Effects of dehorning by amputation on oxidant-antioxidant status in mature cattle

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SUMMARY

Dehorning of cattle is a routine husbandry practice which improves the safety of stock handlers and reduces injury, bruising of carcasses and damage to hides, but methods of dehorning are probably painful and stressful. The aim of the study was to evaluate the stress response and the oxidant/antioxidant equilibrium against dehorning process by amputation performed under local anaesthesia in mature cattle. Blood malondialdehyde (MDA) and glutathione (GSH) concentrations, as well as plasma total antioxidant activity (AOA) and cortisol concentrations were measured in 6 female Holstein cattle one hour before dehorning procedure and immediately after. Circulating MDA, NO and cortisol concentrations were dramatically increased compared to initial values after dehorning whereas AOA and GSH concentrations were significantly depressed. Glucose concentrations significantly increased after dehorning, while the plasma cholesterol concentrations as well as plasma total antioxidant activity (AOA) were measured in 6 female Holstein cattle one hour before dehorning procedure and immediately after. Circulating MDA, NO and cortisol concentrations were dramatically increased compared to initial values after dehorning whereas AOA and GSH concentrations were significantly depressed. Glucose concentrations significantly increased after dehorning, while the plasma cholesterol concentrations significantly decreased. These results demonstrate that MDA, NO, AOA and GSH are the most powerful markers for evaluating the oxidant/antioxidant status in mature cattle and that dehorning was a stressful situation leading to an oxidative stress which can be amplified by strong glucocorticoid secretion.

Keywords: Dehorning, cattle, oxidative stress, lipid per-oxidation, anti-oxidant activity, glutathione, cortisol.

Introduction

Since animal welfare aims a life away from undesired feelings like pain, suffering and stress, absence of stress response is accepted as an indicator for welfare mood in animals [3]. Welfare status of an animal depends primarily on how the animal “feels”, and claims that an animal’s welfare is compromised only to the extent that the animal suffers [18]. Stress has usually been conceived as a reflex reaction that occurs ineluctably when animals are exposed to adverse environmental conditions, and which is the cause of many unfavourable consequences, ranging from discomfort to death [15].

Dehorning is removal of the horns after they have formed from the horn bud [4] and it is a common management practice in the dairy industry. Cattle that are dehorned represent less danger of injury to the farmer and other cows as well as less disruption by boss cows during feeding [33]. On the other hand, dehorning is a painful procedure [29]. The well-being of calves undergoing the dehorning procedure has been of great concern. Recognition and assessment of acute stress and pain following procedures such as dehorning is difficult, although a combination of physiological and behavioural measures has been recommended to more accurately evaluate the animal’s reaction. Dehorning, regardless of the method used, has been shown to affect the animal’s physiology (e.g., increased heart rate and plasma cortisol concentration) and behaviour (e.g., greater frequency of head shaking and rubbing) in a manner typical of an acute stress response [16, 19, 27].

Overwhelming evidences indicate that oxidative stress can lead to cell and tissue injury. However, the same free radicals that are generated during oxidative stress are produced during normal metabolism and thus are involved in both health and disease [45]. A wide variety of reactive oxygen species (ROS) are produced in the course of the normal metabolism in biological systems and they have several important physiological functions, but their accumulation beyond the needs of the cell can potentially damage lipids, proteins, and nucleic acids. The cells possess an intricate net-
work of defence mechanisms to neutralize excessive ROS accumulation, including antioxidant compounds and antioxidant enzymes therefore, under physiological conditions, cells are able to cope with the flux of ROS. Oxidative stress describes a condition in which cellular antioxidant defences are insufficient to keep the levels of ROS below a toxic threshold [38, 53]. The oxidative damage is a crucial aetiological factor implicated in several chronic diseases such as cancer, atherosclerosis, arthritis, neurodegenerative diseases and also in the ageing process [20, 22].

Animal health and growth performance are affected by many factors such as diet, stress, antibiotics and husbandry practices [20]. Since a measure for welfare of the animals has not yet been determined, using the oxidative stress parameters could be a new approach in determining the stress. However there was no published article investigating the effects of dehorning procedures on animal welfare using biomarkers of oxidative stress in mature cattle. The aim of the present study is to evaluate the effect of dehorning by amputation on oxidant-antioxidant status in mature cattle.

Materials and Methods

CHEMICALS

Vanadium (III) chloride (VCl3), NaC1, EDTA Na2, NaOH, thiobarbituric acid (TBA), trichloroacetic acid (TCA), HCl, sulphanilamide (SULF), and N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD) were obtained from Merck (Darmstadt, Germany). Other chemicals used in the study were purchased from Sigma-Aldrich Chemical.

ANIMALS AND PROTOCOL DESIGN

The study was conducted on six female, 3-4 years old, Holstein cattle in the Afyonkarahisar region. All animals were carefully monitored and the study protocol was carried out in accordance with the Helsinki Declaration as revised in 1989. Prior approval to conduct this study was obtained from the Afyon Kocatepe University Animal Ethics Committee. Dehorning was applied to the experimental group including 6 cattle under local anaesthesia. For local anaesthesia a 2% lidocain solution (5 mL) (Vilcain 2%, Vilsan, Turkey) was injected in the corneal nerve of each horn. Fifteen minutes later a skin-prick test was used to test the efficacy of anaesthesia. If there was a response to this stimulus, another 5 mL of local anaesthetic were injected near the corresponding corneal nerve. Thereafter cattle were dehorned as described above. The dehorning procedure was performed in a closed area using the Gigli wire saw. The animals in the experimental group were only placed in the operation stocks. The base of the horn was prepared for the operation. The Gigli wire saw was placed on the base of the horn and the amputation was performed. Haemostasis was achieved by applying thermal cautery to vessels. The procedure was repeated for the opposite horn. Peri-operative antibiotics or anti-inflammatory drugs were not administered. Bandages were not used.

The concentrations of MDA, considered as an index of lipid peroxidation, were measured by the double heating method of DRAPER and HADLEY [17]. The method is based on spectrophotometric measurement of the purple color generated by the reaction of TBA (thiobarbituric acid) with MDA. For this purpose, 2.5 mL of trichloroacetic acid solution (10%, w/v) was added to whole blood (0.5 mL) in each centrifuge tube; the tubes were then placed in a boiling water bath for 15 min. After cooling to room temperature, the tubes were centrifuged at 1000g for 10 min and 2 mL of each supernatant was transferred to a test tube containing 1 mL of TBA solution (0.67%, w/v). Each tube was then placed in a boiling water bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm by using the Shimadzu UV 1601 spectrophotometer. The concentration of MDA was calculated based on the absorbance coefficient of the TBA–MDA complex (ε: 1.56.105 cm/M).

Determination of MDA concentrations

Plasma haemoglobin concentration was estimated by a modified method, based on the cyanmet-haemoglobin method described by BAURE [5]. Haematocrit was determined by the micro-method of STRUMIA et al. [55] using a micro-haematocrit centrifuge and a reader.

Plasma nitric oxide concentrations

Nitric oxide decomposes rapidly in aerated solutions to form stable nitrite/nitrurate products (NOX). Plasma nitrite/nitrate concentration was measured by a modified method of Griess assay, described by MIRANDA et al. [40]. The principle of this assay is reduction of nitrate by vanadium combined with detection by the acidic Griess reaction. Briefly, samples were deproteinized prior to assay. The serum was added to 96% cold ethanol at 1:2 (v/v) and then vortexed for 5 min. After incubating for 30 min at 4°C, the mixture was

centrifuged at 8000g for 5 min and the supernatants were used for the Griess assay. Analysis was done in a micro-titre plate. One hundred microliters of filtrated plasma was mixed with 100 µL of VC13 and was rapidly followed by the addition of the Griess reagents, which contain Sulphanilamide (50 µL) and N-(1-naphthyl) ethylenediamine dihydrochloride (50 µL). The determination was performed at 37°C for 30 min. The absorbance was measured by a microplate reader (Multiskan Spectrum, Thermo Labsystems, Finland) at 540 nm. Nitrite/nitrate concentration was calculated using a NaNO2 standard curve and expressed as µmol/L.

Plasma total Antioxidant Activity (AOA)

The total AOA was determined using the method described by KORACEVIC et al. [32]. The assay measures the capacity of the serum to inhibit the production of TBA reactive substances (TBARS) from sodium benzoate, under the influence of the reactive oxygen free radicals derived from the Fenton’s reaction. The reaction was measured spectrophotometrically at 532 nm. Antioxidants from the added sample cause suppression of the production of TBARS, and the inhibition of the colour development is defined as AOA. A solution of 1 mmol/L uric acid was used as standard.

Blood reduced glutathione concentrations

The blood GSH concentration was measured as described by BEUTLER et al. [6]. Briefly, after haemolysis in distilled water (0.2 mL of blood samples in 1.8 mL of distilled water), the precipitating solution (3 mL) (1.67% w/v meta-phosphoric acid, 0.2% EDTA, 30% NaCl) was added and the mixture was allowed to stand for approximately 5 min and then filtered (Whatman No. 42). Thereafter, a 0.3M disodium hydrogen phosphate solution (8 mL) and 5, 5-Dihioibis, 2-Nitrobenzoic Acid (DTNB) (1 mL) were added to the filtrate (2 mL) and the absorbance was measured at 412 nm in the Shimadzu UV 1601 spectrophotometer. The blank reactant was prepared by a microplate reader (Multiskan Spectrum, Thermo Labsystems, Finland) at 540 nm. Nitrite/nitrate concentration was calculated using a NaNO2 standard curve and expressed as µmol/L.

Plasma cortisol concentrations

Plasma cortisol concentrations were determined using an ELISA kit (Eucardio Laboratory, Inc., Encinitas, CA. 92024, USA).

Plasma glucose and total cholesterol concentrations

Plasma glucose concentrations were estimated by the glucose oxidase/peroxidase method using the commercially available kit (n° G520-480, Teco Diagnostic, USA) and plasma total cholesterol concentrations with the specific assay kit (n° CT F400 CH, Chema Diagnostica, Italy).

STATISTICAL ANALYSIS

All data were presented as mean ± Standard Error (SE) for parametric variables. The comparisons of parameters were performed with the Student’s t-test. Data were analyzed using the SPSS® for Windows computing program (Version 10.0), and P < 0.05 was considered statistically significant [54].

Results

As reported in Table I, the rectal temperature, heart and respiratory rates as well as the haematocrit values were slightly but significantly increased just after the dehorning procedure compared to the initial values (P < 0.05) whereas the haemoglobin concentrations have not significantly differed.

The variations of the biochemical markers during the dehorning procedure are summarized in Table II. While the plasma cholesterol concentrations have significantly decreased after dehorning (P < 0.05), glycaemia and plasma cortisol concentrations have significantly and markedly increased (P < 0.05); the variation factors were +19.08 ± 2.16% and +33.16 ± 0.17%, respectively. Moreover, a remarkable and significant increase of the circulating MDA concentrations compared to the initial values (P < 0.05, factor of variation: 37.51 ± 10.91%) was observed after dehorning. In parallel, the nitric oxide concentrations were also significantly increased during the dehorning procedure by amputation (P < 0.05) but the variation intensity remained weak (variation factor: +2.60 ± 0.02%). On the other hand, the blood GSH concentrations and the total plasma antioxidant activity were significantly depressed by a factor of 11.29 ± 2.72% and 24.89 ± 1.10% respectively.

Discussion

Dehorning of cattle is a routine husbandry practice which improves the safety of stock handlers and reduces injury, bruising of carcasses and damage to hides. Caustic chemicals or cautery are used to disbud younger calves and amputation is used to dehorn older animals. All methods of dehorning are probably painful and stressful [57]. Stress has been defined as the cumulative response of an animal resulting from interaction with its environment via receptors [23]. Biological consequences of stress have an adaptive purpose, intending to keep homeostasis balanced [14, 43]. However, they may lead to the development of a pre-pathological state and, eventually, to a pathological state. The pre-pathological state has been proposed to be clinically defined by changes in biological functions without obvious negative consequences for lifespan [41]. The stress-induced impairment of biological functions can compromise animal well-being, health and life [30, 41]. Some hormones are involved for modifications of biological functions [25]. In this way, it has been reported that stressful conditions would stimulate the hypothalamic-hypophysal-adrenal axis [3] leading to dramatically increasing in the ACTH and cortisol secretions by at least 10 times. Corticosteroids are steroid hormones produced by the adrenal
cortex and cortisol is the main corticosteroid in ungulates and has been traditionally considered as a good stress indicator [43]. In normal conditions, only 10% of blood cortisol is in the free form, which is the active form. At body temperature, 90% of plasma cortisol is bound to proteins whose 70% to the corticosteroid binding globulin (CBG) and albumin [42]. However, Rijnberk and Mol [50] reported that during the stress response, the free cortisol concentrations can increase up to 20-30%. Several studies have demonstrated that plasma cortisol concentrations increase in cattle husbandry practices [13, 51] including cautery disbudding [33, 48] and scoop amputation dehorning [37]. In agreement with that, marked elevations of plasma cortisol concentrations after the dehorning procedure were recorded in the present study, suggesting that dehorning by itself would be a stressful condition, although it was performed under local anaesthesia.

In the same way, the physiological parameters of rectal temperature, heart and respiratory rates are known to be the most relevant on-the-spot diagnostic parameters of the state of an animal’s health. These parameters are of importance in evaluating the adaptability of domestic animals to various environmental stress factors [39]. In study on donkeys during road transportation, it was observed that the heart and respiratory rates increased during the first half of the journey time [49]. In the present study, these physiological parameters were significantly increased after the dehorning procedure.

In ruminants, plasma glucose comes approximately at 44% from organic acid absorption from the rumen (predominantly propionate) and subsequent conversion to glucose in the liver, at 33% from post-ruminal glucose absorption, and at 23% from other carbon sources such as amino acids and subsequent conversion to glucose in the liver. Catecholamines and glucocorticoids increase the glycogenolysis and the gluconeogenesis [20, 31] and secondary the glycaemia. In this study, dehorning procedure increased plasma glucose concentration probably because of the increase of the cortisol secretion. These results confirmed previous studies conducted in stressed animals (gazelle, sheep and deer) [8, 24, 36]. Cholesterol is synthesized mainly in the liver but its concentrations also depend on the diet supply. The lipolytic glucocorticoids stimulate fat mobilization from adipose tissue and increase the circulating concentrations of free fatty acids [26, 28]. Thus, an increase of plasma cholesterol concentrations would be expected after a stress episode and some authors have found such an increase [36, 43]. However, as cholesterol is also involved in corticoid synthesis, plasma cholesterol concentrations would also be reduced during stress [47]. As both increases and decreases of serum cholesterol and triglyceride concentrations have been related to stress [2, 36], plasma cholesterol concentrations significantly decreased, in the present study, after the dehorning process.

Handling stress has been observed to alter numerous blood cell components. The first stage of the stress response is the

### Table I: Rectal temperature, heart and respiratory rates and haematological parameters (haemoglobinemia and haematocrit) measured in female Holstein cattle (n = 6) during the dehorning procedure. Results are expressed as means ± standard errors.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before dehorning</th>
<th>After dehorning</th>
<th>Variation factor (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal temperature (°C)</td>
<td>38.41 ± 0.09</td>
<td>38.7 ± 0.09*</td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats / min.)</td>
<td>69.00 ± 2.44</td>
<td>81.33 ± 5.15*</td>
<td></td>
</tr>
<tr>
<td>Respiratory rate (cycles / min.)</td>
<td>19.00 ± 0.73</td>
<td>23.33 ± 1.28*</td>
<td></td>
</tr>
<tr>
<td>Haemoglobinemia (g/L)</td>
<td>142.0 ± 5.6</td>
<td>145.7 ± 9.2</td>
<td></td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>34.83 ± 0.48</td>
<td>37.83 ± 0.79*</td>
<td></td>
</tr>
</tbody>
</table>

*: P < 0.05.

### Table II: Biochemical parameters and oxidant / antioxidant status in female Holstein cattle (n = 6) during the dehorning procedure. Results are expressed as means ± standard errors.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before dehorning</th>
<th>After dehorning</th>
<th>Variation factor (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.37 ± 0.18</td>
<td>5.20 ± 0.12*</td>
<td>+19.08 ± 2.16</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.69 ± 0.30</td>
<td>5.02 ± 0.36*</td>
<td>-11.86 ± 1.68</td>
</tr>
<tr>
<td>Cortisol (nmol/L)</td>
<td>5.24 ± 0.19</td>
<td>6.98 ± 0.25*</td>
<td>+33.16 ± 0.17</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>2.83 ± 0.29</td>
<td>3.86 ± 0.09*</td>
<td>+37.51 ± 10.91</td>
</tr>
<tr>
<td>NOx (μmol/L)</td>
<td>9.63 ± 0.07</td>
<td>9.88 ± 0.07*</td>
<td>+2.60 ± 0.02</td>
</tr>
<tr>
<td>GSH (g/L)</td>
<td>34.17 ± 2.13</td>
<td>30.37 ± 2.82*</td>
<td>-11.29 ± 2.72</td>
</tr>
<tr>
<td>AOA (mmol/L)</td>
<td>4.31 ± 0.27</td>
<td>3.24 ± 0.25*</td>
<td>-24.89 ± 1.10</td>
</tr>
</tbody>
</table>

*: P < 0.05.

The variation factors were given by the following formula: variation factor (%) = 100 [(Ca – Cb) / Cb] where Ca and Cb were the concentrations observed after dehorning and before dehorning, respectively.
activation of the sympathetic nervous system, stimulating the adrenal medulla and releasing catecholamines. Increases in haemoglobin concentration and packed cell volume are often associated with the spleen contraction caused by the action of catecholamines on α-adrenergic receptors located in the capsule [3, 25]. In the present study, although the haemoglobinemia was not significantly modified, the dehorning procedure has induced strong elevations of haematocrit values.

Among stress-induced negative consequences, the oxidative stress characterized by the accumulation of radical oxygen species (ROS) can affect life and metabolic efficiency [3, 11, 46]. Actually ROS are produced as normal products of the cellular metabolism. However, excessive generation of free radicals can occur due to endogenous biological or exogenous environmental factors, such as exposure to radiation, pollution or chemical substances [20, 21]. When ROS production and various antioxidant systems are imbalanced [34], cellular injury and tissue damage occur, leading to alterations of macromolecules (membrane lipids, proteins and DNA), changes in intracellular calcium and intracellular pH, and finally to cell death [20, 22], mainly throughout the free radical-mediated lipid attack called lipid peroxidation (LP). Since membrane phospholipids are the major targets of oxidative damage, LP is often the first parameter analyzed for proving the involvement of free radical damage and the plasma MDA concentrations have been reported to directly correlate with the damage severity [1]. Although there is no report available on oxidative stress during dehorning procedure, it has been shown that some other management procedures cause oxidative stress. FIDAN et al. [20] reported that the shearing procedure has induced strong elevations of blood MDA concentrations, indicating the occurrence of an oxidative stress in sheep. CALAMARI et al. [9] found an increase of plasma TBARS and a decrease of plasma lipid soluble antioxidants in moderately heat stressed, mild-lactating cows during summer. AVCI et al. [3] suggested that transport might play an important role in oxidative stress by reducing GSH and increasing MDA concentrations in sheep. In the present study, the dehorning procedure has induced strong elevations of blood MDA concentrations indicating the occurrence of an oxidative stress. This result could be explained by the occurrence of free radicals due to the stress factors during the dehorning process.

The antioxidant defence systems include small molecular antioxidants, antioxidant enzymes, and metal chelating agents. Initially, endogenous antioxidant enzymes are induced for removing the continuously generated free radicals but later, the enzyme depletion occurs with oxidative cell damage [58]. The total AOA of body fluids suggests a simultaneous interaction between various antioxidants and is crucial for the maximum suppression of a free radical reaction in extra-cellular compartments [12]. Whereas enzyme activity appears to indicate the antioxidant characteristics of only one antioxidant, total antioxidant activity represents the aggregate antioxidant characteristics of all antioxidants found in the plasma. In addition to AOA, reduced GSH and its metabolizing enzymes constitute a major defence system against ROS-induced cellular damage [10]. GSH serves as a potent cytoplasm reducing agent [35] and is oxidized into GSSG. Thereby, a decrease of the GSH concentrations may reflect depletion of the antioxidant reserve: in this way, a high erythrocyte TBARS content coupled to low erythrocyte GSH concentrations and low GPx (glutathione peroxidase) activity, was evident in rats exposed to a hot stress [56]. Various related functions may consequently be impaired such as the diminution of the reducing and detoxification capacities, of the protein biosynthesis and the immune function, and the accumulation of lipid peroxidation products [20, 21]. Moreover, high glucocorticoid concentrations have been reported to decrease blood glutathione concentrations and erythrocyte SOD activity in rats [44]. Coupled to the MDA accumulation in blood, the circulating GSH concentrations were deeply depressed in cattle after dehorning in the present study. This observation confirms the existence of LP phenomena during dehorning and the GSH depletion observed would aggravate the intensity of the oxidative injury. In parallel, the increased cortisol secretion evidenced here would also contribute to the GSH deficiency. In this study, the increase of plasma cortisol in response to the dehorning stress could be an additional factor responsible for amplifying the oxidative stress reflected by increased MDA concentrations and decreased plasma AOA and GSH content in erythrocytes.

The role of nitric oxide (NO) appears controversial because a tissue dysfunction or injury could occur after inhibition of NO. However, high NO production has been suggested as a cause of tissue injury [7]. Stimulation of tissue NO production is also associated with adverse events such as hypotension, inhibition of intermediary metabolism, and the production of the potent oxidant peroxynitrite following radical–radical reaction with superoxide [52]. The bioavailability of NO is reduced with the increased content of superoxide radical, which transforms NO to peroxynitrite [59]. In this way, plasma NO concentrations were significantly increased after the dehorning procedure in the present study.

Although this is a small study, the results show that the dehorning procedure, although it was performed under local anaesthesia, will be considered as a stressful condition as evidenced by the strong increases in circulating cortisol, MDA and NO concentrations associated with decreases in AOA and GSH concentrations. Although the other investigated physiological, haematological and biochemical parameters were also significantly affected by the dehorning procedure by amputation except for haemoglobinemia, they are not considered as specific markers of stress. Furthermore, the increase of plasma cortisol concentrations in response to dehorning could be an additional factor responsible for the oxidative stress amplification. A more complete identification of the physiological changes during dehorning could be beneficial for further researches in terms of correct management practices within cattle industry. Nevertheless, the determination of the oxidative stress parameters could be a new approach for evaluating stress in cattle and antioxidant treatment could be proposed for alleviating the dehorning stress.

References


