Small Ruminant Research 156 (2017) 50–56

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

The combined use of ozone therapy and autologous platelet-rich plasma as an alternative approach to foot rot treatment for sheep. A preliminary study

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ARTICLE INFO

Keywords:
Foot rot
Sheep
Ozone therapy
PRP
Oxidative status

ABSTRACT

The aim of this study was to evaluate the efficacy of ozone therapy and platelet rich plasma (PRP) in the treatment of acute foot rot. The study was carried out on 10 sheep suffering from foot rot compared to a control group of 5 healthy sheep. The therapeutic scheme consisted of two steps: baseline treatment by local ozone application and then, in the case of non-healing, the application of PRP. We analyzed the effectiveness of the combined treatment, as well as the potential toxicity of ozone therapy to the patients. For this purpose, the parameters of oxidative stress, antioxidant defense and systemic inflammatory response were evaluated in the course of the study. Complete recovery was achieved after local ozone application in six of the ten sheep. The remaining four animals also healed after the subsequent PRP therapy. Furthermore, plasma concentrations of fibrinogen and haptoglobin did not change significantly during therapy. All of the observed fluctuations in oxidative parameters were mild and transitory, antioxidant/oxidant balance (AOB) calculated on the basis of antiradical capacity (AC) indicated a higher value just after ozone therapy (128.69 ± 66.01%), whereas AOB calculated on the basis of reducing power (RP) was lower at this time point, with an insignificant increase in the last measurement. Growth factors in PRP were detected using the MALDI TOF method. Our results indicated that ozone therapy did not evoke haematological changes, alterations in acute phase proteins and oxidative status. We demonstrated that the local application of ozone and PRP proved to be an effective ovine foot rot treatment that does not require the conventional use of antibiotics and disinfectants. However, due to the relatively high costs and time requirements of this system, it is potentially most suitable for small, organic farms.

1. Introduction

Ovine foot rot is a widely distributed, highly contagious disease of the interdigital tissue, especially the skin and hoof matrix, caused by *Dichelobacter nodosus* together with *Fusobacterium necrophorum* as a complicating factor. In severe cases, foot rot is a debilitating disease causing severe lameness combined with reduced wool and meat production and decreased fertility (Ansari et al., 2014).

Different treatment regimens have been introduced to treat sheep affected by foot rot. Currently, the method of choice is the parenteral administration of antibiotics in combination with footbaths in bactericidal solutions and a topical antibiotic aerosol spray. However, this system of treatment poses some disadvantages, the overuse of antibiotics leads to the development of drug resistance, several of the chemical solutions used for foot bathing are toxic both to the human personnel administering the treatment and the environment. Also, given the increasing number of organic farms where the use of antibiotics is strictly limited, there is a need for a viable alternative therapy for foot rot (Koćisowa et al., 2006; Chanyalew and Alemu, 2014). For these reasons, we propose to introduce ozone therapy to avoid the application of more toxic factors. It is an alternative method of treatment, which may help to limit the use of antibiotics in raising livestock.

Ozone is a powerful oxidant which promotes oxidative stress, but may simultaneously limit some factors released by inflammatory cells as well as activating the antioxidant endogenous system (Ozbay et al., 2017). To date, ozone therapy has been successfully used to treat many disorders including infected wounds, chronic skin ulcers, early-stage gangrene and advanced ischemic diseases (Guven et al., 2009). In

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http://dx.doi.org/10.1016/j.smallrumres.2017.08.015
Received 11 June 2017; Received in revised form 17 August 2017; Accepted 18 August 2017
Available online 24 August 2017
0921-4488/

veterinary medicine, ozone has been used both in small animal practice and in buiatrics, especially in the treatment of reproductive system disease (Duričić et al., 2015). According to these authors, ozone treatment has been successfully applied to treat many disorders, among others *urovagina*, *pneumovagina*, acute *metritis*, *endometritis* and *mastitis* in the form of an intrauterine application of ozone paillettes.

It has been proven that ozone increases the activity of antioxidant defense composed of glutathione peroxidase, superoxide dismutase (SOD), and catalase against reactive oxygen species (ROS) (Guven et al., 2009). As a component of alternative or complementary therapy, ozone is used for a brief period of time to obtain desirable biological effects i.e. to eradicate infection, improve oxygen delivery to anoxic tissue, and upregulate antioxidant systems to reduce chronic oxidative stress (Du Plessis et al., 2008; Sagai and Bocci, 2011). Furthermore, ROS generated by ozone therapy may enter the cells and, by activating the nuclear factor kappa B, induce cytokine production in cells leading to enhanced immune response (Du Plessis et al., 2008).

In our study, an additional therapy for foot rot included the preparation and use of platelet rich plasma (PRP) applied locally on the lesions. PRP has been previously utilized to enhance the healing of wounds, bone, muscle, ligaments, tendons etc. (Castillo et al., 2011). We assumed that it may be useful for the treatment of limited interdigital dermatitis (grade 1 according to the scoring system described by Raadsma and Egerton, 2013), and allow us to avoid local antibiotic therapy or foot bathing in toxic agents. To date, there remains a lack of standardization of the method for the preparation of PRP, before clinical use, the obtained autologous blood-derived preparation has been carefully evaluated.

The treatment scheme consisted of two steps. The first step involved a baseline treatment by local ozone application followed, in the case of non-healing lesions, by the application of PRP. Since ozone therapy as a new approach, involves certain problems, including a high level of tissue toxicity resulting from oxidation and lipid peroxidation leading to changes in membrane permeability as well as enzyme inactivation, it should be carefully evaluated before being introduced as a broad clinical application (Elvis and Ekta, 2011).

Thus, the aim of our study was to evaluate the effectiveness and safety of ozone therapy as an alternative method of acute ovine foot rot treatment without the use of antibiotics. We assessed the parameters of oxidative stress, antioxidant defense and systemic inflammatory response in sheep treated with ozone.

2. Materials and methods

2.1. Animals and treatment scheme

The study was conducted using fifteen adult female Polish lowland sheep (Uhruska sheep Bezek flock, PON) weighing approximately 40–50 kg, and aged between 1 and 2 years. Ten sheep with suspected foot rot based on clinical signs (FR group) were admitted to our clinic from Bezek Experimental Farm, five additional healthy sheep of the same breed and of a similar age were kept at the Clinic and served as a control group. All the animals were fed, housed and cared for in accordance with the relevant EU directives concerning animal welfare. Acute foot rot diagnosis was based on typical clinical signs (lameness, lesion with interdigital inflammation, hoof horn separation, characteristic foul smell, absence of hyperplasia of the sole, grade 3–4 according to Raadsma and Egerton, 2013) and confirmed by microbiological tests. Swabs for microbiological analysis were collected from lesions of intradigital skin. Samples were cultured on Eugon agar enriched with 5% defibrinated sheep blood for the isolation of *D. nodosus* and Fusobacterium selective agar (FSA) with 5% defibrinated sheep blood, without antibiotic supplements. Colonies were evaluated under a microscope (Olympus, Japan) after the incubation of plates in anaerobic jars at 37 °C for 5 d. *D. nodosus* and *F. necrophorum* were confirmed on the basis of colony morphology, haemolytic properties, incubation needs, and Gram staining (Aguiar et al., 2011, Ozgen et al., 2015).

The clinical parameters: rectal temperature, pulse and respiratory rate were recorded. Each sheep from the FR group was treated in accordance with the following scheme:

1. Cleaning and gentle removal of the necrotic tissue
2. Application of dressing: cotton gauze was applied to pad the foot and the interdigital space, then the foot was wrapped in bandaging and covered with polyethylene foil as waterproofing
3. Next, the foot dressing was infused with ozonated saline solution. The therapeutic solution was prepared using a medical generator for ozone therapy ATO-3 (Metrum Cryoflex Poland), which supplied 500 mL of 0.9% NaCl with a concentration of ozone of 70 mg/mL
4. After 20 min, the bandages were removed.

The above procedure was repeated three times at weekly intervals. Before each ozone administration, a careful clinical examination of the foot was performed. To minimize the harmful effects of ozone for both people and animals, all procedures were performed outdoors.

In the group undergoing PRP treatment, the interdigital space was irrigated with 0.9% NaCl, and then activated PRP was applied by spraying. Next, a protective dressing was applied using sterile gauze and bandages. The dressing was changed every four days for 14 days.

2.2. Blood sampling

Blood was drawn at following time points: First T0 − immediately after foot cleaning and before ozone treatment, second T1- immediately after the last stage of ozone therapy, and finally T2 − one week after the last stage of ozone therapy. When the ozone therapy was complete, all sheep were clinically examined and four animals with persisting lesions (grade 1 on the Egerton scale) were selected for further treatment with PRP.

Blood samples for haematological assays were collected at T0 and T2 from the jugular vein into tubes containing EDTA as an anticoagulant. Next, complete blood cell counts, including platelet counts, were performed using the Vet EXIGO analyzer (Boule Medical AB). Blood obtained at the respective time points (T0, T1, T2) was used for biochemical analyses. Additionally, at the second stage of the experiment, approximately 10 mL of blood from each of the four sheep assigned for further treatment was used for the preparation of autologous PRP.

2.3. Assessment of acute phase proteins (APP)

The fibrinogen level was determined using the heat precipitation method (Szponder and Wessely-Szponder, 2010). Diluted plasma was clotted with thrombin and the fibrin formed from the reaction between thrombin and fibrinogen was hydrolyzed by boiling NaOH (10%). Next, the tyrosine content was measured at 720 nm using Folin-Ciocalteu's phenol reagent on the basis of the standard curve. Haptoglobin in plasma was determined spectrometrically after reaction with methemoglobin and guaiacol reagent. The optical density of the solutions was measured at 470 nm.

2.4. Plasma oxidant-antioxidant status

The experiments were carried out using an improved ABTS decolorization assay. The free radical scavenging ability was expressed as a Trolox equivalent in mg per mL (Re et al., 1999). The reducing power (RP) was determined following Oyaizu's method (1986). The analyzed sample (0.5 mL) was mixed with phosphate buffer (0.5 mL, 200 mM, pH 6.6) and potassium ferricyanide $K_3[Fe(CN_6)]$ (0.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. The reactions were stopped with 0.5 mL 10% TCA and centrifugation for 10 min at 6500 × *g*. The