

Behavior of hematological variables in horses treated with ozone therapy (Comportamiento de las variables hematológicas en caballos tratados con ozonoterapia)

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Resumen

El comportamiento de las variables hematológicas (hematocrito, hemoglobina, eritrocitos y plaquetas) fue monitoreado, in vivo, en 12 caballos (6 machos, 6 hembras) con edades comprendidas entre 4 y 20 años de edad (11.8 ± 1), tratados con ozonoterapia. En los tratamientos fueron aplicados 500 o 100 ml de la mezcla oxígeno-ozono (O₂-O₃) por la vía endovenosa, cada tres días, durante 24 días. Los caballos fueron divididos en cuatro grupos: MT500 (tres machos recibiendo 500 ml), MT 1000 (tres machos recibiendo 1000 ml), FT500 (tres hembras recibiendo 500 ml) y FT1000 (3 hembras recibiendo 1000 ml). La ozonoterapia por la vía endovenosa, no causó cambios clínicos en los caballos. Los valores medios mínimos y máximos de las variables hematológicas fueron consideradas dentro de los valores normales de referencia para la especie equina. Hubo una tendencia de aumento en los valores del hematocrito en los caballos tratados con ozonoterapia, en cuanto que el conteo de plaquetas aumentó y disminuyó a lo largo de los tratamientos. De hecho, independientemente del sexo y de la dosis, los caballos respondieron inicialmente con aumento en el conteo de las plaquetas. Este hallazgo parece transitorio, ya que disminuye hacia el equilibrio durante el periodo en estudio. Eritrocitos alcanzaron medias más altas en las hembras que en los machos; pero la ozonoterapia no causó cambios en la hemoglobina. La exposición de caballos sanos a 500 y 1000 ml de la mezcla terapéutica de ozono por la ruta intravenosa no causó cambios clínicos.

Palabras claves: caballos, hematocrito, hemoglobina, eritrocitos, plaquetas.

Abstract

The behavior of blood hematological variables (hematocrit, hemoglobin, erythrocytes and platelets) was monitored *in vivo* in 12 crossbred horses (6 males and 6 females) with ages varying between 4 and 20 years old (11.8 ± 1) treated with ozone therapy. Treatments were carried out by applying 500 or 100 ml of the mixture oxygen-ozone (O_2-O_3) by intravenous route, every three days, during 24 days. Horses were assigned to four groups: MT500 (three males receiving 500 ml), MT1000 (three males receiving 1000 ml), FT500 (three females receiving 500 ml) and FT1000 (three females receiving 1000 ml). Ozone therapy by intravenous route caused no clinical changes in the horses. Minimum and maximum mean values of the hematological variables were within the ones considered as normal reference for the equine specie. There was a tendency to increase hematocrit values in horses treated with ozone therapy, whereas platelet count increased and decreased over the treatments. In fact, independently of gender and dose, horses respond initially with increases in platelet count. This finding appears to be transient as it decreases towards the equilibrium later in the study period. Erythrocytes had higher means in females than in males, but the ozone therapy caused no changes in hemoglobin. Exposure of healthy horses to 500 and 1000 ml of the therapeutic ozone mixture by intravenous route caused no clinical changes.

Keywords: horses, hematocrit, hemoglobin, erythrocytes, platelets, ozonized autohemotransfusion.

Abbreviations: M, male; F, female; MT500, males receiving 500 ml; MT1000, males receiving 1000 ml; FT500, females receiving 500 ml; FT1000, females receiving 1000 ml; O_2-O_3 , oxygen-ozone; EDTA, ethylenediaminetetraacetic acid; SCL, superior control limit; ICL, inferior control limit; PAF, platelet-activating factor; PLA_2 , phospholipase A_2 ; PLC, phospholipase C; PLD, phospholipase D.

Introduction

Since time immemorial, humans have been searching for alternative treatment with economically viable and efficient therapies. Ozone therapy has been widely used in human medicine in Europe, Asia and Cuba. It is now attracting the attention of veterinary medicine in these same regions as well as Latin America and the United States, as an efficient and low cost alternative or complement to traditional therapeutic protocols.

Ozone therapy uses a mixture of oxygen-ozone (O_2-O_3) obtained by passing pure oxygen through a high voltage and high frequency electric discharge. Ozone is reported to improve oxygenation and body metabolism (Recio Del Pino et al., 1999), as well as to have bactericidal, fungicidal and viricidal activities and improve blood circulation (Guerra et al., 1999). Blood perfusion through capillaries is improved by a slight blood pressure elevation, together with the rheologic properties of blood, increasing the capacity of

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distribution and absorption of oxygen in erythrocytes (Pérez et al., 1997). This effect leads to a remarkable improvement of microcirculation with consequent tissue oxygenation, which favors regeneration. Ozone also reduces platelet adherence, as well as acting as antialgic, anti-inflammatory and stimulant of the reticulo-endothelial system (Hernández and González, 2001). Ozone therapy in humans can be carried out by intravenous, intramuscular, subcutaneous, or intra-articulate routes, as well as by vaginal and rectal insufflation. It can still be applied in the form of autohemotransfusion (removal and ozonization of blood that later will be given back to the patient) and as topical treatment (with gas or ozonized oil) (Guerra et al., 1999). In veterinary circles, the most commonly used forms of application are topical and intramammary (Ogata and Nagahata, 2000, Camps et al., 2003). The respiratory route is not recommended as it can cause endothelial lesions, increase in permeability and local inflammation (Noa et al., 1991).

Little information on the effects of ozone therapy on hematological variables in equines is available, nevertheless, there are a few studies published on human red cells (Bocci, 2004), bovine white cells (Ogata and Nagahata, 2000, Terasaki et al., 2001, Ducussin et al., 2003, Ohtsuka et al., 2006) and guinea pig (*Cavia porcellus*) platelets (Wright et al., 1994) treated with the gas mixture. Thus, the objective of this work was therefore to monitor the behavior of hematological variables (hematocrit, hemoglobin, erythrocytes and platelets) in horses during ozone therapy by intravenous route.

Materials and Methods

Twelve crossbred horses (Quarter Horse and Pure Bred Andalusian and Quarter Horse and Peruvian), 6 males and 6 females, between 4 and 20 years old (11.8 ± 1) were used in the experiment. The selection criteria used for of these animals included: a clinically normal physical examination; hematological and blood biochemistry values within normal reference ranges for the species, and females neither pregnant nor lactating.

All horses were semi-confined in open stalls inside a small picket fence to receive the ozone application. They were fed star grass (*Cynodon nlemfuensis*) hay and ration (protein: 15%, minimum fat: 3%, maximum moisture: 13%, maximum fiber: 10% and maximum ash: 10%) and were given *ad libitum* water.

All the horses were administered vermifuge 30 days before the experiment started. The open stalls were covered with a roof, reducing the possibility of thermal stress, changes in heart and respiratory rates and changes in the variables of blood biochemistry that would be assessed in the study.

Before initiating the therapy, the animals were sensitized to ozone using the technique described by Scrollavezza et al. (1997). A 250 ml blood sample

was collected from the jugular vein in phlebotomy bags containing 3.8% sodium citrate as anticoagulant. The blood sample was treated with 250 ml of ozone at $30 \mu\text{g}\cdot\text{ml}^{-1}$ (Table 1) using a Meditronica® ozoniser (Horse acupuncture: Carretera 42 #7 A sur 92, Apto. 1203, Balsos de Oviedo, Medellín, Colombia) connected to a portable 2000 lb oxygen tank. Autohemotransfusion was performed immediately after the *ex vivo* blood ozonization.

Three days after sensitization, was initiated the treatment of horses by intravenous route of the $\text{O}_2\text{-O}_3$ mixture, administered in 250 ml of 0.9% sterile sodium chloride.

Two treatments were performed: T500 consisted of six horses, three females (F) and three males (M) that received maximum volume of 500 ml of the gas mixtures (Table 1). In the second treatment (T1000), the other six animals (three females and three males) received a growing dose of the gas mixture, however the maximum volume was 1000 ml (Table 1).

Table 1. Protocol of ozone therapy used in horses during sensitization (autohemotransfusion) and treatments (intravenous route), at different application periods

	Sensitization (OAH)	Treatment: 500 ml of ozone (O_3) (T500)										PP
	Pre- ozonization period (day zero)	250 ml ($\text{O}_2\text{-O}_3$)	Time	3	6	9	12	15	18	21	24	27
(days)												
		$\text{O}_2\text{-O}_3$	500	500	500	500	500	500	500	500	500	
		Treatment: 1000 ml of ozone (O_3) (T1000)										
		Time	3	6	9	12	15	18	21	24	27	
		(days)										
	250 ml ($\text{O}_2\text{-O}_3$)	$\text{O}_2\text{-O}_3$	500	750	1000	1000	1000	1000	1000	1000	1000	---

OAH: ozonized autohemotransfusion; PP: post-ozonization period.

The applications were applied every three days, totaling eight applications per treatment. In this way, four work groups were formed: MT500 (three males receiving 500 ml), FT500 (three females receiving 500 ml), MT1000 (three males receiving 1000 ml) and FT1000 (three females receiving 1000 ml). The final ozone dose the horses were given was 35 and $70 \mu\text{g kg}^{-1}$, in the treatments 500 and 1000 ml, respectively.

Clinical and laboratory variables

Heart and respiratory rates, rectal temperature, capillary refill time and mucous membrane color of all animals were taken every day. In addition, blood samples were collected to determine hematocrit, hemoglobin, erythrocytes and total platelet count. These variables were analyzed before (time 0) sensitization, as well as immediately before each session of ozone therapy and at three times (1, 6 and 24 hours) after ozone therapy. Additionally, a new recording was taken three days after ending treatments on the 27th day (Table 1).

Samples of 5 ml of blood were collected by jugular puncture using standard vacuum glass tubes containing EDTA as anticoagulant. Erythrocyte count (cells $\times 10^6 \cdot \text{ml}^{-1}$), as well as hemoglobin ($\text{g} \cdot \text{dl}^{-1}$) and hematocrit (%) determination were performed using a semi-automatic Cell Dyn 400 blood analyzer (Rankin Biomedical Corporation: 14515 Mackey Road, Ste. 100 Holly, MI 48442, USA) The platelet count was determined in blood smears under an Olympus CX31 microscope (International Medical Equipment: 170 Vallecitos De Oro, San Marcos, CA 92069, USA).

Behavior of haematological variables (hematocrit, hemoglobin, erythrocytes and platelets) over the experimental time (0, 3, 6, 9, 12, 15, 18, 21, 24 and 27 days) was monitored by the Shewhart xbar chart ($\alpha=0.0027$) for each experimental group (MT500, MT1000, FT500 and FT1000). Comparison among groups was based on superior (SCL) and inferior (ICL) control limits. Groups presenting means within these limits were considered similar, and these variations were considered of random order.

Mean values above SCL or below ICL indicated that the experimental group was different from the others ($\alpha=0.0027$) at the evaluation day. But the effect, or lack of it, of the application period on the hematological variables was assessed by the Durbin-Watson test ($\alpha=0.05$), when it detected a self-relationship of dependence or independence, respectively.

Results

All horses used in this research were obtained from the agricultural school, where the study was conducted. Animal feeding was the same in all treatments, before and during the study. Nutritional status and presence of helminthes did not affect the present study; only horses presenting good nutritional scores and previously submitted to a strategic control of helminthes were used in the experiments.

No horse experienced discomfort or showed change in behavior during ozone therapy. The mean values for body temperature, heart and respiratory rates were 37.01°C , 34 beats per minute and 24 breaths per minute, respectively. The mucous membranes remained pink and moist and the capillary refill time was a constant two seconds.

During ozone therapy, means of minimum and maximum hematocrit values were 34.9% and 44.87%, respectively. There was, however, a growing effect ($p < 0.05$) on hematocrit concentration (Fig. 1a) in the tested combinations. This effect was less significant in the horses treated with the lowest dose (500 ml), which had lower means ($p < 0.0027$) than the horses treated with the highest dose (1000 ml) on the last day of evaluation.

When monitoring hemoglobin during the therapy, the means of minimum and maximum concentrations were 9.08 g.dl⁻¹ and 11.2 g.dl⁻¹, respectively. Stability and similarity were found ($p > 0.0027$) among means of the four groups, with no occurrence of important changes ($P > 0.05$) over the period of application (Fig. 1b), except for the last two evaluations of females treated with 1000 ml.

The means of minimum and maximum erythrocyte concentrations during ozone therapy were 7.26 x10⁶.µl⁻¹ and 9.88 x10⁶.µl⁻¹, respectively.

There was a tendency for increase ($p < 0.05$) in the means of erythrocyte concentration only for the group F1000 (Fig. 2a), which together with the group F500 had higher means ($p < 0.0027$) than the males treated with 500 or 1000 ml, at the end of the experiment.

During the ozone therapy, the means of minimum and maximum platelet counts were 7 and 12.42 platelets per field, respectively.

Following the first ozone administrations, there was a large increase ($p < 0.0027$) in platelet count (Fig. 2b) in all the horses, regardless of gender or dose of gas mixture based on values above SCL for the random variation. Interestingly, there was a marked reduction in the platelet count after the fifth and sixth applications that reduced the values to near baseline. Following this time period there was a random fluctuation in platelet count, and the values kept constant ($p > 0.05$) until the end of ozone administrations, except for the mean of the group MT1000 that was slightly above ($p < 0.0027$) SCL, which was not considered relevant.

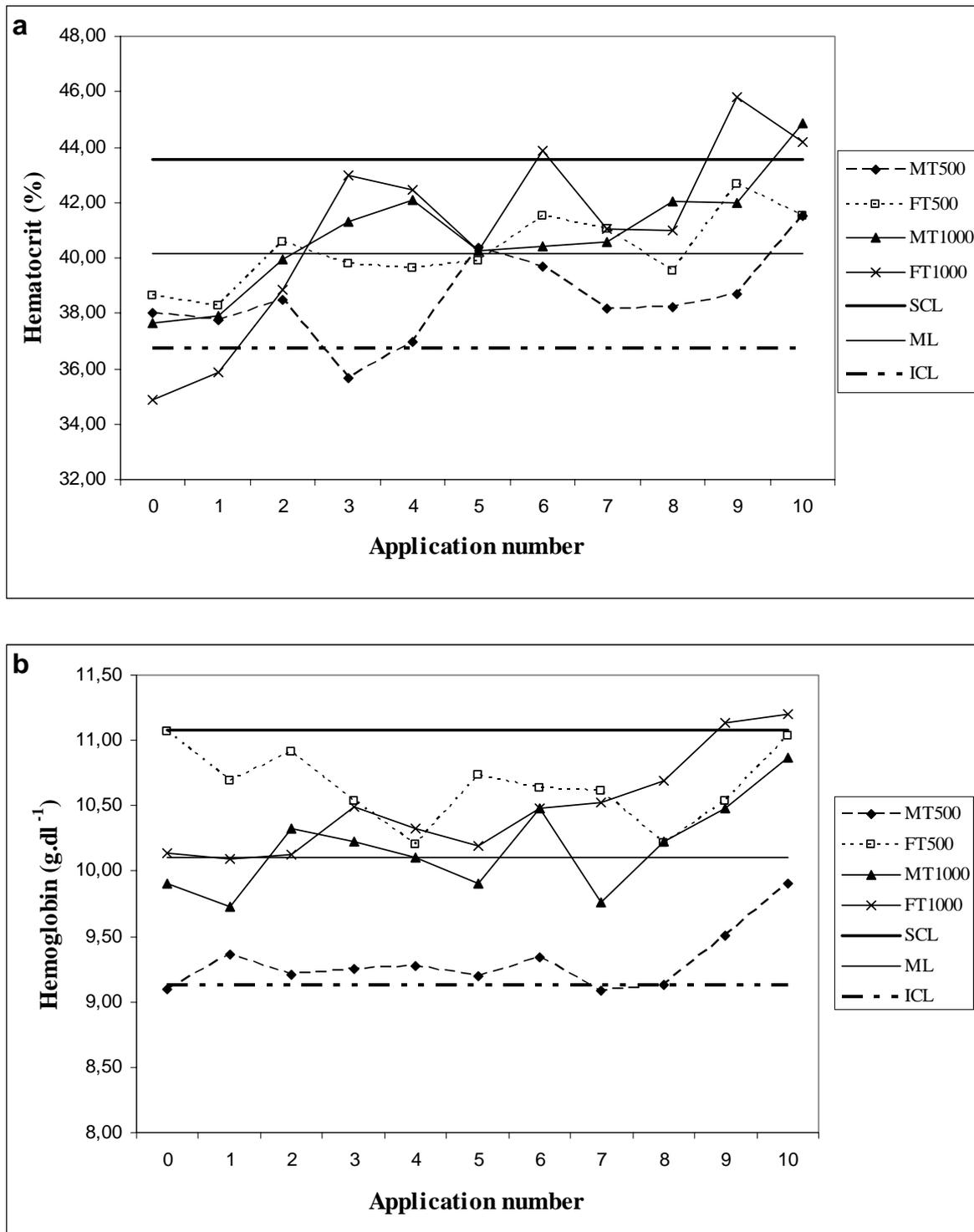


Fig. 1. Means for hematocrit (%) (a) and hemoglobin (g.dl⁻¹) (b) concentrations in MT500 (males treated with 500 ml), FT500 (females treated with 500 ml), MT1000 (males treated with 1000 ml) and FT1000 (females treated with 1000 ml), over the period of ozone application. SCL: superior control limit, ML: medium limit, ICL: inferior control limit (Applications 1-9). Application zero: corresponds to the beginning of the experiment, when the animals still had not been exposed to the gas mixture. Application 1: sensitization of animals. Application 10: mean values of sampling results three days after the last ozone application.

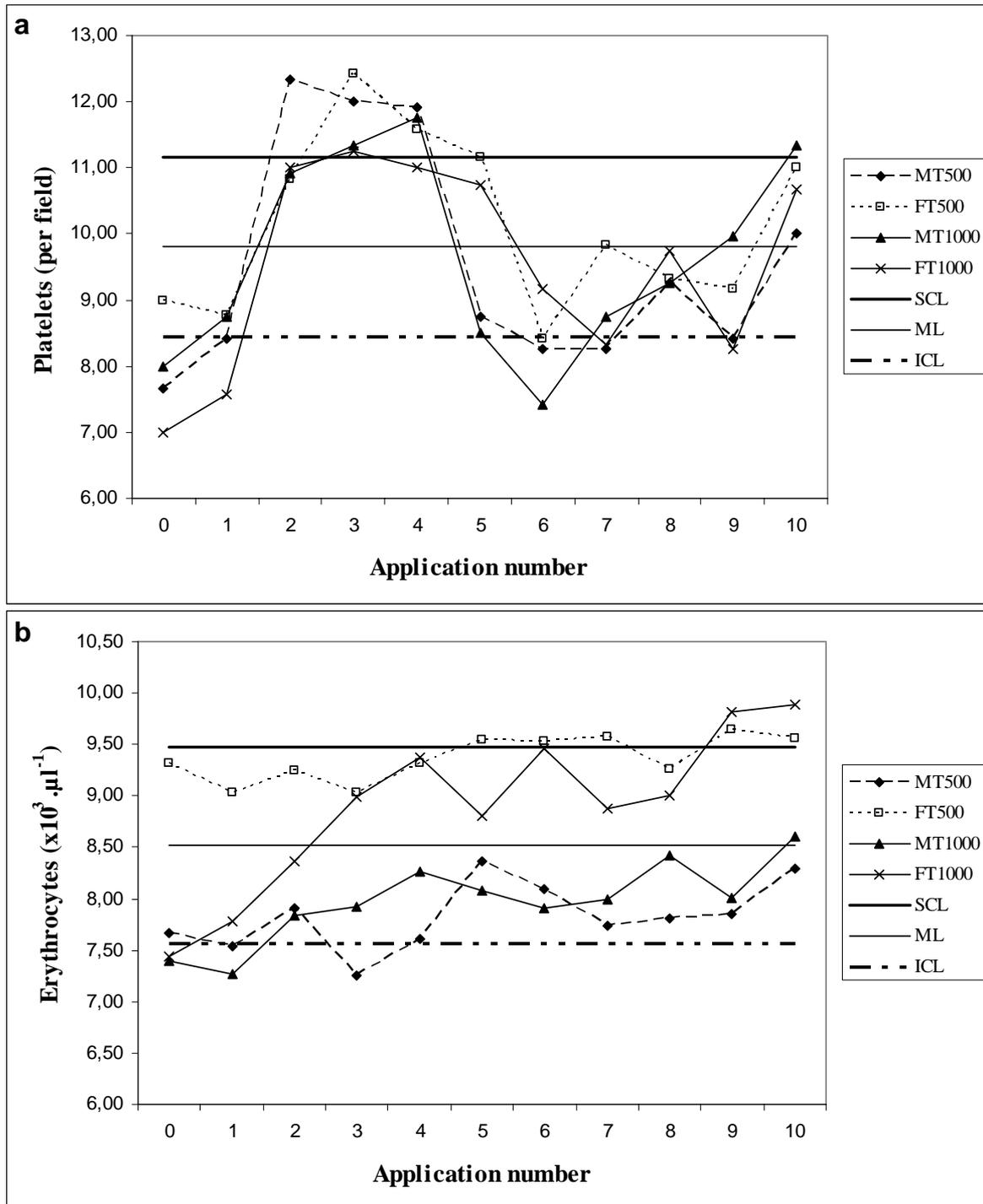


Fig. 2. Means for platelets count (per field) (a) and erythrocytes ($\times 10^6 \cdot \mu\text{l}^{-1}$) (b) concentrations in MT500 (males treated with 500 ml), FT500 (females treated with 500 ml), MT1000 (males treated with 1000 ml) and FT1000 (females treated with 1000 ml), over the period of ozone application. SCL: superior control limit, ML: medium limit, ICL: inferior control limit (Applications 1-9). Application zero: corresponds to the beginning of the experiment, when the animals still had not been exposed to the gas mixture. Application 1: sensitization of animals. Application 10: mean values of sampling results three days after the last ozone application.

Discussion

During the experiment, the physical variables were within normal reference range for the equine species (Speirs, 1999).

Independent of the studied group, the mean of minimum and maximum hematocrit percentages during ozone therapy were within the reference range (26-53%) for equines (Schalm et al., 1975, Jain, 1993). In spite of the difference ($p < 0.0027$) between the groups that received 500 and 1000 ml, the results indicated that hematocrit values increased with ozonization, showing a possible accumulative and dose-dependent effect. A similar accumulative and dose-dependent effect has been described in humans (Bocci, 1994, Valacchi and Bocci, 1999), rats (Plopper et al., 1994), guinea pigs (Wright et al., 1994) and horses (Deaton et al., 2005) with the response defined by the dose and the time of exposure to the gas mixture. Verrazo et al. (1995) and Giunta et al. (2001) found no changes in the hematocrit concentration of human patients with peripheral arterial obstructive disease treated with ozone therapy. Nevertheless, Verrazo et al. (1995) detected increase in erythrocyte filterability, and a decrease in blood viscosity 15 minutes after the first and 24 hours after the last treatments.

The means of minimum and maximum hemoglobin concentrations during ozone therapy were within the normal reference range (8-19 g.dl⁻¹) for the equine species (Schalm et al., 1975, Jain, 1993). The highest means ($p < 0.0027$) presented by the females treated with 1000 ml at the end of the therapy may be explained by their relatively higher values throughout the experiment.

The slight increase in hemoglobin concentration found in the group F1000 may be caused by the ozone. Other causes of increased hemoglobin concentration would be more intense physical activity (Pérez et al., 1997, Martínez et al., 2000, Householder and Douglas, 2005, Stoiber et al., 2005), because of the higher demand for oxygen (Mancini et al., 2003). However, in this study the physical exercise was standardized during the whole experimental period so this factor should not have played a role in the increase noted.

Independently of the studied group, the means of minimum and maximum erythrocyte concentrations during ozone therapy were within normal reference (5.5-13 x10⁶.µl⁻¹) for horses (Schalm et al., 1975, Jain, 1993). Schalm et al. (1975) reported that female Quarter Horses can have mean concentration of red blood cell variables (erythrocytes among them) higher than the males. The difference in the response to ozone therapy observed in this study between males and females is highly evident, with the latter being more responsive to ozone application and producing higher erythrocyte counts independent of the amount of gas mixture. This is in contrast to what Schalm and collaborators reported in 1975. It is interesting to note that Moran & Araya (2003) reported that under normal conditions, it would be more common to

find higher concentration of erythrocytes in males, since testosterone stimulates erythropoiesis. This information reinforces the effect of the ozone therapy on the females of this study.

The animals did not undergo other activities or effort greater than they were conditioned to. Moreover, the people in charge of managing the horses during the experiments were the same along all the experimental period, with no occurrence of climatic changes as well. In this way, we discarded the possibility of a likely increase in erythrocyte and hematocrit values due to factors pointed out by the literature such as stress, fear, activities the animals were not used to or climatic changes that could cause alterations to these variables (Pérez et al., 1997, Martínez et al., 2000, Householder and Douglas, 2005, Gill and Wanska, 1978) derived from splenic contraction. This study indicates that the increase was caused by the action of the several applications of the oxygen-ozone mixture.

During the ozone therapy, the means of minimum and maximum platelet counts were, independently of the group, within normal reference range (5-17.5 platelets per field) for the equine species (Schalm et al., 1975, Jain, 1993). Platelets play an important role in blood fluidity, hematocrit, circulating erythrocytes and total plasma protein concentration in the blood and plasma (Birchard, 1997, Stoiber et al., 2005). According to Zee & De Monte (2001), ozone acts by reducing blood and plasma viscosity in humans through a reduction of plasma macromolecules and blood clotting. This action can be confirmed by the increase in thrombin time (TT), von Willebrand factor (vWF) and plasminogen (tPa), as well as decrease in fibrinogen.

The direct action of ozone on the erythrocyte membrane causes an increase in its fluidity and decrease in cell adhesion, with consequent lower blood viscosity (Bulies, 1996). The increase in number of platelets per field observed in the animals of this study may be caused by the release of platelet-activating factor (PAF). Wright et al. (1994) described results from the selective ozone effect on cell membranes, inducing mainly cleavage of arachidonic acid and producing this lipid mediator through the action of phospholipases (PLA₂, PLC, PLD).

There was no difference in behavior of variables in relation to gender or dose, which is different from findings reported by Wright et al. (1994) of an *in vitro* study on platelets of guinea pigs treated with ozone. The authors discussed the action of ozone on platelet membranes, pointing out an increase in cell permeability that acts as a sign for the activation of fibrinogenic processes. This can be a direct response to the gas mixture or to by-products of the ozone reaction. Blood fluidity might be the initial physiological response to ozone exposure, just as it is described in the literature (Verrazo et al., 1995) for humans. In horses, however, the results from the present study indicate that some unknown factor may be responsible for the increase in platelet concentration, which is possibly opposed to the findings reported for other species.

In conclusion, this study shows that there is a tendency to increase hematocrit values in horses treated with ozone therapy, whereas independently of gender and dose, horses respond initially with increases in platelet count. This finding appears to be transient as it decreases towards the equilibrium later in the study period. Additionally ozone therapy induces higher erythrocyte concentration in females, with no changes in hemoglobin. Furthermore exposure of healthy horses to 500 and 1000 ml of the therapeutic ozone mixture by intravenous route causes no clinical changes.

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This work was approved by the Ethic Committee of Veterinary Department of the Federal University of Viçosa (Process number 67/2005), and the assays is in agreement with the Medical Veterinary Professional Ethics Code, with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation and with actual Brazilian legislation, as well as the guiding principles in the use of animals in toxicology.

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