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Effect of short-term water restriction on oxidative and inflammatory status of sheep (*Ovis aries*) reared in Southern Italy

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ABSTRACT

Water restriction (WR) may impair productive and reproductive performances in livestock. Our major aim was to assess physiological changes associated with a short-term WR in sheep reared in Southern Italy, in order to develop novel methods for evaluating/monitoring the biological implications of such environmental stress. Control animals received water ad libitum (24 h/day) throughout the experimental period. Treated animals were exposed to progressive water restriction (WR) (days 0–4: 12–6 h/day; days 4–8: 3 h/day), followed by a re-adaptation phase (days 9–10). Plasma concentrations of nitro-tyrosine (N-Tyr) and carbonyl (PC) derivatives were measured as markers of protein oxidation. Lipid hydroperoxides (LPOs) were titrated as index of lipid oxidation. Haptoglobin (Hpt) and cortisol were titrated as markers of systemic inflammation and endocrine stress, respectively. Polypeptide profiling of serum samples was performed by MALDI-TOF-MS analysis. Plasma N-Tyr concentration was higher in WR group at day 8 and 10, and significantly increased from day 4–8. Both PC and LPOs levels were higher in WR animals at each time point, and increased until day 8. The concentration of the inflammatory marker Hpt was higher in WR at day 4 and 8, and progressively increased until day 8. MALDI-TOF-MS ascertained decreased levels of apolipoprotein A-II (ApoA-II) and increased levels of platelet factor 4 (PF4) in WR animals at day 8. Taking into consideration the limitations due to the involvement of these redox status and inflammatory biomarkers in other stressing events, these results suggest that N-Tyr concentration might contribute to assess whether a condition of severe WR is occurring, while PC and LPOs may be used for identifying WR animals, and for evaluating the extent of deprivation. The concentration of Hpt, PF4 and ApoA-II might be used in combination for identifying dehydrated animals, and for monitoring the restoring of homeostasis.

1. Introduction

A proper water supply is essential for animal welfare (European Union, Council Directive 98/58/EC 2009), and is crucial for optimizing milk production, growth rate and reproduction in livestock (de Araújo et al., 2010; Schlink et al., 2010). Indeed, an insufficient supply of water can be a critical limiting factor for animal performance. Although ruminants can get and use water coming from feed and oxidative metabolism, marked differences in the efficiency of water utilization exist among species. Indeed, most mammals can die when body water loss is higher than 15%, while ruminants, especially sheep, can tolerate a water loss greater than 20% (Jaber et al., 2004). However, water deprivation or restriction, due to high temperatures and/or low food and water availability, represents a stress condition strongly affecting body weight, specific blood parameters, and productive performances of sheep (Abdelatif et al., 1994; Alamer and Al-Hozab, 2004; Jaber et al., 2004). As water is essential for life and cell homeostasis, its deprivation was reported to induce oxidative stress both in plants and humans (Faraco et al., 2014; Halliwell and Gutteridge, 2015). Oxidative stress-related processes are associated with modifications of physiological and metabolic functions (Halliwell and Gutteridge, 2015), and are involved in the aetiology of several diseases and metabolic disorders (Bernabucci

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hpt, haptoglobin; LDH, lactate dehydrogenase; LPOs, lipid hydroperoxides; nanoLC-ESI-Q-Orbitrap-MS/MS, nanoliquid chromatography coupled to electrospray-quadrupole-Orbitrap tandem mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; N-Tyr, protein-bound nitro-tyrosine; PC, protein-bound carbonyls; WR, water restriction; SCE, sister chromatide exchange

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et al., 2002, 2005; Castillo et al., 2005).

The main objective of this study was to investigate the physiological changes associated with a water restriction (WR) condition in sheep, in order to develop novel analytical tools for evaluating/monitoring the impact of such environmental stress, also in light of the prevised increased water scarcity in the planet (Coping with water scarcity, FAO 2012). To this purpose, we used an integrated approach based on different experimental methods. In particular, we focused on the analysis of biochemical parameters and oxidative status in blood from female individuals, as redox homeostasis provides information on animal health and metabolic status (Bernabucci et al., 2005; Castillo et al., 2005, 2006). In particular, the extent of oxidative damage to proteins was evaluated by measuring plasma concentration of protein-bound nitro-tyrosine (N-Tyr) and carbonyl (PC) derivatives. On the other hand, the extent of the oxidation of polyunsaturated fatty acids was assayed by measuring the concentration of lipid hydroperoxides (LPOs) in plasma (Roomi and Hopkins, 1970; Porter et al., 1995). Haptoglobin (Hpt) and cortisol levels in water-deprived sheep were also measured as inflammation (Quaye, 2008) and endocrine stress indices, respectively. Finally, a proteomic profiling of sheep serum samples was also carried out in order to monitor the protein profile changes associated with WR, thus obtaining putative polypeptide biomarker candidates for the occurrence of a dehydration condition.

2. Materials and methods

2.1. Materials

Bovine serum albumin fraction V (BSA), chemicals of the highest purity, rabbit anti-human Hpt IgG, goat anti-rabbit IgG-horseradish peroxidase linked (GAR-HRP) and rabbit anti-DNP IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polystyrene 96-wells plates were from Nunc (Roskilde, Denmark). Nitrated BSA, lipoperoxide titration kit (Cayman Chemical, Ann Arbor, USA) and rabbit anti-N-Tyr IgG (Covalab, Villeurbanne, France) were from Vincibiochem (Vinci, Italy). The dye reagent for protein titration was from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). Cortisol (sheep) ELISA kit was purchased from Abnova (distributed by Prodotti Gianni, Milan, Italy).

2.2. Animals and management

The study was carried out on eighteen lactating multiparous sheep (age < 4 years) reared in a farm located near to Naples (Southern Italy). Animals were kept under temperate conditions in individual pens on straw, and each pen was equipped with an individual feed through and water bucket. The animals were fed a commercial feed (calculated composition: 89% dry matter, 17% crude protein, 0.70% salt and UFL 0.87 kg t.q.) in an amount equal to 500 gr/head/day. Animals were randomly assigned to the treatment group (WR; N = 18) or to the control group (C; N = 18). In the control group, water was offered ad libitum (24 h/day) throughout the experimental period (ten days). In the treatment group, water was restricted as follows: in period 1 (experimental days 0–4), animals were adapted to WR regime by limiting access to water gradually from 12 to 6 h per day. During the second period (experimental days 4–8), animals of the treatment group had access to water for 3 h/day. In the final period (experimental days 9 and 10), water was offered ad libitum (24 h/day) to restore homeostasis. Individual water intake was recorded daily by weighing buckets at the beginning and after water administration. A correction for water evaporation was carried out using a control bucket located in an adjacent area not accessible to animals. During the trial, the mean temperature and humidity values were 13.8 ± 0.5 °C and 68.00 ± 5.4%, respectively; thus, animals did not underwent heat stress. Animals were managed according to the local farm-production practices. Blood sampling was carried out kindly, and always by the same veterinarian, for avoiding animal suffering and stress. This research project was approved by the Animal Ethics Committee of ISPAAM-CNR (Reference number 0000559).

2.3. Blood and milk sampling

Early in the morning, blood samples were collected into normal or heparinized tubes (7 mL) at day 0, 4 (water gradually limited to 6 h/day), 8 (water restricted to 3 h/day), and 10 (re-adaptation phase). They were then rapidly centrifuged at 500 × *g* for 20 min, at 4 °C, for obtaining corresponding serum or plasma samples, which were then subjected to further analytical determinations. At the same days of blood sampling, duplicate milk samples (30 mL) were collected in sterile tubes earlier at the morning milking.

2.4. Determination of blood and milk biochemical parameters

Plasma concentration of cholesterol, total proteins, albumin, bilirubin, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), calcium, glucose and creatinine levels were measured with a Spotchem System 4410 automated analyser, using reactive strips for cardiac and kidney profiles, according to the manufacturer's instructions (Menarini Diagnostics, Italy). Non-fat dry matter (NFDM), density, freezing point, pH and conductivity (Ce) of milk samples as well as the corresponding content of fat, total proteins, minerals and lactose were measured by a Fourier-transformed IR spectrophotometer (MilkLab Pro, MilkLab, USA).

2.5. Determination of protein-bound N-Tyr levels

N-Tyr levels were measured by ELISA according to a published procedure (Spagnuolo et al., 2012). Briefly, plasma samples were diluted (1:300, 1:1000, 1:2000, and 1:5000 v/v) with coating buffer (7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6), and incubated in a microtitre plate, overnight, at 4 °C. Standard curves were obtained with serial dilutions of nitrated BSA. N-Tyr was detected by incubation (1 h, 37 °C) with rabbit anti-N-Tyr antibody (1:750 dilution in 130 mM NaCl, 20 mM Tris-HCl, 0.05% Tween 20, pH 7.3) supplemented with 0.25% BSA, followed by GAR-HRP (1:2000 dilution). Colour development was monitored at 492 nm. Protein concentration in each sample (previously diluted 1:50 v/v in 130 mM NaCl, 20 mM Tris-HCl, pH 7.4) was measured with the Bradford assay (Bradford, 1976); data were reported as nmol of N-Tyr per mg of proteins.

2.6. Determination of protein-bound carbonyl and lipid hydroperoxides levels

PC levels were measured by ELISA as previously reported (Cigliano et al., 2014). Plasma samples were diluted (1:1000, 1:3000, 1:6000 and 1:12,000) with 10 mM sodium phosphate buffer, pH 7.0, containing 140 mM NaCl, and placed in a microtitre plate, overnight, at 4 °C. PC levels were detected by incubation with rabbit anti-DNP antibody diluted 1:1000 with PBS supplemented with 0.2% gelatine and 0.05% Tween 20 (1 h, 37 °C), followed by addition of GAR-HRP antibody (diluted 1:4000 as the primary antibody). Colour development was monitored at 492 nm (Spagnuolo et al., 2003). A six-point standard curve of oxidized BSA was included in each plate. A blank for DNP reagent in PBS without protein was subtracted from each absorbance measurement. Protein concentration in each sample was determined, and data were reported as nmol of carbonyls per mg of proteins.

LPOs concentration was measured by a colorimetric quantitative assay, using the Lipid Hydroperoxide Assay kit (Cayman Chemical), according to the manufacturer's instructions.