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Original Communication

Stress-induced modifications of trace elements in sheep serum

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ABSTRACT

Stress and its relationship to the occurrence of diseases have long been recognized. Stress is induced by parturition, lactation, weaning and transport, and decreases the ability of the animals to respond immunologically to antigens that they encounter. Furthermore, researches have indicated that stress can alter the metabolism of trace minerals. In this work, we investigated the mineral concentration in the serum of sheep under pharmacological induced stress. Two homogeneous groups of 6 dairy sheep each were used, one negative control (CT) and one injected with ACTH (adrenocorticotropic hormone) to mimic biological stress. The groups were fed with a restricted amount of the same basal diet. Blood samples were collected before (T0), and after 3 (T3) and 51 (T51) h from the first ACTH injection. We attempted to quantify elemental serum contents using synchrotron radiation total reflection x-ray fluorescence (SR-TXRF) at the Brazilian Synchrotron Light Laboratory. The analysis indicates that the ATCH activity causes significant variations in K, Ca, Cu and Zn concentrations. Our results indicate that stress can affect serum microelement concentrations and further analyses are required to investigate the physiological meaning of Ca and Cu variations.

KEYWORDS: trace elements, serum sheep, TXRF, ACTH

INTRODUCTION

Scientific research on the factors affecting the well-being of sheep is rather recent, as are studies of strategies to minimize the adverse effects of environmental challenges and improper management practices on animal welfare. Recently, an increased attention to welfare conditions of animals in intensive and semi-intensive production systems is being given, especially in dairy sheep breeds, due to the growing concern of consumers about the life conditions of animals, and the issuing of a number of rules and laws on the safety of animal products and well-being of livestock [1].

Among the factors affecting welfare, animal transportation has received an increasing attention, regardless of how it is organized. Transport implies a sudden change in the living conditions together with an adaptation effort by the animals, which subsequently modifies the quality level of their welfare and leads therefore to a typical acute stress condition [2]. The study of the effect of transport on stress and sheep welfare became a paramount issue within the larger context of implementation and enforcement of EU legislation. The EU Regulation 1/2005 concerning animal

protection during transport states, among others stipulate that the travel time for sheep should be lower than 8 h, or 14 h under special circumstances with 1 h break followed by another 14 h journey. Animal welfare during transport may be influenced by many stress factors such as vehicle movement, engine noise, loading/unloading maneuvers [3], vehicle microclimate [4] or the informational flow between animals and their environment.

Chronic stress occurs when animals are unable to deal with a persistent stressor with species-typical responses, or when several stressors are present concurrently. This type of stress is most frequently considered in intensive systems, but it may also be a welfare concern for extensively managed species, such as sheep [5].

In order to monitor the effects of stress on animal health, recent studies have analyzed well known biochemical markers in blood such as cortisol. In addition, the concentrations of chemical elements in blood can also be evaluated to check physiological conditions and their variations to reflect, for instance, acid-base alterations, renal functionality, or responses to infections. At the same time minimal variations in blood element concentrations, although within the range of physiological normal values, may be useful to understand the individual responses to stress conditions or to drug treatments. Among the others, blood microelements like Cu and Zn are recently receiving increased attention due to the emerging complexity of biological mechanism in which they are involved.

In this work, elements in serum samples of sheep were analyzed to monitor their variations during cortisol-induced stress after ACTH injections. Thanks to the quantitative sensitivity of Total Reflection X-Ray Fluorescence (TXRF), minimal variations were also revealed, suggesting the utility of this approach in combination with conventional analyses to investigate health conditions and to disclose new aspects of the influence of diet on the individual state and homeostatic regulation.

METHODOLOGY

Experimental protocol

Twelve dairy sheep, homogeneous for age and body conditions, were randomly assigned to two groups and fed twice a day with the same basal diet (1 kg/head day of pellet) formulated to cover maintenance requirements (Table 1). Animals were allocated into separated boxes with straw bedding and had free access to the same water source (city water supply) [6-8]. Animal health was assessed by clinical examination before and during the whole experimental period. All procedures were carried out in respect of the Italian legislation on animal care (DL n.116, 27/1/1992). After an adaptation period of 24 days the sheep of ACTH group were injected with 0.5 mg of an ACTH agonist (Tetracosactrin acetate; Synacthen, Novartis, Varese, Italy) i.m. every 12 h for 48 h (5 injections), to induce cortisol secretion, whilst the sheep of CT

Table 1. Ingredients, dry matter (DM) content (%), chemical composition (% of DM) and mineral and energy content of basal diet.

	Control
Ingredients (%)	
Corn	15.00
Soybean s.e., 44%	10.00
Hay pellet	50.00
Wheat bran	3.80
Sugar beet pulp, dehy	12.00
Breweries	4.00
Molasses	2.80
Min. Vit. supplement	2.40
Total	100.00
Chemical composition	
DM (%)	88.80
Crude protein (% DM)	14.90
Ether extract (% DM)	2.80
Ashes (% DM)	12.00
NDF (% DM)	37.40
Starch (% DM)	14.30
Calcium (% DM)	0.70
Phosphorus (% DM)	0.45
Potassium (% DM)	0.90
Sodium (% DM)	0.24
Iron (mg/kg DM)	109.00
Copper (mg/kg DM)	12.00
Zinc (mg/kg DM)	107.00
NE_L (Mcal/kg DM)	1.32

group received the same dose of saline and served as negative control. Blood samples were collected from jugular vein in the morning, before ACTH treatment (T0), 3 h after the first ACTH injection (T3), and 3 h after the last ACTH injection (T51). Blood samples were collected into tubes either without anticoagulants, or with Li-heparin and centrifuged (1500 x g/10 min) within one h from collection. Plasma and serum samples were stored at -20°C until analyses.

Unless otherwise indicated, all chemicals employed for biochemical analyses were obtained from Sigma-Aldrich (Milan, Italy). Plasma cortisol was determined with a commercial kit for radio-immunological assay (SORIN SpA, Vercelli, Italy), using the method modified by Bertoni *et al.* (2002). Glucose was determined using kits purchased from Instrumentation Laboratory (IL Test). Ceruloplasmin (CuCp) was analyzed using methods described by Bertoni *et al.* (1998), adapting them to the ILAB 600 conditions. Zinc was determined by commercial kit (Wako Chemicals GmbH, Neuss, Germany) according to manufacturer's instructions.

Sample preparation for TXRF analysis

Serum samples were submitted to the standard chemical digestion by adding nitric acid in order to avoid organic matrix interferences [9, 10]. Deionized and de-mineralized water (Milli-Q) was employed for rinsing the materials.

To perform quantitative analysis, an internal standard was added to the sample to correct the system instability, such as oscillation in the X-ray generator, emission of X-rays by the anode, X-ray detection, and operational mistakes, such as inhomogeneous positioning of the samples. In this work, Ga (102.5 μg μL^{-1}) was used as internal standard at a concentration of 9.09 μg μL^{-1}. Small amount (5 μL) of the final solution was pipetted on the Perspex support for later evaporation under an infrared lamp.

Experimental setup

The SR-TXRF measurements were performed at the X-Ray Fluorescence (XRF) beamline at Brazilian Synchrotron Light Laboratory (LNLS), Campinas, São Paulo, Brazil [11]. All measurements were performed under normal conditions of pressure and temperature (760 mm Hg and 273.15 K) and a geometry excitation standard was used. All samples were analyzed by exciting them with a white beam with maximum energy of 20 keV. Fluorescent photons were detected with a Ge detector of 165 eV at 5.9 keV of resolution with 8 mm beryllium window thickness, 30 mm^2 active area, coupled to an amplifier module and a multi-channel analyzer. The samples were excited for 100 seconds and the x-ray spectra obtained were evaluated by the software Quantitative X-ray Analysis System (QXAS) distributed by the International Atomic Energy Agency (IAEA) in order to obtain the X-ray intensities for each element and the associated uncertainty [12]. The setup can be seen in Figure 1.

Quantitative analysis

In the TXRF technique, there is no occurrence of the absorption effect and enhancement as in Energy Dispersive X-ray Fluorescence (EDXRF) or Wavelength Dispersive X-ray Fluorescence (WDXRF) and the correction of the matrix effect due to the small thickness of the sample and high energy of the X-rays usually used for the excitation is not required [13, 14]. Therefore, the quantitative analysis is performed with the equation:

$$I_i = S_i C_i \quad (1)$$

where I_i is the net intensity of the X-rays (cps) of the characteristic K or L line of the element i; C_i is the concentration (ppm or $\mu g.mL^{-1}$) of the element i; and S_i is the elementary sensitivity of the system for the element i (cps/ppm).

Through Equation (1), the relationship between the intensity of the element i and the internal standard (Ga) can be established:

$$\frac{I_i}{I_{Ga}} = \frac{S_i}{S_{Ga}} \frac{C_i}{C_{Ga}} \quad (2)$$

The concentration of the element of interest is calculated using the equation:

$$C_i = \frac{I_i}{I_{Ga}} \frac{C_{Ga}}{S_R} \quad (3)$$

Where: $S_R = \frac{S_i}{S_{Ga}}$ is the relative sensitivity (in relation to the element used as internal standard).

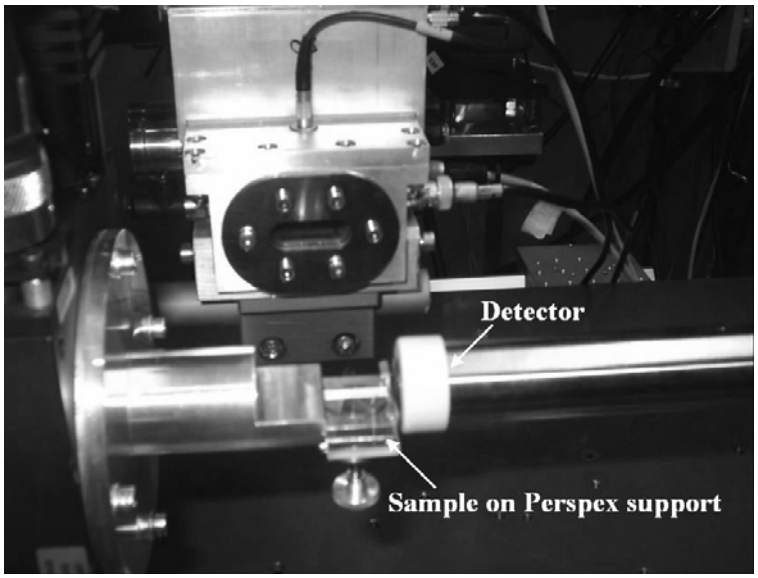

Figure 1. System for positioning the sample in the XRF beamline at LNLS.

Since a high mathematical correlation between the elementary sensitivities and the atomic numbers of the elements exists, it is possible to estimate the sensitivity for an element detected in the sample and not contained in the standard solution, based on the elementary sensitivities of the elements contained in the standard solution and, consequently, to estimate its concentration in the sample.

Statistical analysis

Biochemical data were analysed using the repeated measure analysis of the general linear model procedure of SPSS (1997) [15] with trend analysis, which produces the same results of the SAS mixed model procedure. Factor terms included in the model were groups (ACTH, CT), animal within the group (Subject), time (T0, T3 and T51), and the interaction of the group and time.

Statistical comparison of the mean concentrations of microelements between experimental groups at each time (T1, T2 and T3) was performed using Student's two-tailed t-test. P values lower than 0.05 were considered significant.

RESULTS AND DISCUSSION

Blood analysis

The samples collected from the sheep were analyzed for cortisol, CuCp and Zn (Tables 2 and 3).

Plasma concentrations of cortisol in the control (CT) animals did not change during the experimental period and were within the physiological range. Instead, cortisol levels dramatically increased after ACTH treatment ($P = 0.000$). Glucose concentrations were also increased ($P < 0.05$) as a consequence of ACTH treatment, but the increase was more gradual than for cortisol. Contrast analysis allowed to underline that the effect of ACTH is statistically significant only in T3 ($P < 0.01$).

The concentration of CuCp did not significantly change between times of sampling and groups, even though a clear trend was evident from the data reported in Table 3. The variations were not statistically significant in the multivariate analysis due to the high inter-individual variability. However, since the changes were within the physiological range, the increase of CuCp may reflect an unknown mechanism of action of the drug and needs further investigations.

Zn concentrations significantly decreased between times of sampling in the ACTH group, being influenced by ACTH treatment [16-18].

TXRF analysis

Figure 2 shows the typical x-ray spectra of serum samples. The result for the relative sensitivities ($S_R = S_i/S_s$) with the corresponding fittings for the K series is shown in Figure 3.

Table 2. Results from cortisol analysis.

	Cortisol (mmol/L)		
	T1	**T2**	**T3**
ACTH	83.17 ± 19.78	271.83 ± 76.48	421.00 ± 84.27
CTR	33.97 ± 22.16	43.43 ± 16.32	46.43 ± 23.49

Table 3. Results from CuCp and Zn analysis.

	CuCp (μg/mL)			Zn (μg/mL)		
	T1	**T2**	**T3**	**T1**	**T2**	**T3**
ACTH	0.18 ± 0.02	0.19 ± 0.02	0.21 ± 0.05	0.80 ± 0.08	0.83 ± 0.11	0.55 ± 0.10
CTR	0.17 ± 0.02	0.18 ± 0.01	0.18 ± 0.01	0.81 ± 0.09	0.75 ± 0.14	0.75 ± 0.09

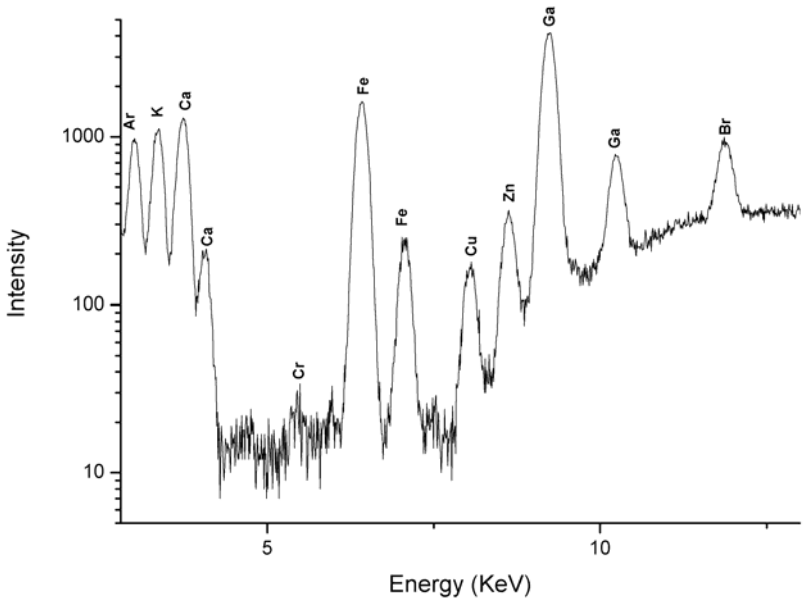

Figure 2. Typical x-ray spectrum of a serum sample.

The major elements K, Ca, Cu and Br were detected in all the analyzed samples. Figures 4 to 7 report the mean and standard deviation of the concentrations found for each elements at each time of sampling (T1, T2 and T3) within each group.

Potassium

Potassium is the cation with the highest concentration in the intracellular fluid (within cells) but with low concentration in the extracellular fluid, as blood. This element participates in osmolarity and pH control in the body fluids and in many other essential functions. The average concentration of this microelement in the control group (244 μg/mL) agrees with those reported by Long *et al.* [19]. ACTH is a hormone that stimulates the adrenal gland and leads to a reduction of serum K levels. In the groups of sheep treated with ACTH, the concentrations of K decreased at T3 in comparison to those measured at T1 and T2. The higher mean K concentrations detected at T1 for the ACTH group should probably reflect some hemolysis and can be considered as an artifact (very frequent in the blood analyses of

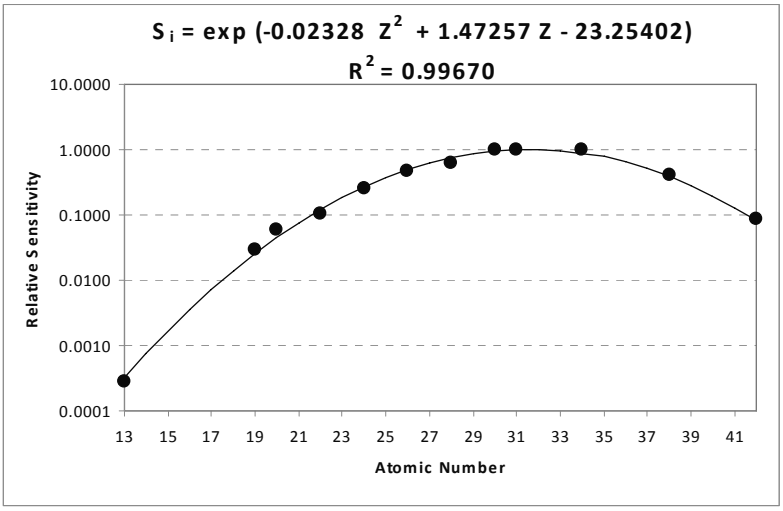

Figure 3. Relative sensitivity curve (SR) for the K series by SR-TXRF.

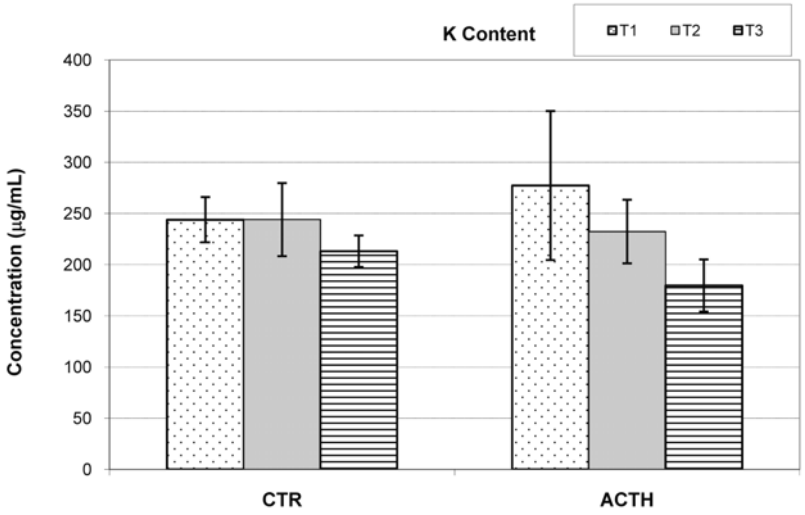

Figure 4. Average concentration of K for all analyzed groups in relation to the sampling time.

this element), particularly considering the normal values found in the same animals after 3 hours (T2).

Calcium

Calcium is the most abundant mineral in the body and its concentrations in the plasma reflect the calcium and inorganic phosphate metabolism and are maintained in a strict range of physiological variation. ACTH in some conditions may sporadically influence Ca levels as a collateral effect, since it affects the renal excretion of phosphates. In our analyses no Ca variations related to ACTH were observed, in agreement with the lack of detectable discomfort in the animals. The mean serum concentration of calcium in the samples of the control group ranged from 86 to 111 µg/mL, a value similar to that (116 µg/mL) reported by Becker and Smith (1950) [20] and within the range reported by Dukes (1955) [21], from 90 to 120 µg/ml. Slightly different concentrations were reported by Norris and Chamberlin (1929) [22] and Stewart and Holman (1944) [23] (99 µg/mL for sheep and wethers or 106 µg/mL for lambs and 107 µg/ml for sheep).

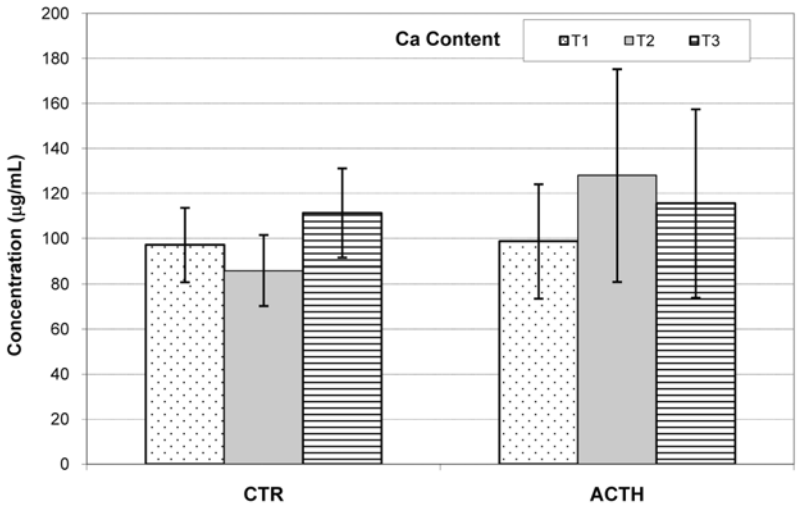

Figure 5. Average concentration of Ca for all analyzed groups in relation to the sampling time.

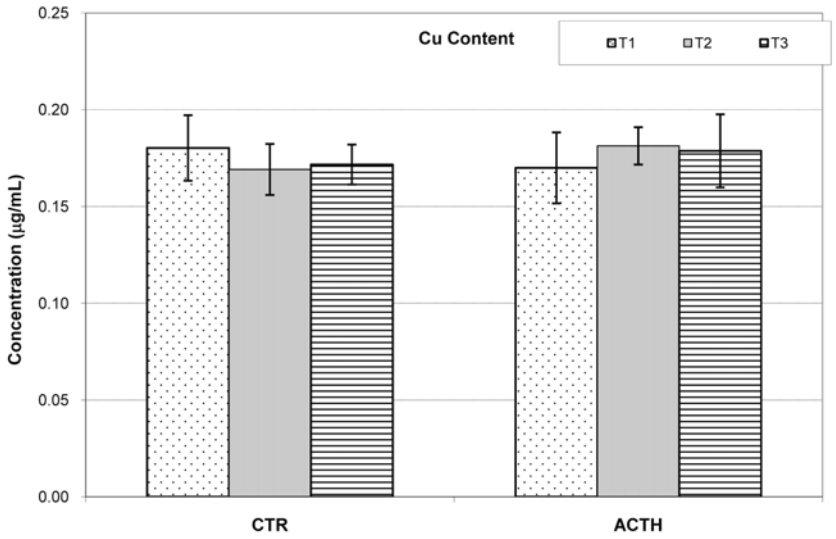

Figure 6. Average concentration of Cu for all analyzed groups in relation to the sampling time.

Copper

Copper is a microelement clinically monitored in blood in the routine laboratory analysis for the diagnosis of certain pathological conditions. Conventional analyses measure the ceruloplasmin (CuCp), which is the main serum transport protein for this element (binding up to 90% of blood Cu). Copper is essential for life and is required for the normal chemical and physical processes that occur in the body. Copper is necessary for normal iron metabolism, red blood cell formation and production of skin and hair pigments, or melanin. Copper is also essential for the formation of myelin, a compound that supports and protects the central nervous system, for wool production, and is involved in the animal's immune response to disease challenges [24].

By SR-TXRF analysis, the mean Cu concentration detected for control group was 0.17 µg/mL, which was similar to the plasma value (0.17 µg/mL.) measured by biochemical analysis. No significant differences in Cu level were found after ACTH administration.

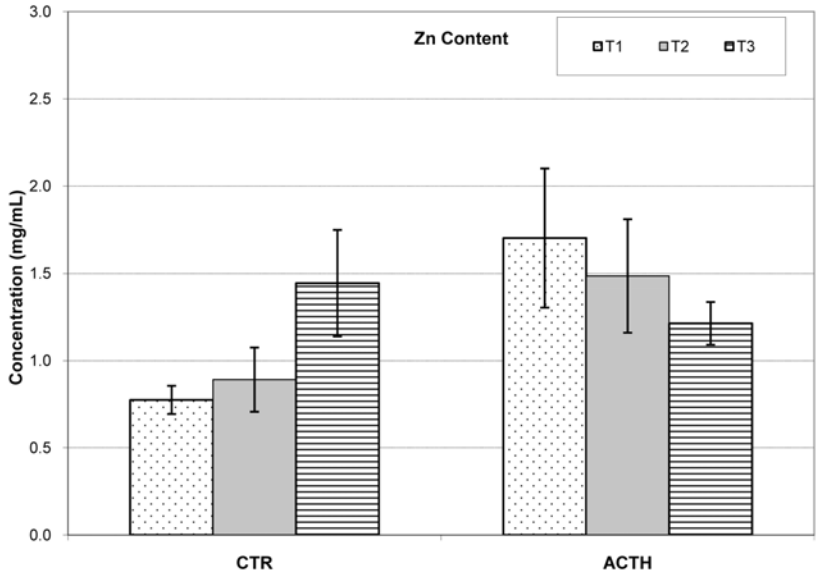

Figure 7. Average concentration of Zn for all analyzed groups in relation to the sampling time.

Zinc

Zinc is essential for the function of large number of enzymes. In particular, Zn has been reported to be important in biochemical processes involved in nucleic acid metabolism and cell division [25]. For this reason, Zn has long been recognized as an important nutrient for spermatogenesis and male fertility also in farm animals. However, more recently the role of zinc in immune response has received an increasing attention. Many investigators have suggested that deficiency of this mineral in cattle impairs appetite and growth, before reducing immune competence. Significant data related to this concept is currently lacking for cattle, but studies in sheep [26] and mice [27] support this supposition. In this study, the biochemical analysis showed a decrease in Zn concentration mainly at T3, probably as a consequence of the ACTH injection, in agreement with the previous research [28].

CONCLUSIONS

The interactions between trace minerals, animal welfare and disease resistance are extremely complex. Many factors can affect animal's response to trace mineral supplementation, such as the length of administration, the amount of the trace mineral in the diet, stressing conditions of an animal associated with pregnancy, lactation and transport, supplementation of dietary antagonists and environmental factors.

In this study, we have provided evidences that ACTH induced stress can affect the serum concentration of microminerals. We have also demonstrated the applicability and advantages in quantitative sensitivity of using SR-TXRF to monitor minimal serum elemental changes during pharmacological and nutritional treatments, which can also be applied for personalized medicine.

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