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Effects of transrectal medicinal ozone in horses – clinical and laboratory aspects

[*Efeitos do ozônio medicinal transretal em cavalos – aspectos clínicos e laboratoriais*]

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ABSTRACT

Ozone (O_3) therapy has been used for medical procedures for centuries; however, there are no extensive studies on its utilization in horses. This study aimed to evaluate the application of transrectal O_3 on horses by physical and laboratorial evaluation, and production of reactive oxygen species (ROS). Sixteen healthy horses were separated in two groups: a control group (CG) and a group treated with O_3 (TG). The TG animals received 1L of an oxygen and O_3 mixture transrectally. The initial dose was 10µg/ml for the first two applications, 15µg/ml for the following two applications, and 20µg/ml for the next six applications. The CG animals received 1L of oxygen transrectally. In TG animals no variations in the physical examination were detected; furthermore, TG animals did not exhibit changes in biochemical evaluation results, fibrinogen concentrations, or ROS production. TG animals had increased red blood cell counts, hemoglobin concentrations, and packet cell volume values in comparison to the baseline and CG values. We could infer that O_3 affected the red blood cell counts and improved rhetological properties of the blood. The transrectal application of O_3 in horses is safe and can indirectly improve the oxygenation and metabolism of tissues.

Keywords: antioxidant, red blood cell counts, horse, ozone therapy

RESUMO

A utilização medicinal do ozônio (O_3) é secular, contudo não existem estudos expressivos de sua utilização em equinos. O objetivo deste estudo foi avaliar o efeito da aplicação transretal de O_3 em equinos por meio da avaliação física, laboratorial, e produção de espécies reativas de oxigênio (EROs). Dezesseis equinos hígidos foram separados em dois grupos: grupo controle (GC) e grupo tratado com O_3 (GT). O GT recebeu por via retal 1L da mistura de oxigênio e ozônio, sendo a dose inicial de 10µg/ml por duas aplicações, 15µg/ml por mais duas aplicações e 20µg/ml por seis aplicações. O GC recebeu 1L de oxigênio via transretal. No GT não foram observadas alterações no exame físico, bem como não foram observadas alterações na avaliação bioquímica, concentração de fibrinogênio e produção de EROs. O GT apresentou aumento no número de hemácias, na concentração de hemoglobina, e nos valores de hematócrito em relação aos valores basais e GC. Podemos inferir que o O_3 alterou os valores de eritrócitos e melhorou as propriedades reológicas do sangue. Conclui-se que a aplicação transretal de 0_3 em equinos é segura e pode melhorar indiretamente a oxigenação e metabolismo dos tecidos.

Palavras-chave: antioxidantes, hemograma, cavalos, ozonioterapia

INTRODUCTION

Ozone (O_3) for medical procedures was first used by a German army chief surgeon in 1915 during the First World War. Medicinal O_3 was used to treat post-traumatic gangrene, frozen feet and decubitus ulcers in German soldiers (Bocci, 2005), to prevent local infections during medical procedures, and to control wound infections (Merin *et al.*, 2007). The first O_3 generator created by physicist Joachim Hansler led to an increase in the development of O_3 therapy in the medical field (Elvis and Ekta, 2011).

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The objective of O_3 therapy is to provoke controlled acute, adequate, and transitional oxidative stress without causing chronic oxidative stress. Repeated, small oxidative shocks activate and stimulate the antioxidant system (Bocci, 2005), consequently creating resistance to oxidative stress by inducing the antioxidant system (Manoto *et al*., 2016). Ozone therapy involves a mixture of oxygen (O_2) and O_3, following the proportions of no less than 95% of O_2 and not more than 5% of O_3 (Bocci, 2006). A high concentration of O_3 is toxic, yet doses from 10 to 80µg/ml are considered a therapeutic window that produces immunomodulatory, anti-inflammatory, bactericidal, antiviral, antifungal and analgesic effects, among other benefits (Schwartz *et al.*, 2012). Ozone is immediately neutralized by antioxidants present in the blood when applied in concentrations lower than 10µg/ml; therefore, this concentration is biologically inefficient and does not reach the minimum value of the therapeutic window (Sagai and Bocci, 2011).

Ozone dissolves in a few minutes if it comes in contact with a biological fluid such as whole blood, plasma, urine, or lymph, among others (Bocci, 2006). When this happens, essential messengers are created to perform therapeutic actions. These messengers consist of reactive oxygen species (ROS), mostly hydrogen peroxide, and a mixture of lipoperoxidation products (Sagai and Bocci, 2011). These products increase the auto-regulation capacity, stimulating the natural mechanisms responsible for organismal and cellular protection. Contrastingly, high doses (>45µl/ml) create an inflammatory response (Viebahn-Hänsler *et al*., 2012). For this reason, O_3 is considered a pro-drug, a stimulus, and it is not possible to measure its active principle (Sagai and Bocci, 2011).

One of the first actions of O_3 therapy is the activation of a glycemia process via an increase of adenosine triphosphate (ATP) and 2,3-diphosphoglycerate. Consequently, a sigmoid oxygen-hemoglobin curve is shifted to the right, increasing O_2 for ischemic tissues (Sagai and Bocci, 2011). The release of cysteine proteins and aldehydes influence the Keap-1/Nrf2 pathway, facilitating the release of Nrf2; Nrf2 will then go to the nucleus of the cell and connect to electrophilic response elements (EpREs) that are responsible for the transcription of a number of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase (Chow *et al*., 2003).

Ozone causes several actions in organisms (Sagai and Bocci, 2011; Jani *et al.*, 2012; Schwartz and Sánchez, 2012). First, oxygenation and blood circulation increase in ischemic tissues because of the presence of nitric oxide and carbon monoxide, increased glycosylation, plasticity, and a rightward deviation of the oxygen-hemoglobin dissociation curve of erythrocyte levels. Second, a general increase in metabolism occurs due to oxygen improvement, causing an improvement in glycose use, an increase in the production of ATP, an improvement in protein metabolism, and a direct oxidizing effect on unsaturated lipids that induces repair mechanisms. Third, O_3 improves angiogenesis and may also boost bone marrow stem cell implantation. Fourth, O_3 increases antioxidant cellular enzyme levels including glutathione peroxidase, glutathione reductase, catalase, and superoxide dismutase.

Fifth, O_3 facilitates a moderate induction of the immune system by activating neutrophils, releasing cytokines, and increasing growth factor release through platelet activation. Sixth, O_3 has an anti-inflammatory and analgesic effect evident through the decreased production of inflammatory mediators, inactivation of pain metabolites through oxidation, lesion repair enhanced by improved microcirculation, toxin elimination, and the resolution of painful physiological disturbances. Seventh, O_3 exhibits excellent disinfectant properties when used topically. Finally, O_3 contributes to a sense of well-being, probably due to the stimulation of the neuroendocrine system through the release of hormones and neurotransmitters.

There are several studies demonstrating medicinal O_3 use in laboratory animals; however, in veterinary medicine, there is little data regarding its use. In the literature, there are only a few studies with horses (Alves *et al*., 2004; Ballardini, 2005; Garcia Liñero *et al.*, 2009; Vendruscolo *et al*., 2018) and bovines (Ogata and Nagahata, 2000; Zobel *et al*., 2012). In view of the promising applications of O_3 therapy, concomitant with the scarcity of studies in veterinary medicine, the objective of this study

was to evaluate the effects of the application of transrectal medicinal O_3 on healthy equines by physical examinations, complete blood counts, biochemical evaluations, fibrinogen concentration analyses, and ROS production capacity assessments. Our hypothesis is that the utilization of transrectal O_3 will not stimulate inflammatory processes or cause hematological and biochemical alterations in horses, which would demonstrate its safety. In addition, we hypothesize that O_3 will improve cellular responses to oxidative stress.

MATERIALS AND METHODS

The research project was approved by the Ethics in the Use of Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo (FMVZ-USP), according to protocol number 6720220516. Sixteen healthy Arabian geldings were used, with an average age of 36 months (2.5-4 years). The animals were allocated in stalls and were fed with approximately 2.20 pounds of commercial grain twice a day; coast cross hay and water were available *ad libidum*. The horses were dewormed after the adaptation period of one month before the experimental period.

The equines were split randomly into two groups: a treatment group (TG) and a control group (CG). In the TG, after manual rectal emptying, the animals received a rectal application of 1L of the O_2/O_3 mixture in crescent doses: the initial dose was 10µg/ml for the first two applications, 15µg/ml for the following two applications, and 20µg/ml for the next six applications for a total of 10 applications. In the CG, animals received 1L of oxygen after manual rectal emptying in the same dosages as the treatment group. The applications were performed three times a week for a period of 22 days.

The O_3 was generated just before the transrectal application with the aid of an Ozone & Life® (São José dos Campos, SP, Brazil) generator in the dosages previously described; the generator regulated the O_2 flow and the electric discharge meter. The gas exited directly from the generator through equipment extensors and urethral probes and was administered directly into the rectum with the aid of palpation during the calculated time, according to the flow of the valve, so that 1L of the mixture was administered. The physical examination consisted of measuring the heart rate, respiratory rate, rectal temperature, intestinal sounds through auscultation to determine motility, capillary refill time (CRT), and inspecting the mucosa. The physical examination was done at time 0 (control) to select the animals and daily until the end of the experiment; exams were always performed in the morning by the same evaluator.

The venom blood samples (3ml) were taken weekly on days 0, 7, 14, 21, and 28 (seven days after of the last treatment), from the left jugular vein using a vacuum system (Vacutainer BD®, Becton Dickinson, São Paulo, São Paulo, Brazil), disposable 40 × 9mm needles and tubes, one with 50µl of ethylenediaminetetraacetic acid (EDTA) K3 as an anticoagulant and another without an anticoagulant. The samples were put on ice and processed on the same day by the research laboratory at the Department of Internal Medicine of FMVZ-USP.

Using the automatic hematological analyzer (model ADVIA 2120, Siemens, Healthcare Diagnósticos S.A., São Paulo, Brazil), the following parameters were analyzed: erythrocyte count, leukocyte count, hemoglobin level, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and leukocyte differentiation.

Hepatic profiles (total protein, albumin, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), as well as indirect and direct bilirubin) and renal profiles (creatinine and urea) were analyzed using an automatic biochemist analyzer (model Labmax 240, Labtest, Lagoa Santa, Minas Gerais, Brazil). The fibrinogen was analyzed using Foster`s technique (1959), adapted by Jan (1986), which involves denaturing the protein at 56°C (132.8°F) and performing a refractometer reading.

After the blood was collected in tubes with sodium heparin, the samples were divided into aliquots for subsequent analyses. Each sample was divided into three 12 x 75mm test tubes made of transparent polystyrene for the following analyses: natural fluorescence, basal stress, and fluorescence after phorbol myristate acetate (PMA) activation. A 25µl sample was

required from each test tube for oxidative stress analysis. Only the basal stress and PMA test tubes received 200µl of the dichloro-dihydrofluorescein-acetate (DCFH) reagent (4091-99-0, Sigma, St. Louis, MO, USA) at a final concentration of 55µM. This reagent becomes green if a ROS is present in the sample and if it is excited with 488nm. In tubes for PMA evaluation, 100µl of the PMA reagent (16561-29-8, Sigma, St. Louis, MO, USA) was added at a concentration of 90.9ng/ml or approximately 150nM.

The reagent acts as a chemical stimulus for oxidative stress functioning via protein kinase C (PKC) and phospholipase D (PLD) in neutrophils. Phosphate-buffered saline (PBS) was added to all tubes to bring the final volume to 1.1ml. All tubes were incubated for 20 minutes at 37°C (98.6°F). Two ml of ammonium-chloride-potassium (ACK) lysing buffer was used after incubation to break apart red blood cells. After a 5 minute break, the tubes were centrifuged at 400g for 7 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 200µl of PBS for flow cytometry analysis.

The samples were verified by a flow cytometer (BD FACSCalibur, Beckton and Dickson, Sparks, Maryland, USA) using the acquisition software (BD CellQuest Pro 4.0.2; Beckton and Dickson, Sparks, Maryland, USA). The cell populations were defined by size-for-complexity graphs (FSC × SSC) on a linear scale. The populations of larger size and complexity were characterized as neutrophils and were used to define parameters of acquisition and subsequent analyses. White tubes were used to determine the basal fluorescence, while the basal stress was applied to detect green fluorescence positivity in the FL1 detector, without exceeding the possible limits. The tube with PMA was used to detect the fluorescence after cell activation, and the relation of basal stress and after PMA was used to determine the activation index. Seven thousand data points for the neutrophil population were saved for each file (animal/time). The files were analyzed with software (FlowJo LLC; Ashland, Oregon, USA). The program was asked to calculate the Geometric Mean on the FL1 channel, and the values obtained were exported to Microsoft Excel spreadsheets.

Data were analyzed for normality using the Kolmogorov-Smirnov test. The unpaired t-test was used for comparing TG data with CG data. Comparisons of post-treatment and baseline values were performed by variance analyses, followed by Tukey's post-hoc tests. Statistical analyses were performed using GraphPad Instat 3 (La Jolla, CA, USA). Statistical significance was set at $P< 0.05$.

RESULTS

Heart rate, respiratory rate, rectal temperature, intestinal motility, CRT, and mucosa inspection measurements did not change significantly between groups or between measurements within in each group ($P> 0.05$). The only notable observation was an increased defecation frequency in TG animals.

The number of red blood cells increased in the TG animals in relation to the baseline in all time points ($P= 0.049$) and compared to the CG animals on day 14 (D14) ($P= 0.049$) and D21 ($P= 0.045$) (Figure 1a). Hematocrit increased in the TG animals in relation to the baseline and in relation to CG animals on D7 ($P= 0.047$). In the CG, there was a decrease in the hematocrit in relation to the basal values on D14 ($P= 0.029$) (Figure 1b). Hemoglobin concentration increased in the TG in relation to the baseline after D7 ($P= 0.049$); however, only on D14, the hemoglobin concentration was higher in the TG animals than in the CG animals ($P= 0.019$) (Figure 1c). Platelet levels increased in the TG compared to baseline measurements on D21 ($P= 0.048$).

Still, there was not a statistically significant difference between TG and CG animals ($P> 0.05$) (Figure 1d). The MCHC increased significantly in the CG in relation to the baseline after D7, and in relation to the TG on D7 ($P= 0.001$), D14 ($P= 0.001$), and D21 ($P= 0.049$) (Figure 2a). MCV decreased in the CG in relation to the baseline after D7 ($P= 0.0006$). The MCV did not change in the TG. MCV increased in the CG in relation to TG on D14 ($P= 0.006$) (Figure 2b). Neutrophil counts decreased in the TG in relation to the CG only on D14 ($P= 0.033$), and there was no significant difference in the eosinophils, monocytes, lymphocytes, and white blood cell counts between the groups or between days in each group ($P> 0.05$).

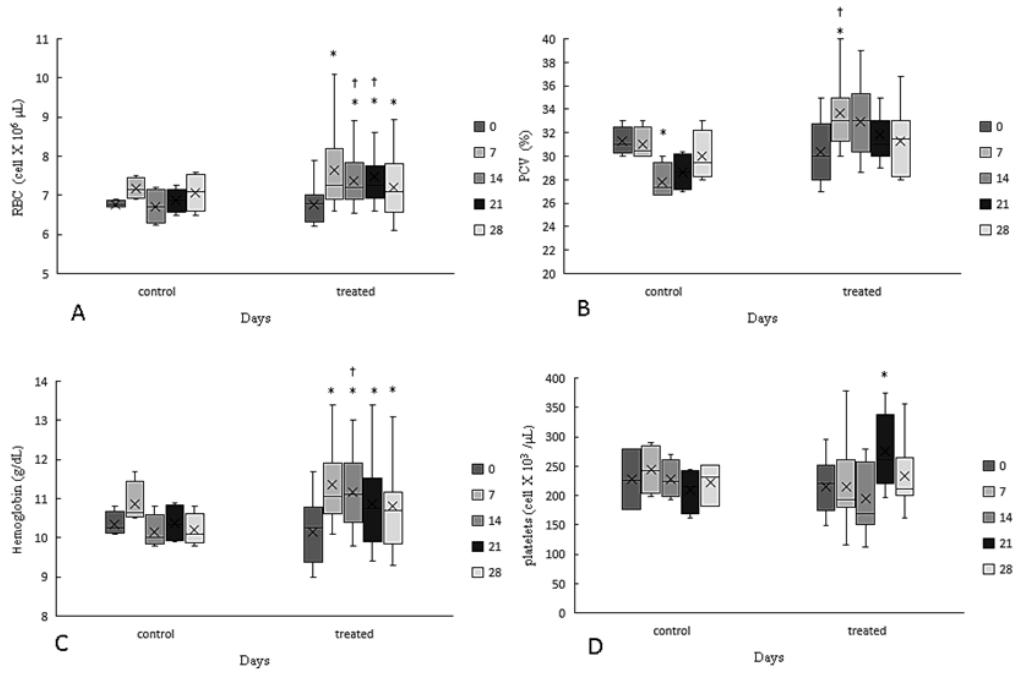

Figure 1. Quantitative data are given as box plots showing medians, means, and first and third quartiles for the O_3 treated and control groups. (A) Red Blood Cell Count (RBC) (cell x 10^6/µL); (B) Packet Cell Volume (PVC) (%); (C) Hemoglobin concentration (g/dL); (D) Platelet (cell x 10^3/µL). *Statistically significant differences compared to baseline values (P< 0.05). †Statistically significant differences compared to the control group (P< 0.05).

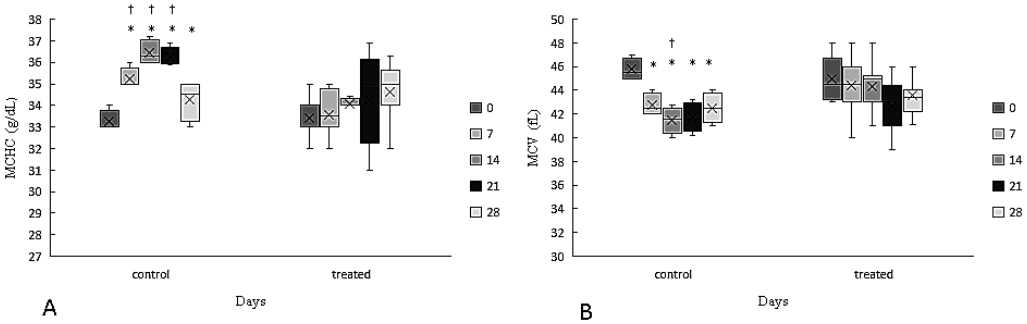

Figure 2. Quantitative data are given as box plots showing medians, means, and first and third quartiles for the O_3 treated and control groups. (A) MCHC (g/dL); (B) MCV (fL) *Statistically significant differences compared to baseline values (P< 0.05). †Statistically significant differences compared to the O_3 treatment group. MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume.

There was no significant difference regarding the plasmatic fibrinogen concentration between the groups, within each group, or in relation to the respective baselines (P< 0.05). Creatinine concentration increased in the TG in comparison to the baseline values only on D21 (P= 0.0001), however no significant increase was observed in relation to the CG (P> 0.05) (Figure 3a). Urea concentration did not change in the TG between days (P> 0.05) but was decreased in relation to the CG on D28 (P= 0.039). There was a decrease in urea concentration in the CG in comparison to the baseline values on D7 and D21 (P= 0.006) (Figure 3b).

Total protein concentration increased in the CG in relation to the baseline values on D7, D14 and D21 (P= 0.025); however, no difference was

observed between TG and CG (P> 0.05). There was no significant difference in TG total protein values (Figure 4a). Albumin concentrations did not change in the TG or CG (P> 0.05) (Figure 4b). Total bilirubin concentration increased in the CG in comparison to the baseline values after D7 (P= 0.0001), and in relation to the TG on D28 (P= 0.002) (Figure 4c). There was no significant difference regarding direct bilirubin (Figure 4d), AST and GGT concentration in both groups (P> 0.05).

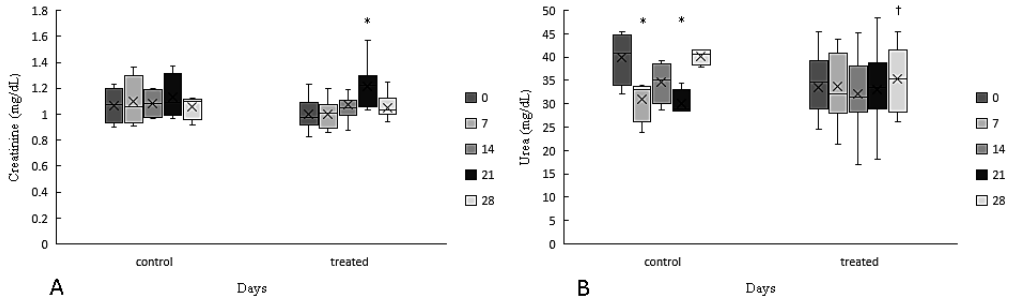

Figure 3. Quantitative data are given as box plots showing medians, means, and first and third quartiles for the O_3 treated and control groups. (A) Creatinine (mg/dL); (B) Urea (mg/dL) * Statistically significant differences compared to baseline values (P< 0.05). †Statistically significant differences compared to the control group.

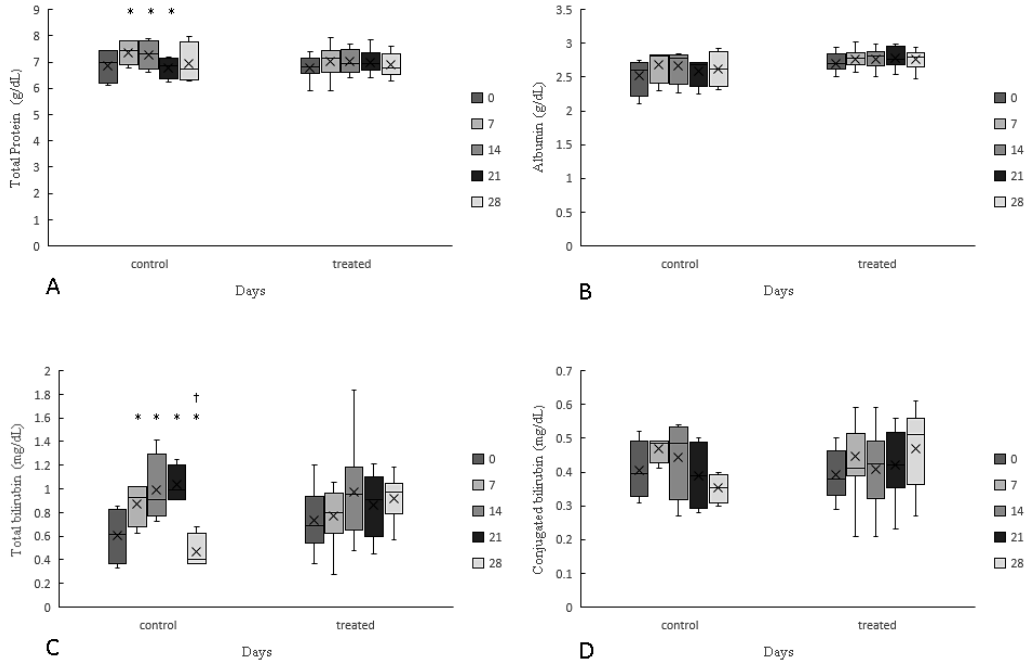

Figure 4. Quantitative data are given as box plots showing medians, means, first and third quartiles for the O_3 treated and control groups. (A) Total protein (g/dL); (B) Albumin (g/dL); (C) Total bilirubin (mg/dL); (D) Conjugated bilirubin (mg/dL). *Statistically significant differences compared to baseline values (P< 0.05). †Statistically significant differences as compared to the O_3 treated group (A).

An increase in the neutrophil activation index values (oxidative stress/basal stress) was observed in the CG in relation to the baseline on D11, D21, and D28 (P= 0.004). There was no significant difference between the days in the TG (P> 0.05). There was an increase in neutrophil activation index values in TG animals in relation to CG animals on D0 (P= 0.001) and D28 (P= 0.025) (Figure 5).

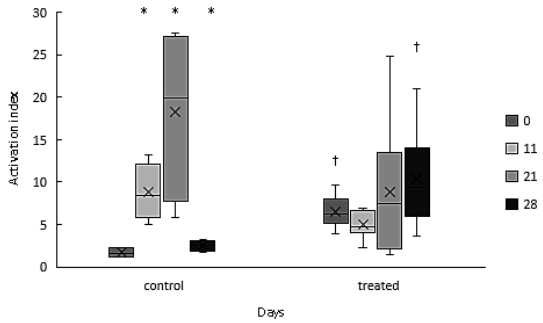

Figure 5. Neutrophil activation index values. Quantitative data are given as box plots showing medians, means, first and third quartiles for each experimental group. *Statistically significant differences compared to baseline values (P< 0.05). †Statistically significant differences compared to the control group.

DISCUSSION

Payr and Aubourg (1936) were the first to suggest a rectal insufflation of a gas mixture with O_3 in the colon, because it is a simple route of administration with a low cost. Currently, this technique is commonly used in humans around the world because it can be easily performed, has a low cost, and is mostly safe. The O_3 effect on red cells, including an increase in rheology proprieties and flexibility, were observed after an O_3 application during autohemotherapy in humans (Bocci, 1994; Gérard, 2001; Menéndez, 2008; Sousa, 2009). Other O_3 effects involve pH intracellular alterations and increased 2,3-diphosphoglycerate that create a right shift in the oxygen-hemoglobin dissociation curve, consequently increasing oxygen delivery to the tissues (Giunta *et al*., 2001; Sagai and Bocci, 2011).

The present study utilized the typical number of applications used in clinical treatments to test the safety of the technique in equines. The horses did not show any signs of discomfort or present behavioral changes during the rectal O_3 applications. The only prominent observation was increased defecation, probably caused by the mild rectal ampoule gas distension that facilitated a motility stimulus. The physiological parameters were within normal ranges during the treatment, similar to what was observed by Haddad (2009) in a study that used 500 and 1000ml of physiological saline with O_3 for an intravenous application on healthy horses.

In a study by López (2007) that used O_3 autohemotherapy in doses of 500 and 1000ml in horses, a significant increase in red blood cells was detected; this result is similar to observations in the present study. Therefore, it can be suggested that the results obtained in equines resemble human studies; that is, humans and horses respond to O_3 treatment in a similar way in both autohemotherapy and rectal insufflation (Menéndez, 2008; Sousa, 2009).

Besides the increased number of red blood cells, the TG presented an increased hematocrit and hemoglobin concentration concomitant with the maintenance of total protein and albumin values. On other hand, CG animals had decreased hematocrit levels, MCVs, and consequent increase in MCHC; however, measured values were within the reference parameters according to Carvalho *et al.* (2016). By generating red blood cells with a greater amount of hemoglobin and the consequent capacity of oxygen transport throughout the treatment, we can infer that O_3 contributes to improved oxygenation (Sagai and Bocci, 2011). Similar results were described by Bocci (1994) and Re (2008), who also observed improvements in the rheological properties of the blood accompanied by an increase in the red blood cell flexibility.

López (2007) and Penido (2010) affirmed that O_3 increased immunological system activity, stimulating the white cells and increasing leukocyte activity to detect pathogens. In our study, the number of leukocytes did not show any alterations, but this could be because the animals were healthy and without challenges during the experimental period. Thus, there was no stimulation before or during the experiment to activate defense mechanisms or rebalance them.

It has also been shown that the effects of O_3 metabolites may lead to increased white cell activity, helping to strengthen the body's defenses (Silva *et al*., 2009), which was not observed in the present study by flow cytometry analysis.

The utilized rectal insufflation doses did not provoke detectable inflammatory processes by the laboratorial exams since the plasma values of fibrinogen and protein remained unchanged throughout the experimental period. It also did not affect the renal function of the horses as the urea and creatinine values remained within the reference range for the species (Neves *et al*., 2014). We can also infer that transrectal O_3 insufflation did not interfere with the hepatic profile. The values of total and direct bilirubin had a non-significant increase in relation to the baseline values and remained within the reference range (Zobel *et al*., 2012). This effect occurs due the action of the aldehyde-albumin complex that increases heme-oxygenase-1, thus generating the release of carbon monoxide and bilirubin responsible for vasodilation and antioxidant activity *(*Bocci *et al*., 2011). It may also be caused by lysis of the old and rigid red blood cells that may occur during O_3 therapy, resulting in improved rheological properties of the blood.

AST remained stable in a study by Ajamieh *et al.* (2002) who used rectal O_3 insufflation to treat ischemia/reperfusion and in a study by Haddad *et al.* (2009) who used O_3 autohemotherapy. These results corroborate with the results of the present study. Flow cytometry was used to analyze leukocyte behavior and activation index changes. According to Bocci (2011), O_3 therapy affects the neutrophils by increasing their function, making them more efficient and easier to activate. In the present study, this phenomenon was not observed as there was no change in neutrophil activity; in contrast, we observed that the control group was more susceptible to challenges with PMA. Therefore, we can suppose that O_3 acted as a modulator of these reactions.

CONCLUSIONS

Transrectal O_3 application in equines is safe; it does not lead to harmful clinical and laboratory alterations. In addition, we can infer that transrectal O_3 therapy can indirectly improve tissue oxygenation and metabolism as well as modulate ROS production.

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