**Effects of shearing procedures on oxidant-antioxidant status in Chios sheep**

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SUMMARY

The aim of the study was to evaluate the stress response and the oxidant / antioxidant equilibrium against shearing process in sheep. Blood malondialdehyde (MDA) and glutathione (GSH) concentrations, plasma cortisol, glucose and cholesterol concentrations as well as plasma total antioxidant activity (AOA) were measured in 18 female Chios sheep one hour before a traditional shearing procedure and immediately after. Circulating MDA and cortisol concentrations were dramatically increased compared to initial values after shearing whereas GSH concentrations were significantly depressed. The variations of the other biochemical parameters were not significant. These results demonstrate that MDA and GSH are the most powerful markers for evaluating the oxidant / antioxidant status and that shearing was a stressful situation leading to an oxidative stress which can be amplified by strong glucocorticoid secretion.

Keywords: Shearing, sheep, oxidative stress, malondialdehyde, glutathione, cortisol.

RÉSUMÉ

Effets de la tonte sur des paramètres de stress oxydatif chez le mouton de race Chios

Le but de cette étude a été d’évaluer l’existence d’un stress et l’équilibre oxydants / antioxydants lors de la tonte chez le mouton. Les concentrations sanguines en malondialdéhyde (MDA) et en glutathion (GSH), la cortisolemie, la cholestérolémie et la glycémie ainsi que l’activité plasmatique totale anti-oxydante ont été mesurées 1 heure avant une tonte traditionnelle et immédiatement après chez 18 brebis Chios. Les concentrations circulantes en MDA et en cortisol ont été considérablement augmentées par rapport aux valeurs initiales à l’issue de la tonte tandis que celles en GSH ont diminué de façon significative. Les variations des autres paramètres biochimiques n’ont pas été significatives. Ces résultats démontrent d’une part que le MDA et le GSH sont les 2 marqueurs les plus pertinents pour évaluer le stress oxydant / antioxydant et d’autre part que la tonte est une situation stressante conduisant à un stress oxydatif qui peut être amplifié par une forte sécrétion de glucocorticoïdes.

Mots clés : Tonte, mouton, stress oxydatif, malondialdéhyde, glutathion, 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 [23]. 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 [19].

Shearing sheep contributes directly to the welfare of both animal and the owner. Shearing is necessary to enhance the physical welfare of the animal, as domestic sheep do not shed their wool naturally [32], but can negatively affect the welfare of the animal if performed in an inappropriate way and time. Shearing can result in thermal stress when animals are exposed to wet conditions, severe cold, or intense sunshine coupled with high temperatures. Failure to shear ewes before confinement for lambing, even in the winter, may result in moisture and health problems in the barn. Shearing process causes stress on the animal, because circulating corticoid concentrations increase [13] regardless of the method used, and noise, heat, and contact of the clippers induce this reaction. In addition, the traditional method of up-ending sheep for shearing (resting on rump in upright position) contributes to stress [36, 63]. Some shearers restrain sheep by binding their legs, a procedure which is stressful in itself [17], and may result in injuries, but a comparison of the overall stressfulness of this method with up-ending has not been made. Shearing is less stressful if done quickly [44], bearing in mind cuts resulting from hurried or careless shearing add to stress [35].

The physiological condition of the organism is affected by internal and external factors and a physiological and behavioural response occurs towards these stimuli. Under the stress provoking conditions, many physiological and biochemical changes such as activity of the sympathetic nervous system, increase of the glycaemia based on the hormonal changes occur in the body [3]. According to the reported studies on different species, stress causes a significant increase of the heart rate [43], of the adrenal cortical activity [62] and
infectious related mortality and morbidity. Moreover, it is indicated that stress increases the oxidative stress which affects the survival and the metabolism efficiency [3, 15, 58]. 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 [57]. Under normal circumstances, the generated reactive oxygen species (ROS) are detoxified by the antioxidants present in the body and the generated ROS and the present antioxidants are in equilibrium. However, owing to ROS overproduction or inadequate antioxidant defence, this equilibrium is hampered favouring the ROS upsurge that culminates in oxidative stress. The ROS readily attack and induce oxidative damage to various bio-molecules including proteins, lipids, lipoproteins and DNA [25, 42]. 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 [46].

The objective of this study was to determine the stress response of Chios sheep during shearing process as well as to evaluate the consequences on the oxidant-antioxidant status.

Materials and Methods

CHEMICALS

The chemicals used in the study were purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Co. St. Louis, MO, USA).

ANIMALS AND PROTOCOL DESIGN

The study was conducted on 18 female, 18-24 months old, Chios sheep 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. The shearing procedure was performed in a closed area using traditional method with shearsers. Sheep were fasted and penned in a clean area before shearing. Any faeces or other debris that might be present in the coat was initially removed. The sheep were shorn using mechanical hand pieces. The wool was removed beginning by removing the sheep's belly wool. After setting the sheep on its rump, the belly was first shorn. The wool was removed starting from the wool-free area inside the sheep’s right hind leg to the wool-free area just beside the sheep’s right fore leg. In the same direction, all the wool from the sheep’s right hind leg to the wool-free area was removed. The wool was removed starting from the wool-free area inside the left hind leg, the blows started near the crotch and proceed out to the hoof. Once the entire fleece has been removed from the sheep, the fleece was thrown, clean side down, on to a wool table by a shed hand.

Heart and respiratory rates were recorded during the shearing procedure. Blood samples were taken from each animal, an hour before and just after the shearing procedure, by puncture of the jugular vein into heparinized tubes for measuring malondialdehyde (MDA), reduced glutathione (GSH), glucose, total cholesterol and plasma cortisol concentrations as well as the plasma total antioxidant activity (AOA). The circulating MDA and GSH concentrations were measured from whole blood (2 mL) whereas the other biochemical parameters were determined on plasma obtained after blood centrifugation (1 000g, 10 min at room temperature) and stored at -30°C.

BIOCHEMICAL ANALYSES

Blood malondialdehyde (MDA) concentration

The circulating MDA concentration, an index of lipid peroxidation, was measured by the double heating method of DRAPER and HADLEY [22]. The method is based on the spectrophotometric measurement of the purple colour generated by the reaction of thiobarbituric acid (TBA) with MDA. Briefly, 2.5 mL of a trichloroacetic acid solution (10% w/v) was added to the whole blood (0.5 mL) and the mixture was placed in a boiling water bath for 15 min. After cooling to room temperature and centrifugation (1000 g for 10 min at 4°C), the supernatant fraction (2mL) was transferred to a test tube containing 1 mL of the TBA solution (0.67%, w/v). Each tube was again placed in a boiling water bath for 15 min, cooled to room temperature, and finally the absorbance was measured at 532 nm using a Shimadzu UV 1601 spectrophotometer. The MDA concentration was calculated based on the absorbance coefficient of the TBA-MDA complex (ε =1.56.105 cm⁻¹.M⁻¹).

Blood reduced glutathione (GSH) concentration

The blood GSH concentration was measured as described by BEUTLER et al. [5]. 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-Dithiobis, 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 with the phosphate solution (8 mL), the 3/5 diluted precipitating solution (2 mL) and DNTB (1 mL) and a GSH standard solution (0.4 g/L) was used as a reference.

Plasma total Antioxidant Activity (AOA)

The total AOA was determined using the method described by KORACEVIC et al. [45]. 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 352 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.

**Plasma Cortisol concentration**

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 [65].

**Results**

As reported in Table I, the heart and respiratory rates were slightly increased during the shearing procedure compared to the initial values.

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Before shearing</th>
<th>After shearing</th>
</tr>
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<tbody>
<tr>
<td>MDA (µmol/L)</td>
<td>2.98 ± 0.21*</td>
<td>6.43 ± 0.21*</td>
</tr>
<tr>
<td>GSH (g/L)</td>
<td>188.2 ± 11.3*</td>
<td>110.4 ± 4.3*</td>
</tr>
<tr>
<td>AOA (mmol/L)</td>
<td>4.63 ± 0.20</td>
<td>4.93 ± 0.28</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>3.98 ± 0.18</td>
<td>3.87 ± 0.48</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>3.36 ± 0.21</td>
<td>3.74 ± 0.33</td>
</tr>
<tr>
<td>Cortisol (mmol/L)</td>
<td>518.1 ± 38.1*</td>
<td>757.9 ± 67.9*</td>
</tr>
</tbody>
</table>

**TABLEAU III : Factors of variations (%) of the biochemical parameters (blood MDA and GSH concentrations, plasma cortisol, glucose and cholesterol concentrations and plasma total antioxidant activity) investigated after a traditional shearing in female Chios sheep (n = 18).**

**Discussion**

Stress has been defined as the cumulative response of an animal resulting from interaction with its environment via receptors [27]. Biological consequences of stress have an adaptive purpose, intending to keep homeostasis balanced [18, 55]. 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 change in biological functions without obvious negative consequences for lifespan [52]. The stress-induced impairment of biological functions can compromise animal well-being, health and life [39, 52]. Some hormones are involved for modifications of biological functions [30]. In this way, it has been reported that stressful conditions would stimulate the hypothalamo-hypophysal-adrenal axis [3] leading to dramatically increasing 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 [55]. However, various factors such as the great inter-individual variability, the existence of circadian, ultra-circadian and seasonal secretory patterns and the effects of the sampling method may affect serum cortisol concentrations [38, 53, 67].

Because of this great inter-individual variability and the difficulty to relate corticoid variations and animal welfare,
some authors have considered cortisol as a poor welfare indicator [52, 64]. 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 [54]. However, Rijnberk and Mol [61] reported that during stress response free cortisol concentrations can increase up to 20-30%. Nevertheless, several studies have demonstrated that plasma cortisol concentrations increase in sheep in response to a stressor [4, 12, 51], like shearing process [36, 50, 60]. In agreement with that, marked elevations of plasma cortisol concentrations after the shearing procedure were recorded in the present study, suggesting that shearing by itself would be a stressful condition.

Among stress-induced negative consequences, the oxidative stress characterized by the accumulation of radical oxygen species (ROS) can affect life and metabolic efficiency [3, 15, 58]. Nowadays the interest of ROS in biology and medicine has been increased because of their strong relationship with aging and disease processes [11]. 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 [25]. For example, pesticides have been reported to cause alteration in antioxidants or free radical scavenging systems [54]. When ROS production and various antioxidant systems are imbalanced [47], 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 [21, 26]. The lipid attack mediated by free radicals, named as lipid peroxidation (LP), is a complicated radical chain reaction leading to the formation of various products including lipid hydroperoxides, conjugated dienes and thiobarbituric acid-reactive substances (TBARS) such as malondialdehyde (MDA) which can be measured and used as markers for LP [20, 24, 41].

Since membrane phospholipids are the major targets of oxidative damage, lipid peroxidation is often the first parameter analysed for proving the involvement of free radical damage and the plasma MDA concentrations have been reported to directly correlate with the severity of stress [1]. Although there is no report available on oxidative stress and shearing procedure, it has been shown that some other management procedures cause oxidative stress. CALAMARI et al. [10] 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, shearing procedure has induced strong elevations of blood MDA concentrations (they have been multiplied by a factor 2.15), 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 shearing process.

On the other hand, the antioxidant defence systems include small molecular antioxidants, antioxidant enzymes (like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR)) and metal chelating agents. HALLIWELL and GUTTERIDGE [34] define an antioxidant as “any substance that when present at low concentrations, compared to those of an oxidizable substrate, significantly delays, or inhibits, oxidation of that substrate”. Among the various antioxidants, the reduced GSH and its metabolizing enzymes provide the major defence against ROS-induced cellular damage [14]. GSH serves as a potent cytoplasm reducing agent [48] 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 activity, was evident in rats exposed to a hot stress [66]. 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 [25]. Moreover, high glucocorticoid concentrations have been reported to decrease blood glutathione concentrations and erythrocyte SOD activity in rats [56]. Coupled to the MDA accumulation in blood, the circulating GSH concentrations were deeply depressed in Chios sheep after shearing in the present study. This observation confirms the existence of LP phenomena during shearing and the GSH depletion observed would aggravate the intensity of 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 shearing stress could be an additional factor responsible for increasing the oxidative stress in sheep after shearing procedure as reflected by the decreased GSH content in erythrocytes.

As far as antioxidant enzymes are concerned, they are initially induced during the oxidative stress but an enzyme depletion occur later resulting in aggravation of the oxidative cell damage [68]. Consequently, it would be sometimes difficult to correlate antioxidant enzyme activities with the intensity of the oxidative stress. Because of the synergy between the various antioxidants crucial for an optimal suppression of free radicals [16], measurement of the total antioxidant activity (AOA) of plasma which represents all antioxidants found in plasma has been proposed [9, 16]. However, only a weak and not significant increase of the plasma AOA was recorded during shearing in the present study whereas marked variations of blood MDA and GSH concentrations were noticed in parallel, suggesting that this parameter is not enough sensitive or that other antioxidant systems would interfere and partially compensate the GSH depletion. In the present study, the reason of the unchanged plasma AOA after shearing is probably the availability of many non specific and major antioxidants in plasma such as vitamin E, ascorbic acid, uric acid, bilirubin and protein thiols [29].

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 [6, 30, 40] and secondarily the glycaemia. The plasma glucose concentration has been reported to be increased
in stressed animals (gazelle, sheep and deer) [8, 28, 37, 49]. However, in this study, shearing procedure unchanged plasma glucose concentration. HARTMANN [37] reported that, maximum glucose concentration is achieved two hours after ACTH injection, and then this parameter decreases to basal values in domestic ruminants. This report may also indicate that the effects of cortisol to increase the plasma glucose concentrations take time. Consequently, in this study increased cortisol could not affect glycemia because shearing procedure last about half hour. Because of the various factors that affect glycemia and its complex regulation, this biochemical marker is not considered as a sensitive measure for welfare or stress detection [7, 55, 69].

Cholesterol is synthesized mainly in the liver but its concentrations also depend on the diet supply [33]. The lipolytic glucocorticoids stimulate fat mobilization from adipose tissue and increase the circulating concentrations of free fatty acids [31, 33]. Thus, an increase of plasma cholesterol concentrations would be expected after a stress episode and some authors have found such increase [49, 55]. In the present study, a moderate elevation of 11% of the cholesterolemia was observed after shearing. This result is in agreement with AVCI et al. [3] which reported that the plasma cholesterol concentration didn’t change in transported sheep. However, as cholesterol is also involved in corticoid synthesis, plasma cholesterol concentrations would also be reduced during stress [59]. As both increases and decreases of serum cholesterol and triglyceride concentrations have been related to noise and physical activity) will be considered as a stressful parameters [2, 49], these biochemical parameters are considered to poor indicators for stress and welfare [7].

As a conclusion, the shearing procedure (mediated by noise and physical activity) will be considered as a stressful condition as evidenced by the strongly increased circulating cortisol and MDA concentrations associated to decreased GSH concentrations, the other investigated parameters (glucose and cholesterol concentrations, plasma total antioxidant activity) being not enough sensitive markers. Furthermore, the increase of plasma cortisol concentrations in response to shearing could be an additional factor responsible for oxidative stress. A more complete identification of the physiological changes during shearing could be beneficial for further researches in terms of correct management practices within sheep industry. Nevertheless, the determination of the oxidative stress parameters could be a new approach for evaluating stress in sheep and antioxidant treatment could be proposed for alleviating the shearing stress.

References


