

# Blood vitamin C, vitamin A, $\beta$ -carotene, ceruloplasmin, glutathione and malondialdehyde concentrations in cows with sub-clinical mastitis treated with intramammary antibiotics

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## SUMMARY

Antioxidant status (blood vitamin C, vitamin A,  $\beta$ -carotene, glutathione and ceruloplasmin concentrations) and oxidative stress markers (malondialdehyde concentration) were analyzed in Holstein Frisian cows with subclinical mastitis (n = 24), the mammary infections being confirmed by CMT and bacteriological tests. According to the effectiveness of the intramammary antibiotherapy (200 mg Cephalexin and 100 000 IU Kanamycine), cows were divided into "cured" (n = 15, both CMT tests and bacteriological analysis of milk performed on days 14 and 21 were negative) and "not cured" (n = 9) (infection was still evidenced on days 14 and/or 21). Plasma MDA and blood antioxidant concentrations were determined on Days 14 and 21 post-treatment and compared with values obtained in healthy cows (n = 13). Blood vitamin C and glutathione concentrations in controls and cured cows were significantly higher than those of uncured cows. A significant rise in mean plasma vitamin A concentration was observed following intramammary therapy in cured cows, but a similar trend was also evident in the control group. No significant difference was observed in plasma  $\beta$ -carotene, malondialdehyde and ceruloplasmin concentrations neither between groups or in repeated samplings of individual groups. It was concluded that subclinical mastitis significantly alters the antioxidant capacity in plasma of cows and that an efficient intramammary treatment partially restore the antioxidant status.

**Keywords:** Cow, oxidative stress, antioxidants, subclinical mastitis, intramammary antibiotherapy.

## RÉSUMÉ

**Concentrations sanguines en vitamine C, vitamine A,  $\beta$ -carotène, céruoplasmine, glutathion et malondialdéhyde chez les vaches atteintes de mammite subclinique traitées par antibiothérapie**

Le statut anti-oxydant (concentrations sanguines en glutathion, vitamines C et A,  $\beta$ -carotène et céruoplasmine) ainsi que la survenue d'un stress oxydatif (concentrations plasmatiques de malondialdéhyde) ont été évalués chez des vaches Frisonnes x Holstein atteintes de mammites subcliniques (n = 24), l'infection mammaire étant confirmée par un test CMT et une analyse bactériologique du lait. Selon l'efficacité de l'antibiothérapie intramammaire (200 mg Cephalexin and 100 000 IU Kanamycine), les vaches ont été réparties en 2 groupes: celui des "guéries" (lorsque les tests CMT et les analyses bactériologiques ont été négatifs à J14 et J21 après le traitement) et celui des "non guéries" (l'infection a été encore détectée à J14 et/ou J21). Les concentrations plasmatiques de MDA et le statut sanguin en antioxydants ont été évalués à J14 et J21 après le traitement et comparés aux valeurs obtenues chez des vaches saines (n = 13). Les concentrations sanguines en vitamine C et en glutathion ont été significativement plus élevées chez les contrôles et les animaux guéris que chez les vaches non guéries. La concentration moyenne en vitamine A a augmenté significativement à l'issue du traitement chez les vaches guéries, cependant la même tendance a également été observée chez les contrôles. Aucune variation significative des concentrations en MDA,  $\beta$ -carotène et en céruoplasmine n'a été décelée entre les groupes ou entre les différents temps de prélèvement. En conclusion, la capacité anti-oxydante du plasma est significativement altérée lors de mammite sub-clinique chez la vache et un traitement local efficace permet de restaurer au moins partiellement le statut anti-oxydant.

**Mots-clés :** Vache, stress oxydatif, anti-oxydants, mammite subclinique, antibiothérapie intramammaire.

## Introduction

Subclinical mastitis in cows does not cause any observable symptoms or changes in milk or in the udder, whereas decreased quality and quantity of milk production due to the functional disturbance of the udder parenchyma, are the main economical drawbacks. Furthermore, cows with sub-

clinical mastitis also exist as continuous reservoirs of infectious mastitis agents in the herd. In the scope of preventive approach in bovine mastitis, currently antibiotic therapy is considered as a part of the mastitis control schema which is composed of a series of control steps such as good management, feeding, udder and milking hygiene, dry cow therapy etc. It has been reported that cure rates obtained by the intramam-

mary antibiotic therapy in subclinical mastitis affected cows during lactation are mainly depended on the type and virulence of the pathogens while the most resistant type of microorganism is *Staphylococcus aureus* [13]. Other bacteria like *S. aureus*, *Corynebacterium bovis*, *Coagulase Negative Staphylococci*, *Pseudomonas aeruginosa* and Gram negative bacteria in a lesser extent cause subclinical mastitis. Antibiotics are the second factor for determination the therapy response in mastitis. Antibiotics which have been used for intramammary therapy of mastitis include penicillin G, amoxicillin, ampicillin, erythromycin, cephalosporins, cloxacillin, dihydrostreptomycin, neomycin, novobiocin and oxytetracyclin [17, 21]. These agents are used either alone or in combination inside intramammary preparations. While most of the bacteria are resistant to either one or more types of antibiotics, antibiotic sensitivity tests can be advocated in choosing the right agent [34]. Finally natural defence mechanisms also may play an important role in eliminating infections. Antioxidants are reported to enhance the neutrophil-killing ability and other immune functions during clinical and subclinical mastitis [14, 29].

Many researches have provided information about the effects of antioxidants (Vitamins A, C, E, Selenium and  $\beta$ -carotene) on the occurrence, existence and the course of infection, by measuring them in blood and in milk from cows with clinical and subclinical intramammary infections (IMI) [4, 9, 11, 12, 18, 23]. Dietary supplementation with vitamin E and selenium has induced beneficial effects on the udder health. Moreover, plasma vitamin E concentrations in mastitis cows were found below to those of healthy cows [4]. CHEW *et al.* [9] have reported that plasma vitamin A and  $\beta$ -carotene concentrations are negatively correlated with somatic cell counts of cows with mastitis. In the same way, plasma glutathione (GSH) concentrations were found to be negatively correlated with somatic cell counts in dairy cows [11, 23]. In a recent report, KLECZKOWSKI *et al.* [18] have observed that serum vitamin C concentrations are significantly lower in cows with subclinical mastitis than in healthy cows and that subclinical mastitis were associated to decreased antioxidant concentrations in the blood. Ceruloplasmin (CERU) is an extracellular antioxidant which acts as a free radical scavenger [32]. CHASSAGNE *et al.*, [8] have studied the risk factors of early lactation mastitis in cows and reported that high ceruloplasmin concentrations were associated in cows with an increased incidence of mastitis in the early lactation. Malondialdehyde (MDA) is a degradation product of lipid peroxidation and is a marker of oxidative stress [7].

The objective of this study is defined in two steps. The first is to determine whether there are alterations in the blood antioxidant concentrations in cows with subclinical mastitis or not. And the second is whether this oxidative stress is reversible by intramammary antibiotherapy.

## Material and Methods

### ANIMALS AND SELECTION CRITERIA

Thirty seven Holstein Frisian 2.5 – 7 years old (average age: 4.5 year approximatively) cows belonging to a commercial

dairy herd, were used in this study. Main constituents of the diet were corn silage, alfalfa and hay as roughage while commercial milking cow feed were used as concentrates. The cows had free access to feed and water all the time. The ratio of concentrates in the total ration was adjusted according to the lactation status as early, mild and late lactating cows, and was ranged between 21 to 28 % on the dry matter basis. The cows were housed in a tie stall barn which is bedded with sand, and milked twice daily at approximately 11 and 13 hour intervals. The study was conducted within a two month period between May and June.

### EXPERIMENTAL PROTOCOL

In order to minimize the metabolic and nutritional differences due to the stage of lactation, the cows chosen for the study were distributed evenly to both control and assay groups. Herd records were used to identify the cows that had a clinical mastitis occurrence in their history. The California Mastitis Test (CMT) was performed in the milking parlour prior to the morning milking. Thirteen cows gave negative CMT reactions and served as controls. Cows which exhibited a positive CMT score in 2 or more quarters constituted the assay group (n = 24) and were submitted to intramammary antibiotic treatment. In parallel, milk samples were aseptically collected into sterilised glass tubes. The papillary duct of the teat was disinfected with 70 % alcohol and the tubes were held with a 45° vertical angle to the teats. These samples were immediately analysed in laboratory for bacteriological isolation and identification of the major pathogen germs. These tests have confirmed CMT results and were used for choosing antibiotics. According to the results of bacteriological and antimicrobial susceptibility tests, an intramammary lactation antibiotic combination with 200 mg Cephalexin and 100 000 IU Kanamycine was administered to cows of the assay group for three consecutive milking.

Post treatment samples for bacteriological analysis were collected on days 14 and 21 and were again analysed by CMT. The cows of the assay group for that the 2 post-treatment samples were both CMT and bacteriologically negative were considered as cured (n = 15) otherwise cows were considered as not cured (n = 9). Milk samples were also collected from the control cows at the same times (days 14 and 21) in order to confirm that there is no new intramammary infection occurrence.

In parallel, duplicate blood samples were obtained from the jugular vein of each animal (control and assay cows) in both EDTA and silicone covered vacutainer tubes on day 0 (before the onset of intramammary therapy), on days 14 and 21 after the antibiotic treatment. Plasmas were separated by EDTA blood centrifugation at 1700g for 10 min at room temperature and GSH, MDA and ascorbic acid concentrations were immediately measured in plasma. Sera were harvested after blood clotting (at 24°C, 30 min.) and centrifugation (at 24°C, 1700g, 10 min.), frozen and kept under -20°C until the day of the measurement of ceruloplasmin, Vitamin A and  $\beta$ -carotene concentrations.

## ANALYTICAL PROCEDURES

### *Milk analysis*

CMT: the CMT is a simple method to estimate the DNA content in milk. It is based on the use of an anionic detergent, Na-lauryl sulphate (SDS), which dissolves cell membranes and nuclei. Consequently DNA is released and it forms a transient gel with the detergent, which the viscosity is proportional to the DNA content of the sample. Besides, a pH indicator, the bromocresol purple, is included in the reagent and it gives a purple colour in the presence of alkaline mastitic milk [26]. According to the colour change and viscosity of the formed gel, the CMT score is considered as negative (-), suspect (?), or positive (1+ to 3+).

*Germ isolation and identification:* individual milk samples were cultured by streaking 100  $\mu$ l on a Petri plate containing Blood Agar with 7 % sheep blood and MacConkey Agar. The plates were incubated at 37°C under aerobic condition for 2-3 days. The biochemical identification was carried out as described in the previous studies [22].

*Antibiotic Susceptibility Tests:* for antibiotic susceptibility testing, isolates were suspended in trypticase soy broth (TSB, Merck), and the suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard. Drug susceptibility testing was performed by the agar disk diffusion method [5]. The following disks were used: penicillin G (10 IU); ampicillin (10  $\mu$ g); streptomycin (10  $\mu$ g); danofloxacin (5  $\mu$ g); Kanamycin (30  $\mu$ g), cephalexin (45  $\mu$ g); neomycin (30  $\mu$ g); erythromycin (15  $\mu$ g) and cefoperazone (15  $\mu$ g). Isolates were categorized as susceptible (S), intermediate (I), and resistant (R) based upon interpretive criteria developed by the Clinical and Laboratory Standards Institute (CLSI) [30].

### *Blood analysis*

Serum ceruloplasmin concentration was estimated according to the method of SUNDERMAN and NUMOTO [27]. A coloured oxidation product is formed from ceruloplasmin and p-phenylenediamine and the rate of formation of this product is proportional to the concentration of serum ceruloplasmin.

The plasma lipid peroxidation was measured by the TBA method as described by YOSHIOKA *et al.* [33]. Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, was considered as an index for the peroxidation reaction. The absorbance of the reaction product of MDA with TBA was measured at 532 nm. Quantification was based upon a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

The plasma ascorbate (vitamin C) concentration was measured by the phosphotungstic acid method of KWAY [19].

The plasma glutathione (GSH) concentrations were measured with 0.1mM 5,5'-dithiobis-2 nitrobenzoic acid following the method of BEUTLER *et al.* [6]. The amount of reduced product, thionitrobenzene, was measured spectrophotometrically at 412 nm.

The vitamin A and  $\beta$ -carotene concentrations were measured according to the spectrophotometric method of SUZUKI and KATHO [28].

### *Statistical Analysis*

Animals were grouped as cured (n = 15), not cured (n = 9) and controls (n = 13). The evaluation of values within a group throughout the experiment was analyzed by use of ANOVA for repeated measures, followed by the Tukey's test while intergroup differences and relationships were compared with One-Way ANOVA. Differences were considered as significant when p values were less than 0.05. Correlations between parameters were calculated with Pearson correlation test.

## Results

The CMT results have allowed the diagnosis of subclinical mastitis in 24 cows and with bacteriological tests, 6 major pathogen germs were identified (Table I). Furthermore, 5 cows were polyinfected (at least 2 micro organisms were identified in the same milk). Among pathogens, *Corynebacterium bovis*, *Staphylococcus aureus* and coagulase negative *staphylococci* presented the highest incidence (33.3 %, 30.0 % and 26.7 % respectively). The mammary infection for a given pathogen germ involved 2 to 3 quarters of the udder, and a total of 78 quarters for the 24 subclinical mastitis affected cows (3.25 quarter / cow in average) were infected. Results of the antibiotic susceptibility tests have revealed the sensitivity of micro organisms to kanamycine-cephalexine combination. Following antibiotic therapy, 15 cows were cured microbiologically. Coagulase negative staphylococci (CNS), Streptococci and Pseudomonas were completely killed by antibiotic treatment whereas 33 % of *Staphylococcus aureus* infections (3 / 9) and 60 % of *Corynebacterium bovis* (C. bovis) infections (6 / 10) were resistant to the intramammary antibiotherapy.

Table 2 presents the evolution of plasma MDA concentrations and blood antioxidant concentrations according to time after antibiotic treatment in the control and assay groups. Before intramammary therapy (on day 0), plasma MDA concentrations tended to weakly increase in the subclinical mastitis cows (assay group), although the differences with control cows were not significant. On the other hand, plasma GSH concentrations were significantly decreased in affected cows ("cured" and "not cured" groups) compared to the healthy controls (p <0.05). Although no significant difference was evidenced for blood vitamin C and  $\beta$ -carotene concentrations between healthy and infected cows, these 2 parameters appeared to be weakly depressed in affected cows. By contrast, subclinical mastitis cows (not cured group) showed higher serum ceruloplasmin and vitamin A concentrations than the 2 other groups but differences were not statistically significant.

After intramammary antibiotherapy (on days 14 and 21), plasma MDA concentrations seemed to decrease in the "cured" group (from  $15.13 \pm 1.67$  on day 0 to  $11.42 \pm 1.16$  mmol/L on day 21) whereas they remained relatively stable in the "not cured" group. They have also slowly declined in the control group. Nevertheless, any significant difference between groups at days 14 and 21 and according to time could be observed. In parallel, serum ceruloplasmin concen-

Microorganism	Before treatment		After treatment	
	Infected cows	Infected quarters	Infected cows	Infected quarters
<i>Staphylococcus aureus</i>	9	27	3	9
<i>Coagulase Negative Staphylococci</i>	8	24	-	-
<i>Corynebacterium bovis</i>	10	20	6	16
<i>Streptococcus dysgalactiae</i>	1	3	-	-
<i>Streptococcus agalactiae</i>	1	2	-	-
<i>Pseudomonas aeruginosa</i>	1	2	-	-
<b>Total</b>	<b>30*</b>	<b>78</b>	<b>9</b>	<b>25</b>

\* Five cows were infected with more than one type of microorganism

TABLE 1: Type and intensity of intramammary infections before and following intramammary antibiotherapy (intramammary lactation antibiotic combination with 200 mg Cephalexin and 100 000 IU Kanamycine).

Parameters		Groups			P (group effect)
		Control (n = 13)	Cured (n = 15)	Not Cured (n = 9)	
MDA (mmol/L)	Day 0	14.62 ± 1.56	15.13 ± 1.67	15.19 ± 1.43	NS
	Day 14	13.11 ± 1.11	12.76 ± 1.03	15.17 ± 1.53	NS
	Day 21	11.21 ± 1.17	11.42 ± 1.16	13.59 ± 1.26	NS
	P (time effect)	NS NS	NS		
GSH (mg/L)	Day 0	121.2 ± 9.6 <sup>aA</sup>	90.0 ± 10.1 <sup>bA</sup>	87.9 ± 7.4 <sup>b</sup>	< 0.05
	Day 14	153.5 ± 11.0 <sup>aB</sup>	131.8 ± 18.5 <sup>abB</sup>	90.6 ± 10.4 <sup>b</sup>	< 0.05
	Day 21	170.3 ± 16.3 <sup>aB</sup>	190.6 ± 13.9 <sup>aC</sup>	111.0 ± 11.0 <sup>b</sup>	< 0.05
	P (time effect)	< 0.05 < 0.001	NS		
Vitamin C (µg/L)	Day 0	11.3 ± 1.7	9.1 ± 0.6 <sup>A</sup>	9.4 ± 1.6	NS
	Day 14	13.5 ± 1.6	12.4 ± 1.4 <sup>B</sup>	8.1 ± 1.3	NS
	Day 21	14.0 ± 1.6	13.6 ± 1.1 <sup>aB</sup>	8.2 ± 1.1 <sup>b</sup>	< 0.05
	P (time effect)	NS < 0.05	NS		
Vitamin A (µg/L)	Day 0	262.8 ± 1.0 <sup>A</sup>	272.6 ± 8.4 <sup>A</sup>	288.8 ± 20.8	NS
	Day 14	317.8 ± 30.7 <sup>AB</sup>	322.8 ± 29.3 <sup>AB</sup>	298.7 ± 21.9	NS
	Day 21	349.7 ± 27.1 <sup>B</sup>	355.1 ± 21.5 <sup>B</sup>	327.2 ± 28.4	NS
	P (time effect)	< 0.05 < 0.05	NS		
β-carotene (µg/L)	Day 0	955.3 ± 42.7	981.7 ± 46.8	938.0 ± 65.4	NS
		1056.5 ± 66.0	1124.1 ± 98.8	956.9 ± 60.3	NS
	Day 21	1135.2 ± 58.3	1150.6 ± 90.2	1062.0 ± 65.6	NS
	P (time effect)	NS NS	NS		
Ceruloplasmin (mg/L)Day 0	Day 0	241.3 ± 25.2	247.6 ± 18.5	287.5 ± 34.1	NS
	Day 14	266.0 ± 31.0	304.2 ± 26.2	346.7 ± 36.7	NS
	Day 21	327.2 ± 40.8	310.7 ± 28.9	394.9 ± 46.4	NS
	P (time effect)	NS	NS	NS	

\* Different superscripts a,b,c, in the same row indicate significant differences (p < 0.05: group effect).

\* Different superscripts A,B,C, in the same column indicate significant differences (p < 0.05: times effect).

TABLE 2: Evolution of plasma MDA concentrations and of blood antioxidant concentrations (plasma GSH and vitamin C concentrations; serum ceruloplasmin, vitamin A and β-carotene concentrations) according to time (day 0: before the intramammary antibiotic treatment, days 14 and 21 after treatment) in healthy cows (control group, n = 13) and in subclinical mastitis affected cows effectively cured ("cured" group, n = 15) or not ("not cured" group, n = 9) after the treatment (intramammary lactation antibiotic combination with 200 mg Cephalexin and 100 000 IU Kanamycine). Results are expressed as means ± standard errors.

trations slowly increased according to time in the 3 groups and remained always higher but not significantly in the “not cured” group. Progressive increases of vitamin A and β-carotene concentrations were observed according to time in both groups: this time effect on serum vitamin A concentrations was significant (p<0.05) in the control and in the “cured” group. On days 14 and 21 lowest values were recorded in the “not cured” group but no inter-group significant difference was found. In the same way, plasma vitamin C concentrations remained significantly lowered in the “not cured” group compared to the 2 other groups (“control” and “cured” cows) on day 21 (p<0.05), whereas they have gradually and significantly increased in the “cured” group (p<0.05). Whatever the time point, plasma GSH concentrations were dramatically depressed in “not cured” cows compared to the healthy controls (p<0.05). By contrast, in “cured” cows, they rapidly increased according to time (p<0.001) like in the control group (p<0.05).

The degree of oxidative stress produced by the different types of micro organisms is presented in Table III. No significant differences were observed in MDA, GSH, Vitamins C, A and β-carotene concentrations of cows infected with different types of germs. Ceruloplasmin concentrations were significantly higher in the “not cured” cows infected with *S.*

*aureus* or *C. bovis* compared to “cured” cows (p < 0.05) on day 21 but maximal concentrations of this parameter were observed in “cured” cows infected with Coagulase negative staphylococci and other micro organisms on day 21 too.

Correlations were calculated between biochemical parameters. Negative correlations between MDA and antioxidant concentrations (GSH, vitamin C and vitamin A) were observed in the control group on Day 0 (r = - 0.81, p < 0.01; r = -0.59, p < 0.05 and r = - 0.43, p < 0.05, respectively) and on Day 21 (between MDA and GSH or vitamin A concentrations, r = -0. 62 and r = -0.58, respectively, p < 0.05), whereas vitamin A and β-carotene concentrations positively correlated (r = 0.42, p<0.05). In the “cured” group, MDA concentrations were also negatively correlated with GSH concentrations on Day 14 (r = -0.90, p < 0.05). By contrast, positive correlations between antioxidant concentrations were evidenced in infected cows: GSH concentrations were positively associated with β-carotene concentrations on Day 14 (r = 0.58, p < 0.05) and with vitamin A concentrations on Day 21 (r = 0.63, p < 0.05) in the “cured” group and the concentrations of the vitamins A and C were positively correlated on Day 21 in the “not cured” group (r = 0.75, p < 0.05).

Parameters	Pathogen germ in infected cows															
	<i>S. aureus</i> (n = 9)					<i>C. bovis</i> (n = 10)					CNS (n = 8)			Others (n = 3)		
	Day	Cured (n = 6)		Not cured (n = 3)		Day	Cured (n = 4)		Not cured (n = 6)		Day	Cured		Day	Cured	
		0	Day 14	Day 21	Day 14		Day 21	0	Day 14	Day 21		Day 14	Day 21		0	Day 14
MDA (mmol/L)	16.9 ±2.6	11.7 ±1.7	11.6 ±2.3	13.6 ±2.6	11.9 ±2.6	13.8 ±1.1	15.0 ±1.3	11.3 ±1.3	15.9 ±2.0	14.4 ±1.5	15.5 ±1.7	12.4 ±1.5	12.3 ±1.9	13.4 ±0.7	15.8 ±1.4	11.1 ±2.9
GSH (mg/L)	88.8 ±9.1	145.4 ±28.5	198.7 ±28.1	102.2 ±27.4	109.5 ±16.2	93.4 ±10.1	95.8 ±27.4	178.8 ±21.7	84.9 ±9.6	111.8 ±15.4	93.9 ±11.6	123.1 ±26.8	153.4 ±24.3	53.6 ±14.9	66.1 ±19.1	129.1 ±34.5
Vit C (mg/L)	9.8 ±1.7	9.5 ±2	13.5 ±1	8.5 ±1.2	9.7 ±1.5	9.0 ±0.7	10.9 ±2.6	15.8 ±4.0	7.9 ±2.0	7.5 ±1.5	9.5 ±1.7	10.3 ±1.4	11.2 ±1.3	9.8 ±0.4	16.4 ±4.6	10.2 ±1.0
Vitamin A (µg/L)	258.6 ±11.7	300.1 ±25.4	352.7 ±40.9	299.7 ±62	366.8 ±35.6	293.2 ±17.7	396.2 ±61.7	381.1 ±46.6	298.2 ±29.9	307.3 ±38.1	284.9 ±23.4	307.7 ±30.1	376.1 ±32.7	260.8 ±23.3	289.2 ±88.4	285.6 ±12.6
β-carotene (µg/L)	967.6 ±85	981.8 ±196.8	1106.9 ±175.8	979.3 ±169.2	1183.4 ±105.5	959.8 ±47.0	1497.4 ±43.9	1099.7 ±203.1	945.7 ±54.1	1001.4 ±76.8	980.2 ±65.9	1136.2 ±119.4	1193.1 ±126.8	930.2 ±39.2	894.6 ±116	1265.7 ±139.9
Ceruloplasmin (mg/L)*	277.9 ±29.9	267.0 ±38.1	310.5 ±33.2 <sup>c</sup>	349.9 ±97.1	396.3 ±120.9 <sup>ab</sup>	248.9 ±31.7	274.0 ±42.8	196.5 ±23.2 <sup>d</sup>	345.1 ±36.7	394.2 ±47.5 <sup>ab</sup>	264.9 ±23.4	387.6 ±24	404.3 ±40.1 <sup>ab</sup>	237.8 ±34.9	369.8 ±74.4	494.4 ±46.6 <sup>a</sup>

*S. aureus*: *Staphylococcus aureus*; *C. bovis*: *Corynebacterium bovis*; CNS: *Coagulase negative staphylococci*  
 \*: p<0.05

TABLE 3: Evolution of plasma MDA concentrations and blood antioxidant concentrations (plasma GSH and vitamin C concentrations; serum ceruloplasmin, vitamin A and β-carotene concentrations) in intramammary infected cows according to the identified pathogen germ and according to time (day 0: before the intramammary antibiotic treatment, days 14 and 21 after the treatment) (intramammary lactation antibiotic combination with 200 mg Cephalexin and 100 000 IU Kanamycine). When the 2 post-treatment milk samples are CMT and bacteriologically negative, cows were considered as cured. Results are expressed as means ± standard errors.

## Discussion

The comparison of the biochemical parameters between healthy cows and subclinical mastitis affected cows suggest the occurrence of a moderate oxidative stress in the infected udder characterized by slight increases of plasma MDA concentrations but also by consumption of antioxidants, particularly of GSH and vitamin C, and that the eradication of pathogen germs by intramammary antibiotherapy succeeded in restoring the blood antioxidant concentrations. However, because a significant time effect was also evidenced in healthy controls for some biochemical parameters (GSH and vitamin A) and a same tendency was observed for the others (vitamin C,  $\beta$ -carotene and ceruloplasmin) the improvement of the antioxidant status maybe not only related to the treatment efficiency but also to other factors such as lactation status, feeding quality or management.

Concentrations of antioxidants may show a great variety between herds and even in individual animals within a herd. Dietary conditions and different analytical techniques may be responsible for these fluctuations. Average vitamin C concentrations in healthy cows have been reported as 5.3 mg/L in one review [1] and in another study as 7.47 mg/L [25]. In the present study, plasma vitamin C concentrations were higher than the above data in both control and mastitis affected cows but still within physiological limits [1] (Average vitamin C concentrations on Day 0 in control, "cured" and "not cured" cows were  $11.3 \pm 1.7$ ,  $9.1 \pm 0.6$  and  $9.4 \pm 1.6$  mg/L respectively). Increased uptake of ascorbate by the immune cells for oxidation may cause a subsequent decrease of the plasma concentrations during acute inflammatory conditions caused by *E. coli* mastitis [31]. RANJAN *et al.*, [25] have observed that plasma vitamin C concentrations were significantly lower in cows with subclinical and clinical mastitis compared to controls and they have stated that this condition might be existed due to over utilization or sequestration of this antioxidant to neutralize the overproduction of reactive oxygen species during inflammatory conditions of the udder. KLECZKOWSKI *et al.* [18] have also observed a marked decrease of Vitamin C concentrations in subclinical mastitis cows compared to healthy cows. In the present study, pre-treatment values were higher in healthy cows but did not differ significantly from cows with subclinical mastitis. This non-significance might be a result of a high vitamin C dietary supply in the herd. However, in "cured" cows, significant increases of plasma vitamin C concentrations according to time can be observed, and on Day 21 both control and cured cows exhibited significantly higher ascorbate concentrations than "not cured" cows. These results are similar to the results of above studies and showed that the efficient therapy of subclinical mastitis cases induced increases of plasma vitamin C concentrations.

The relationships between blood prostaglandins, vitamin E, Selenium and glutathione peroxidase (GSH-Px) activity in cows with mastitis were deeply studied by ATROSHI *et al.* [2, 3]. Blood PGF<sub>2</sub> $\alpha$  concentrations were negatively correlated with GSH-Px activity in mastitis affected cows [2]. They have also demonstrated that both plasma and milk vitamin E concentrations in cows with mastitis were lowered, and that blood vitamin E concentrations were positively correlated

with erythrocyte GSH contents [3]. Similarly, blood Se concentrations and GSHPx activity were higher in cows with low somatic cell counts than in cows with high somatic cell counts [12]. It has been stated that GSH and Se may be involved in protecting tissues from oxidative damage and also involved in phagocytosis by leukocytes [12]. Especially, immune resistance against major mastitis pathogens like *S. aureus* has been reported to be regulated by GSHPx activity. In this study, before antibiotic treatment, plasma GSH concentrations were significantly higher in control cows than in infected cows. Following treatment, they significantly increased in "cured" cows while in the "uncured" cows no difference with pre-treatment values was observed. These findings are in accordance with the above studies. When the intramammary antibiotic preparation used according to the antibiotic susceptibility tests was efficient against major mastitis pathogens, consumption of GSH as major cytoplasm antioxidant was markedly limited and GSH was successfully regenerated from the oxidized GSSG form, leading to enhancement of blood concentrations. By contrast, the persistence of pathogens in the udder maintained local oxidative burst and massive utilization of antioxidants such as GSH.

It has been observed that lower plasma vitamin A and  $\beta$ -carotene concentrations were associated with higher CMT scores and that carotenoid and retinol deficiency in postpartum period lead to higher somatic cell counts during subsequent lactation period in dairy cows [9, 15]. ERSKINE *et al.* [12] have reported a significant decrease of plasma vitamin E concentrations in cows with high somatic cell counts while vitamin A and  $\beta$ -carotene concentrations remained unaltered. Moreover, a vitamin A and  $\beta$ -carotene supplementation during dry period did not significantly avoid the occurrence of new intramammary infections [24]. JUKOLA *et al.* [16] found that vitamin A and somatic cell counts had a minimal relationship and stated that combined effects of antioxidants were enough to compensate a possible deficiency of a single antioxidant. In the present study, vitamin A and  $\beta$ -carotene concentrations in all cows were within physiological limits [1, 20]. Post-treatment mean concentrations of  $\beta$ -carotene and vitamin A were increased in all cows although they remained always weakly depressed in the "not cured" group before and after intramammary therapy. In agreement with previous reports [12, 16, 24], these results suggest that carotenoid and retinol would probably weakly and/or belatedly consumed during oxidative conditions and consequently, that these 2 parameters are not sensitive markers of antioxidant status alterations. The increase of vitamin A concentrations according to time was significant in the "cured" cows, but, because a significant time effect was also observed in healthy controls, these variations were probably not only due to the treatment effect but other factors such as lactation status, feeding or management.

In the acute form of clinical mastitis, ceruloplasmin concentrations together with haptoglobin and alpha 1 antitrypsin were found higher than in normal cows [10]. CHASSAGNE *et al.* [8] have reported a significant increase of ceruloplasmin concentrations in cows with higher early lactation mastitis incidence than in cows with lower early lactation mastitis incidence, in the late stages of previous lactation. The authors' opinion is that high vitamin C and normal vitamin A

and  $\beta$ -carotene concentrations in all cows might be sufficient to minimize the effects of oxidative stress resulting from subclinical mastitis. In the present study, although any inter-group significant difference was observed, serum ceruloplasmin concentrations appeared to be slightly increased in mastitis affected cows, not successfully cured by antibiotics on Days 14 and 21.

RANJAN *et al.* [25] have reported that MDA concentrations in cows with clinical mastitis were significantly higher than in healthy cows while this significance could not be observed between subclinical mastitis and healthy cows. Results of the present study are in accordance with the above research as MDA concentrations in the control, cured and uncured cows were found similar to each other, albeit they tended to remain more elevated in the "not cured" cows.

Negative correlations were observed in the control group on Day 0, between MDA concentrations and GSH, vitamins C and A. This result could be expected as MDA is a marker of inflammation and blood antioxidant reserves are higher in healthy animals. JUKOLA *et al.* [16] have stated that the correlations between the blood antioxidant concentrations studied and the udder health was low. The antioxidant status of animals depends on the total antioxidant reserves when the infection and inflammation starts. Consequently, the consumption of one antioxidant may possibly affect the concentrations of the others. In the present study positive correlations were also observed between vitamin A and  $\beta$ -carotene or GSH concentrations and between vitamins A and C in the various groups for a given time; however these correlations were not markedly pronounced in all groups for all times.

In conclusion, the results of the present study indicate that primarily vitamin C and GSH concentrations are increased in cows with subclinical mastitis following microbiological cure obtained by intramammary antibiotic therapy. Vitamin A and ceruloplasmin might also be involved in this mechanism however more intensive research concerning other perturbations of the health status could be appropriate to elucidate the effects of these compounds.

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