goats after N-acetylcysteine administration suggests that NAC helps the defence against pathogens which evoke homeostatic reactions. Thus, MDA together with glutathione and vitamin C are considered major markers of inflammatory processes and oxidative stress [Grasso *et al*. 1990, Cook-Mills 2002, Corradi *et al*. 2004, Weiss *et al*. 2004, Tsai *et al*., 2005].

The obtained results showed that introducing n-acetylcycteine (NAC) into the diet of lactating goats, decreased concentration of both MDA in blood plasma and GSH in whole blood, and increased the level of vitamin C in goat blood serum, irrespective on the concentration of somatic cells in their milk. It means that NAC increases anti-oxidant capacity and may reduce products of lipid peroxidation in blood of goats. This may lead to the improvement of the quality of milk and health status of milking goats.

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Status oksydacyjny kóz dojnych po doustnym podawaniu N-acetylocysteiny

S t r e s z c z e n i e

Badano zachowanie się wybranych parametrów redox – zawartości witaminy C w surowicy, malonodwualdehydu (MDA) w osoczu i glutationu (GSH) w pełnej krwi kóz dojnych – jako wskaźników stresu oksydacyjnego po 7 dniach doustnego podawania N-acetylocysteiny (NAC). Użyto 20 kóz rasy polskiej białej uszlachetnionej ze stada liczącego 60 zwierząt. Wybrane kozy podzielono na 4 grupy, zależnie od liczby komórek somatycznych (SCC) w mleku (grupa I – poniżej 1×10^6, grupa II – 1×10^6-2×10^6, grupa III – 2×10^6-$4x10^6$ i grupa IV – ponad 410^6/ml. Poziom witaminy C, MDA i GSH we krwi oznaczono jednorazowo na początku badań (dzień 0), a następnie, także jednorazowo, po 7 dniach, w ciągu których codziennie podawano zwierzętom doustnie NAC. Postępowanie takie doprowadziło do spadku poziomu MDA w osoczu i GSH w pełnej krwi oraz wzrostu poziomu witaminy C w surowicy krwi. Autorzy wnioskują, że NAC podawana *per os* zwiększa antyoksydacyjną wydajność organizmu i może ograniczać zawartość produktów peroksydacji lipidów w osoczu.