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Effects of ozone therapy on hematological, biochemical, and oxidative stress parameters of vaquejada athlete horses

[*Efeitos da ozonioterapia sobre os parâmetros hematológicos, bioquímicos e estresse oxidativo de cavalos atletas de vaquejada*]

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ABSTRACT

Ozone therapy is a technique used in several specialties of equine medicine; however, there are few studies on its use in *vaquejada* (cowboy competition) athlete horses. This study aims to evaluate the potential effect of ozone gas administered by two different routes on hematological and biochemical values and the oxidative stress marker in *vaquejada* athlete horses. For this, nine healthy equines that followed a training protocol and underwent two treatments were used with an 8-day wash-out between them. The major ozonated autohemotherapy (MOA) treatment group received a volume of 600ml of the O2-O3 mixture at a concentration of 60 µg/mL, and the rectal insufflation (RI) treatment group received 5mL of gas per kg of body weight at a concentration of 15µg/kg performed every 24h on three consecutive days. Results were significant for RBC, hematocrit, and hemoglobin in the hematological variables, and AST and lactate for biochemical and malondialdehyde variables. No statistically significant differences were found in comparisons between treatment groups. Thus, we can conclude that there is no difference between the two therapies, indicating that the two techniques are effective for the application of ozone therapy in horses competing for *vaquejada.*

Keywords: ozone, lipid peroxidation, antioxidants, equine, sport

RESUMO

A ozonioterapia é uma técnica utilizada em diversas especialidades da medicina equina, contudo, são escassos os estudos de sua utilização em cavalos atletas de vaquejada. O presente estudo tem por objetivo avaliar o potencial efeito do gás ozônio administrado por duas diferentes vias sobre os valores hematológicos, bioquímicos e no marcador de estresse oxidativo em cavalos atletas de vaquejada. Para isso, foram utilizados 9 equinos hígidos que seguiram um protocolo de treinamento e foram submetidos a dois tratamentos, com um wash-out *de 8 dias entre eles. O grupo de tratamento auto-hemoterapia maior ozonizada (AHTMO) recebeu um volume de 600ml da mistura O2-O3 na concentração de 60 µg/mL e o grupo de tratamento insuflação retal (IR) recebeu 5mL de gás por kg de peso vivo, na concentração de 15µg/ kg, realizado a cada 24h em três dias consecutivos. Os resultados demonstraram-se significativos para hemácias, hematócrito e hemoglobina nas variáveis hematológicas, AST e lactato para as variáveis bioquímicas e para o malondialdeído. Não foram encontradas diferenças estatísticas significativas nas comparações entre grupos de tratamento. Assim, pode-se concluir que não há diferença entre as duas terapias, indicando que as duas técnicas são eficazes para a aplicação da ozonioterapia em cavalos competidores de vaquejada.*

Palavras-chaves: ozônio, peroxidação lipídica, antioxidantes, equino, esporte

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INTRODUCTION

Vaquejada is an equestrian sport traditionally practiced in the northeastern region of Brazil (Santiago *et al*., 2013; Bastos *et al*., 2017). The athletic activity developed by *vaquejada* horses is considered high intensity and short duration, making the anaerobic pathway prevail for energy production
(Lopes *et al.*, 2009; Hunka *et al*., 2017; Sousa *et al*., 2018). Physical exercises with these characteristics induce changes in hematological and biochemical parameters and the oxidative metabolism of horse athletes (Balogh *et al*., 2001; Sagai and Bocci, 2011; Sousa *et al*., 2018).

There is an increase in oxygen consumption by muscle mitochondria during sports activity, which will convert the consumed oxygen into carbon dioxide and water. However, 2% to 5% of this oxygen is not converted and will result in the formation of reactive oxygen species (ROS) (Piccione *et al*., 2007; Soares *et al*., 2011; Antunes, 2013; Bottegaro *et al*., 2018). The body balances ROS production and degradation (Wulf, 2001). However, when ROS production increases to the limit where the organism cannot eliminate or neutralize it, a condition called oxidative stress (OS) occurs (Fernandes *et al*., 2012).

In *vaquejada* competitions, horses are exposed to continuous days of exercise with reduced moments of rest, deviating from their regular exercise routine and increasing physical activity during competition days (Lopes *et al*., 2009). This increased exercise load, and the resulting OS can decrease performance during competition due to muscle tissue injury and decreased skeletal muscle force production (Ott, 2021).

Malondialdehyde (MDA) is the best known and most reliable marker of lipid peroxidation, widely used as evidence of oxidative stress installed in the animal's body (Kerksick and Willoughby, 2005; Sara *et al*., 2012).

Ozone therapy is a complementary therapeutic technique that uses an oxygen-ozone mixture (95%-99.95% oxygen and 0.05%-5% ozone) (Schwartz *et al*., 2020). Ozone therapy's therapeutic window ranges in concentrations from 10 to 60μg/ml (Schwartz *et al*., 2020) and produces immunomodulatory (Jaramillo *et al*., 2020), analgesic, anti-inflammatory (Escodro *et al*., 2012; Jaramillo *et al*., 2020), bactericidal, antiviral, and antifungal effects (Schwartz *et al*., 2020).

The effects produced by O3 are a result of its action mechanism, which, through a moderate, adequate, and transient oxidative stress, induces the activation of second messengers, such as nuclear factor related to erythroid 2 (Nrf2) and lipid peroxidation products, such as malondialdehyde and 4-hydroxynonenal, which will increase the synthesis of different antioxidant enzymes and, consequently, promote the growth of the total antioxidant capacity of the organism (Smith *et al*., 2017; Rosseto, 2020; Sciorsci *et al*., 2020; Farias *et al*., 2020).

In this context, this study aims to evaluate the effects of ozone therapy administered by different routes on the hematological and biochemical parameters and on the lipid peroxidation index of vaulting athlete horses.

MATERIAL AND METHODS

The research was approved by the Ethics Committee on Animal Use of the Federal University of Alagoas (Ceua/Ufal), under number 02/2021.

The experiment was conducted at Grupo Aliança de Vaquejada, located in the municipality of Viçosa, Alagoas (geographical coordinates: latitude -9.325494 south, longitude -36.303363 west), in October and November 2021.

Nine animals of the Quarter Horse breed were selected for the experiment, six males and three females, with a mean age of 4.2±0.94 years and a mean weight of 474.89±30.82kg. The feeding program consisted of 6 kg of commercial concentrate per day (14.0% crude protein, 3.5% ethereal extract, and 2.9 Mcal of digestible energy) supplied in three meals (7 am, 12 noon, and 5 pm), 12kg of Tifton (Cynodon dactylon) based volume also divided into three times, plus mineral salt (EQX Pro - Integral Mix®) and water ad libitum.

All animals underwent a previous physical examination consisting of measuring heart rate

(HR), respiratory rate (RR), rectal temperature (T ºC), intestinal motility through auscultation, capillary refill time (CRT), and mucosal inspection. For selection criteria, the scale for evaluating welfare in equine athletes, adapted from Coelho *et al*. (2018), was taken as a basis considered in the Welfare Evaluation Guide of the Brazilian Vaquejada Association (Manual…, 2018), as described in Table 1.

Table 1. Physiological, hematological, and biochemical parameters considered for the inclusion criteria of animals in the research, adapted from Coelho *et al*. (2018)

		Assessment	
	Assessed variable	0	1
1	Body score (BS), with levels from 1 to 9, ranging from very thin to obese, as described by Henneke (1983).	4-6	≤3 ≥7
2	Heart rate (HR) in beats per minute (bpm) measured with a stethoscope in the left thoracic region, caudal to the elbow.	20-50	Above 50
3	Globular Volume (GBV) to detect possible anemia. 28% was considered as the minimum hygiene limit (Gul *et al*., 2007; Dias, 2014; Silva *et al*., 2018).	≥ 28%	< 28
4	Total plasma proteins (TP) to detect possible dehydration, considering values above 8 g/dL as worthy of scoring.	≤8	>8
5	Systemic inflammation, assessed by plasma fibrinogen concentration for being a positive regulatory acute phase protein with a tolerable value of up to 400 mg/dL	≤400	>400
6	Muscle stress through serum measurement of the enzyme creatine kinase -CK, considering the scoring range in values higher than 450 IU/L, following observations by Valberg *et al*. (1993).	≤450	>450
7	Presence of injuries/bleeds on the animals caused by the harness, bridle by "hackamore," or recent accidents (identify in the review).	Absent	Present
8	Presence of pain or claudication (including dorsal tenderness) through classification according to OBEL (1-5), identifying the region.	Absent	Present
9	Presence of stereotypies or aggressiveness, assessing the animal in the stall for two hours to identify which option.	Absent	Present

The observation of all animals was performed by two researchers interspersed between days to verify the occurrence of stereotypes. The animals were assessed in their environment, keeping a distance between the observer and the animal to prevent interest in human presence. The types of behaviors classified as normal (distracted, alert in the station, lying down, muzzle close to the ground, and neighing) or abnormal (aggression, digging, stereotypical stall walking, repeated head movements, wolf dance or bear syndrome or "weaving", aerophagia, wood biting, coprophagia, and trough licking) were considered.

The animal's inclusion in the research was conditioned to obtaining a score between 0-3 in the sum of the scores of the variables described in Table 1 as proof of good-welfare practices applied in their sporting activities, according to Coelho *et al*. (2018) (Table 2).

Table 2. Scoring system of the welfare scale for athletic horses, adapted from Coelho *et al*. (2018)

Score	Conclusions and intervention
0-3	Animals submitted to good-welfare practices in their sporting activities.
4-6	Animals that must undergo reassessment due to compromised welfare (zootechnical, veterinary professional intervention).
7-9	Animals with compromised welfare in need of activity interruption and urgent professional intervention.

The athletes were subjected to the same training system, ensuring standardization in their physical conditioning, constituted as follows: day 1- simulation of *vaquejada* according to the proposal by Santiago *et al*. (2013), where each horse runs three times with a 2-min interval

between each race; day 2- a 60-minute walk in the morning (aerobic work); day 3- same as day 1; day 4- same as day 2; day 5: total rest in paddocks. As described, a training cycle was considered for each set of sequential five-day activities, which was repeated three times during the experimental period.

Blood samples for hematological, biochemical, and malondialdehyde (MDA) evaluations were aseptically collected from peripheral blood by jugular venipuncture. They were then packed into 4mL tubes, two containing ethylenediaminetetraacetic acid tripotassium (EDTA k3) and another containing a clot activator. The tubes were packed in a thermal box with reusable ice packs for storage and transport to the laboratory.

The first collection was performed at baseline (T0), and the animals were at rest for seven days without any physical activity. The next day, after sample collection, the first five-day training cycle was initiated, as described above, with the subsequent inclusion of the animals in the two experimental groups, the first group of greater ozonated autohemotherapy (MOA) and the second group of rectal insufflations (RI). The ozone therapy employed in these groups is fully described in item 2.4. New blood collections were performed after 24h (T1), 48h (T2), 72h (T3), 7 d (T4), and 15d (T5). The wash-out between treatments was eight days, considering seven days of rest for the animals.

The samples collected in one of the tubes with EDTA were used for hematological analyses. The parameters were evaluated in the erythrogram: Red blood cells count (RBC), Hemoglobin level (HG), Hematoctit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin Concentration (MCHC), Platelets count (PLT), plasma total plasma protein concentration (TPP), Fibrinogen (FIB), and in the leukogram, the parameters were evaluated: Leukocytes total count (LEUC), Rods (ROD), Neutrophils (NEU), Lymphocytes (LIN), Monocytes (MON), Eosinophils (EOS), Basophils (BASO). Hematological measurement was performed with the aid of an automated device model BC-30Vet®, Mindray (Shenzen, China) and differential counting on blood smear stained by the Romanowsky method (Panoptic) using a Nikon microscope (E100®) and an Inbras® manual cell counter (ALB300CC). The measurement of fibrinogen was performed by the heat precipitation method in a water bath (Kacil bm-03®) at a temperature of 56°C for three minutes, and the result corresponds to the difference between the protein concentrations pre- and post-precipitation and centrifugation of the sample, measured in mg/dL using a portable Instrutherm® refractometer (RTP-20ATC).

Samples collected in a tube with a clot activator were intended to determine the serum activity of the enzymes: Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Creatine Kinase (CK), Alkaline Phosphatase (ALP), Gamma Glutamyl Transferase (GGT), plasma lactate concentration and the serum concentration of Urea and Creatinine (CREAT). The serum was centrifuged at a speed of (4000 rpm/5 minutes) in a KASVI tube centrifuge (MODEL K14-0815), and the serum was stored in Eppendorf tubes containing 1000 µl each. The concentration of blood lactate and biochemical analyses were measured in the BIOCLIN 2200® device using the enzymatic and kinetic UV method, respectively, using Bioclin Quibasa Química Básica Ltda commercial kits.

MDA levels were measured by high-performance liquid chromatography (HPLC) coupled with UV detection at 270 nm. For analyses, samples were stored on ice and transported to the laboratory, where they were centrifuged at 4,000 rpm for 10 minutes at 4°C to separate plasma from RBCs. The plasma was divided into 1.5mL Eppendorf tubes stored in a freezer at -80°C until analysis.

For analysis in HPLC, the solution was filtered through a sterile 0.22µm pore size Durapore membrane filter. A volume of 50 µL of the filtrate was injected into the HPLC, and the reading was performed at 270 nm. The reading lasts a total of six min, where the MDA retention time is around 2'81". MDA values were assessed from the standard curve using 1,1,3,3-tetramethoxypropane (TMP), a precursor compound of MDA, and expressed as nanomol of MDA.mL-1 of blood.

The MOA group's treatment was adapted from the treatment proposed by Tsuzuki *et al*. (2015). Through left jugular venipuncture, and after trichotomy and antisepsis with alcohol and

iodine at the site, the collection of two CPDA-1 transfusion bags (JP Indústria Farmacêutica SA) was performed. The anticoagulant volume in the bag was adjusted to a 300 ml blood volume per bag. The blood bags were weighed on scales (Toledo Prix 3/14) to standardize the amount of blood collected, and the collection was stopped when the total weight reached 375 g (315 g of blood + 60 g of the package and diluent). The convention that every 1 ml of blood equals 1.05g was used to calculate the total blood weight (YAGI, 2016).

Then, a volume of 300 mL of the oxygen/ozone mixture was infused per bag at a 60 µg/mL concentration, making slight movements to homogenize the mixture. A transfusion line was attached to the bag, and the ozonated blood was slowly reinfused into the animal at a transfusion rate of 3.2mL/kg/hr.

The RI group's treatment methodology was adapted from that proposed by Jaramillo *et al*. (2020). The animals' rectal ampulla was emptied by palpation to increase the contact surface of the ozone with the mucosa. For O2/O3 gas administration, a urethral probe no. 8 was inserted coupled to the ozone generator extender (Ozone & Life® - São José dos Campos, SP, Brazil).

The O2 flow and the electrical discharge meter of the ozone generating machine were regulated so that 5mL of gas per kg of body weight was administered at a concentration of 15µg/kg.

For administering the treatments, the animals were randomly divided into groups. At first, five animals received the MOA treatment, and four received RI, reversing this division after the wash-out described in item 2.3. Only one administration was performed for the MOA treatment, and one application was performed every 24 hours for three days for the RI treatment.

The data were tested for normality using the Shapiro-Wilk test and presented by the media and its quartiles (first and third quartile). The Mann-Whitney test was applied for the analyses between the groups' major ozonated autohemotherapy (MOA) and rectal insufflation (RI). The Friedman test was applied for intragroup comparisons. To assess intragroup differences, the Kruskal-Wallis test was chosen, which can be used in continuous variables or dependent variables of ordinal level, being a viable statistical alternative in situations in which, eventually, the necessary assumptions for the application of the ANOVA's F test are not met, as it does away with the assumptions of normality and homoscedasticity of the samples (Corder and Dale, 2011). Statistical significance was set at $p \leq 0.05$. All analyses were performed in IBM SPSS Statistics® software (Version 22).

RESULTS

Comparative analyses were performed between the MOA and RI groups for hematological (Table 3), leukometric (Table 4), biochemical (Table 5), and MDA (Table 6) variables, showing no statistical differences between them ($p>0.05$).

In the intragroup analyses, comparing the control time (T0) with the other times (24h, 48h, 72h, 7 days, and 14 days), the hematological variables of the MOA group showed statistical differences for red blood cells count ($p= 0.009$) at T2 ($p= 0.028$), T3 ($p= 0.017$), T4 ($p= 0.020$), and T5 ($p= 0.024$), hemoglobin ($p= 0.004$) at times T1 ($p= 0.018$), T2 ($p= 0.044$), T3 ($p= 0.008$), T4 ($p= 0.025$), and T5 ($p= 0.021$), hematocrit ($p= 0.039$) at times T1 ($p= 0.018$), T3 ($p= 0.007$), T4 ($p= 0.035$), and T5 ($p= 0.025$), MCV ($p= 0.020$) at moments T4 ($p= 0.021$), and MCHC ($p= 0.010$) at moments T2 ($p= 0.015$), T3 ($p= 0.011$), and T4 ($p= 0.036$).

In the RI group, the statistical differences were for RBCs ($p= 0.005$) at moments T2 (p= 0.043), T3 ($p= 0.011$), T4 ($p= 0.012$), and T5 ($p= 0.017$), hemoglobin ($p= 0.007$) at moments T1 ($p= 0.042$), T2 ($p= 0.015$), T3 ($p= 0.021$), T4 ($p= 0, 050$), and T5 ($p= 0.011$), hematocrit (0.042) at T1 ($p= 0.049$), T2 ($p= 0.027$), T3 ($p= 0.038$), and T5 ($p= 0.011$), MCV ($p= 0.001$) at moments T3 ($p= 0.038$) and T4 ($p= 0.012$), and MCHC ($p< 0.001$) at moments T3 ($p= 0.008$) and T4 ($p= 0.012$).

Table 3. Median (minimum, maximum) values of the serum hematological profile, before the treatments MOA and RI (T0), and T1 (24h), T2 (48h), T3 (72h), T4 (7d) and T5 (21d) after treatments in vaquejada horses

Variable	Group	T0 Md (1stq-3rdq)	*p* value	T1 Md (1stq-3rdq)	*p* value	T2 Md (1stq-3rdq)	*p* value
RBC ($x10^3/mm^3$)	MOA	7.50(6.60-8.35)a	1.000	7.70(6.75-8.40)a	0.730	8.00(7.20-9.00)b	0.931
	RI	7.50(6.60-8.35)a		7.50(7.05-9.25)a		7.80(7.15-9.10)b	
Hemoglobin (g/dL)	MOA	11.50(9,90-12.60)a	1.000	12.10(11.15-13.50)b	1.000	12.50(10.60-13.80)b	0.730
	RI	11.50(9.90-12.60)a		11.50(10.80-14.35)b		12.20(10.65-13.75)b	
Hematocrit (%)	MOA	36.00(31.00-39.50)a	1.000	37.00(34.50-42.50)b	0.931	38.00(33.00-42.00)a	1.000
	RI	36.00(31.00-39.50)a		36.00(34.00-45.00)b		37.00(33.50-42.50)b	
MCV (fL)	MOA	47.43(46.16-47.84)a	1.000	48.05(47.41-48.31)a	0.931	47.05(45.30-48.27)a	0.605
	RI	47.43(46.16-47.84)a		48.00(47.22-49.63)a		46.37(46.14-47.32)a	
HCM (pg)	MOA	15.12(14.74-15.28)a	1.000	15.39(15.27-15.63)a	0.796	15.14(14.72-15.86)a	0.489
	RI	15.12(14.74-15.28)a		15.33(15.00-15.92)a		15.00(14.75-15.45)a	
MCHC (g/dL)	MOA	31.93(31.86-31.94)a	1.000	31.93(31.86-32.33)a	0.387	32.85(32.09-32.86)b	0.297
	RI	31.93(31.86-31.94)a		31.90(31.79-31.97)a		32.00(31.84-32.88)a	
Variable	Group	T3 Md (1stq-3rdq)	*p* value	T4 Md (1stq-3rdq)	*p* value	T5 Md (1stq-3rdq)	*p* value
RBC ($x10^3/mm^3$)	MOA	8.80(7.60-9.00)b	0.605	8.00(7.75-8.90)b	0.730	8.30(7.65-8.65)b	0.436
	RI	8.70(7.30-10.00)b		8.00(7.25-9.50)b		8.60(7.45-9.30)b	
Hemoglobin (g/ dL)	MOA	13.20(12.30-14.45)b	0.863	11.90(11.10-13.10)b	0.796	12.80(11.40-13.10)b	0.546
	RI	11.80(11.20-15.95)b		12.20(9.95-14.05)b		13.30(11.30-14.10)b	
Hematocrit (%)	MOA	39.00(35.50-42.00)b	0.863	36.00(34.00-41.00)b	0.863	39.00(35.00-41.00)b	0.489
	RI	37.00(34.00-46.00)b		38.00(31.00-43.00)a		41.00(35.50-43.00)b	
MCV (fL)	MOA	45.45(44.22-47.14)a	0.436	45.05(44.52-45.29)b	0.796	46.51(45.15-48.23)a	0.931
	RI	45.07(43.79-46.31)b		45.20(43.52-45.47)b		46.37(45.25-48.21)a	
HCM (pg)	MOA	14.66(14.41-16.45)a	1.000	14.52(14.38-14.96)a	0.863	14.88(14.52-15.41)a	0.796
	RI	15.77(14.50-15.95)a		14.62(14.12-14.91)a		14.94(14.69-15.40)a	
MCHC (g/ dL)	MOA	33.33(32.04-35.21)b	0.666	32.05(31.94-33.01)b	1.000	32.00(31.88-32.46)a	0.863
	RI	33.82(32.49-35.84)b		32.25(32.01-32.92)b		32.00(31.88-32.64)a	

MOA = Major ozonated autohemotherapy; RI = rectal insufflation; Md = median; 1stq = first quartile; 3rdq = third quartile; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; Mann-Whitney test for analysis between MOA and RI groups; Different lowercase letters represent significant intragroup statistical difference ($p \leq 0.05$).

Table 4. Median (minimum, maximum) values of the leukogram, before the treatments MOA and RI (T0), and T1 (24h), T2 (48h), T3 (72h), T4 (7d) and T5 (21d) after treatments in vaquejada horses

Variable	Group	T0 Md (1stq-3rdq)	*p* value	T1 Md (1stq-3rdq)	*p* value	T2 Md (1stq-3rdq)	*p* value
Total leukocytes ($x10^3$/µl)	MOA	9800.00 (7700.00-10200.00)a	1.000	10200.00 (8650.00-10800.00)a	0.488	10300.00 (8600.00-11200.00)a	0.489
	RI	9800.00 (7700.00-10200.00)a		10400.00 (9200.00-11250.00)a		10000.00 (8150.00-10850.00)a	
Rods (/µl)	MOA	0.00a	1.000	0.00a	0.730	0.00a	1.000
	RI	0.00a		0.00a		0.00a	
Segmented ($x10^3$/µl)	MOA	3920.00 (3675.00-4682.00)a	1.000	5037.00 (4170.00-6057.50)a	0.387	4700.00 (3713.00-5595.00)a	0.546
	RI	3920.00 (3675.00-4682.00)a		6216.00 (4143.00-7357.50)a		4514.00 (3412.50-5214.00)a	
Lymphocytes (/µl)	MOA	4897.00 (3468.50-5439.00)a	1.000	4482.00 (3037.50-5067.00)a	0.796	5106.00 (3641.00-5828.00)a	0.796
	RI	4897.00 (3468.50-5439.00)a		4472.00 (3110.50-4729.00)a		5125.00 (32.53.00-5800.00)a	
Eosinophils (/µl)	MOA	249.00 (182.00-343.00)a	1.000	180.00 (87.50-298.50)b	0.436	111.00 (0.00-193.00)b	0.666
	RI	249.00 (182.00-343.00)a		11.00 (0.00-341.00)a		125.00 (44.50-242.50)a	
Basophils (/µl)	MOA	0.00a	1.000	0.00 (0.00-45.00)	0.436	0.00a	0.161
	RI	0.00a		0,00a		57.00 (0.00-112.50)b	
Monocytes (µl)	MOA	244.00 (166.00-268.00)a	1.000	180.00 (126.50-375.00)a	1.000	105.00 (38.00-243.00)a	0.387
	RI	244.00 (166.00-268.00)a		222.00 (57.00-416.00)a		200.00 (104.50-243.50)a	

Variable	Group	T3 Md(1stq-3rdq)	*p* value	T4 Md(1stq-3rdq)	*p* value	T5 Md(1stq-3rdq)	*p* value
Total leukocytes ($x10^3$/µl)	MOA	9800.00 (8700.00-11100.00)a	0.340	9700.00 (8350.00-109500.00)a	0.666	10100.00 (8750.00-11450.00)a	0.863
	RI	10800.00 (9300.00-11850.00)a		9800.00 (8850.00-11050.00) a		9900.00 (9200.00-11300.00)a	
Rods (/µl)	MOA	0.00a	1.000	0.00a	1.000	0.00a	0.730
	RI	0.00a		0.00a		0.00a	
Segmented ($x10^3$/µl)	MOA	4410.00 (4191.00-4769.50)a	0.297	4125.00 (3670.00-5877.00)a	0.605	5076.00 (3495.00-5966.50)a	0.863
	RI	5136.00 (3855.00-5747.50)a		4508.00 (4055.00-5910.00)a		5076.00 (3771.50-5652.00)a	
Lymphocytes (/µl)	MOA	5194.00 (4276.00-6084.50)a	0.730	4800.00 (3069.00-5811.00)a	0.605	5148.00 (3417.00-5611.50)a	0.489
	RI	5350.00 (3835.00-6394.00)a		5238.00 (3624.50-6039.50)a		5529.00 (3334.00-6163.00)a	
Eosinophils (/µl)	MOA	93.00 (0.00-133.50)b	0.666	75.00 (0.00-381.50)b	0.605	108.00 (0.00-309.00)a	0.796
	RI	90.00 (0.00-306.00)a		180.00 (40.00-276.00)a		180.00 (0.00-307.00)a	

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Variable	Group	T3 Md(1stq-3rdq)	*p* value	T4 Md(1stq-3rdq)	*p* value	T5 Md(1stq-3rdq)	*p* value
Basophils (/µl)	MOA	0.00 (0.00-92.50)a	0.258	0.00a	1.000	0.00 (0.00-49.50)a	0.605
	RI	0.00a		0.00a		0.00 (0.00-113.00)a	
Monocytes (µl)	MOA	110.00 (46.50-210.00)a	0.222	166.00 (98.50-470.00)a	0.605	166.00 (0.00-330.00)a	0.863
	RI	192.00 (121.00-218.00)a		202.00 (97.50-295.00)a		188.00 (48.50-255.00)a	

MOA = Major ozonated autohemotherapy; RI = rectal insufflation; Md = median; 1stq = first quartile; 3rdq = third quartile; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; Mann-Whitney test for analysis between MOA and RI groups; Different lowercase letters represent significant intragroup statistical difference ($p \leq 0.05$).

Table 5. Median (minimum, maximum) values of the serum biochemical profile, before the treatments MOA and RI (T0), and T1 (24h), T2 (48h), T3 (72h), T4 (7d) and T5 (21d) after treatments in vaquejada horses

Variable	Group	T0 Md (1stq-3rdq)	*p* value	T1 Md (1stq-3rdq)	*p* value	T2 Md (1stq-3rdq)	*p* value
Platelets ($x10^2$/ mm^3)	MOA	252000 (238000-288500)a	1.000	249000 (159000-280500)a	0.340	180000 (125500-220500)a	0.436
	RI	252000 (238000-288500)a		276000 (189000-355500)a		200000 (148000-263500)a	
TPP (g/ dl)	MOA	6.40(6.20-6.70)a	1.000	6.80(6.80-7.60)b	0.796	7.00(6.70-7.30)b	0.113
	RI	6.40(6.20-6.70)a		7.20(6.40-7.80)a		6.60(6.40-7.10)a	
Fibrinogen (mg/ dl)	MOA	200.00a (200.00-400.00)	1.000	400.00a (300.00-600.00)	0.931	400.00a (200.00-600.00)	0.014
	RI	200.00a (200.00-400.00)		400.00a (200.00-800.00)		200.00a (100.00-250.00)	
Lactate (mg/dl)	MOA	52.00(49.50-62.00)a	1.000	27.00(18.00-31.00)b	0.222	18.00(15.55-20.90)b	0.796
	RI	52.00(49.50-62.00)a		33.00(20.50-34.50)b		19.00(17.15-20.50)b	
AST (ui/ l)	MOA	101.00 (92.00-281.00)a	1.000	325.00 (290.00-400.00)b	0.666	355.00 (311.00-413.50)b	0.605
	RI	101.00 (92.00-281.00)a		366.00 (266.50-422.00)b		362.00 (264.00-372.50)b	
ALT (UI/ l)	MOA	12.00(7.85-14.10)a	1.000	10.80(9.00-14.50)a	0.258	12.00(10.00-28.00)a	0.546
	RI	12.00(7.85-14.10)a		12.80(10.05-18.00)a		11.00(9.25-17.50)a	
CK (UI/ L)	MOA	350.00 (314.00-500.00)a	1.000	313.00 (287.00-443.00)a	0.136	374.00 (311.50-433.00)a	0.863
	RI	350.00 (314.00-500.00)a		440.00 (364.50-542.50)a		389.00 (325.00-417.50)a	
GGT (UI/ l)	MOA	15.60(11.75-22.65)a	1.000	18.20(15.10-19.30)a	0.863	12.00(5.80-20.50)a	0.730
	RI	15.60(11.75-22.65)a		21.90(8.80-25.70)a		14.20(6.80-22.50)a	
ALP (UI/ l)	MOA	172.00 (127.05-188.10)a	1.000	183.00 (146.90-199.00)a	0.931	160.00 (143.00-179.00)a	0.605
	RI	172.00 (127.05-188.10)a		178.00 (131.75-199.75)a		172.00 (122.50-208.50)a	
Creatinine (mg/ dl)	MOA	1.46(1.42-1.59)a	1.000	1.60(1.40-1.68)a	0.863	1.70(1.45-1.80)a	0.796
	RI	1.46(1.42-1.59)a		1.60(1.40-1.70)a		1.50(1.40-1.90)a	
Urea (mg/ l)	MOA	33.00(32.00-36.50)a	1.000	31.00(23.15-35.70)a	0.931	32.00(30.00-39.00)a	0.222
	RI	33.00(32.00-36.50)a		29.00(26.50-34.50)a		30.00(27.80-32.50)a	

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Variable	Group	T3 Md(1stq-3rdq)	*p*value	T4 Md(1stq-3rdq)	*p*value	T5 Md(1stq-3rdq)	*p* value
Platelets ($x10^2$/ mm^3)	MOA	222000 (194000-285000)a	0.605	204000 (186000-265000)a	0.063	190000 (146000-250000)a	0.546
	RI	240000 (199500-331500)a		174000 (155000-209000)b		167000 (120500-263500)a	
TPP (g/ dl)	MOA	6.60(6.10-7.10)a	0.796	6.60(6.20-6.65)a	1.000	6.60(6.50-6.90)a	0.931
	RI	6.60(6.30-6.90)a		6.50(6.10-6.90)a		6.80(6.30-6.80)a	
Fibrinogen (mg/ dl)	MOA	200.00(200.00-450.00)a	0.666	200.00(200.00-350.00)a	0.387	400.00(200.00-500.00)a	0.666
	RI	200.00(200.00-350.00)a		200.00(200.00-200.00)a		400.00(200.00-400.00)a	
Lactate (mg/dl)	MOA	20.00(16.50-29.00)b	0.666	22.00(8.60-28.00)b	0.796	26.00(19.30-29.40)b	0.546
	RI	19.00(16.50-20.50)b		10.50(8.05-28.50)b		25.00(18.50-28.50)b	
AST (UI/ l)	MOA	346.00(264.50-374.00)b	0.796	330.00(281.00-370.00)b	0.931	359.00(310.00-400.00)b	1.000
	RI	345.00 (261.50-373.50)a		349.00 (253.00-372.00)b		367.00 (311.00-431.00)b	
ALT (ui/ l)	MOA	10.00(9.50-29.50)a	0.436	11.00(10.00-17.00)b	0.666	18.00(10.50-37.00)b	0.730
	RI	13.00(10.55-16.50)a		11.00(8.15-19.50)b		22.00(14.50-36.50)b	
CK (UI/ L)	MOA	343.00 (325.00-385.00)a	0.040	370.00 (331.50-487.50)a	0.931	289.00 (266.00-369.00)a	1.000
	RI	409.00 (379.50-421.00)a		369.00 (339.00-545.50)a		290.00 (235.00-730.00)a	
GGT (UI/ l)	MOA	15.60(7.60-17.25)a	0.387	17.00(13.30-18.90)a	0.605	11.10(7.15-19.35)a	0.436
	RI	20.80(9.95-26.50)a		21.00(10.60-24.50)a		18.40(9.05-27.00)a	
ALP (UI/ l)	MOA	168.00 (144.65-211.20)a	0.666	166.00 (142.50-193.50)a	1.000	190.00 (141.50-249.00)a	0.863
	RI	173.00 (138.00-215.65)a		159.00 (132.00-211.00)a		183.00 (136.00-247.50)a	
Creatinine (mg/ dl)	MOA	1.70(1.55-1.75)b	0.605	1.60(1.50-1.70)b	0.931	1,60(1.58-1.92)b	0.963
	RI	1.60(1.50-1.80)a		1.60(1.50-1.80)a		1.60(1.48-2.10)a	
Urea (mg/ l)	MOA	35.00(29.00-40.00)a	0.796	26.00(25.00-28.50)b	0.258	26.00(21.50-31.50)b	0.666
	RI	37.00(32.00-39.50)a		28.00(26.00-29.00)b		29.00(24.00-33.50)b	

MOA = Major autohemotherapy ozonated; RI = rectal insufflation; Md = median; 1stq = first quartile; 3rdq = third quartile; TPP = total plasma protein; AST = aspartate aminotransferase; ALT = alanine aminotransferase; CK = creatine kinase; GGT = gamma glutamyl transferase; ALP = alkaline phosphatase; Mann-Whitney test for analysis between MOA and RI groups; Different lowercase letters represent significant intragroup statistical difference ($p \leq 0.05$).

Table 6. Median (minimum, maximum) values of the malondialdehyde, before the treatments MOA and RI (T0), and T1 (24h), T2 (48h), T3 (72h), T4 (7d) and T5 (21d) after treatments in vaquejada horses

Variable	Group	T0 Md (1stq-3rdq)	*p* value	T1 Md (1stq-3rdq)	*p* value	T2 Md (1stq-3rdq)	*p* value
MDA (µg/ mL x 10^{-6})	MOA	21.57 (14.27-26.01) a	1.000	8.22 (6.52-12.98) b	0.931	6.64 (5.36-15.52) a	0.340
	RI	21.57 (14.27-26.01) a		8.09 (5.97-14.31) b		18.31 (5.58-50.96) a	
Variable	Group	T3 Md (1stq-3rdq)	*p* value	T4 Md (1stq-3rdq)	*p* value	T5 Md (1stq-3rdq)	*p* value
MDA (µg/ mL x 10^{-6})	MOA	7.55 (5.78-10.38) b	1.000	4.91 (3.70-8.62) b	0.340	6.23 (4.26-7.93) b	0.605
	RI	8.82 (5.42-10.78) b		4.66 (3.76-4.98) b		7.36 (5.29-7.92) b	

MOA = Major ozonated autohemotherapy; RI = rectal insufflation; Md = median; 1stq = first quartile; 3rdq = third quartile; MDA = malondialdehyde; Mann-Whitney test for analysis between the MOA and RI groups; Different lowercase letters represent significant intragroup statistical difference ($p \leq 0.05$).

Regarding the data concerning the WBC of the MOA group, a statistical difference was found for eosinophils (p= 0.025) at moments T1 (p= 0.038), T2 (p= 0.038), T3 (p= 0.008), and T4 (p= 0.038). In the RI group, a statistical difference was found for basophils (p= 0.003) at time T2 (p= 0.043).

For the biochemical variables in the MOA group, statistical differences were found for TPP (p= 0.008) at T1 (p= 0.007) and T2 (p= 0.007) , lactate (p= 0.001) at T1 (p= 0.008), T2 (p= 0.008), T3 (p= 0.007), T4 (p= 0.008), and T5 (p= 0.008), AST (p= 0, 001) at time points T1 (p= 0.008), T2 (p= 0.008), T3 (p= 0.021), T4 (p= 0.015), and T5 (p= 0.008), ALT (p= 0.001) at time points T4 (p= 0.015) and T5 (p= 0, 008), creatinine (p= 0.002) at time T3 (p= 0.007), T4 (p= 0.017), and T5 (p= 0.015), and urea (p< 0.001) at time T4 (p= 0.012) and T5 (p= 0.017). In the IR group, statistical differences were found for platelets (p= 0.003) at moment T4 (p= 0.008), lactate (p< 0.001) at moments T1 (p= 0.008), T2 (p= 0.008), T3 (p= 0.008), T4 (p= 0.008), and T5 (p= 0, 008), AST (p= 0.008) at moments T1 (p= 0.015), T2 (p= 0.038), T4 (p= 0.021), and T5 (p= 0.008), ALT (p= 0.015) at moments T4 (p= 0.011) and T5 (p= 0.011), and urea at moments T4 (p= 0.018) and T5 (p= 0.013) (p< 0.001).

In the evaluation of the lipid peroxidation index, through MDA measurement, intragroup RI differences were found between the moments control x 24 hours (p= 0.011), control x 72 hours (p= 0.015), control x 7 days (p= 0.011), and control x 14 days (p= 0.008) (figure 1A). In the intragroup MOA comparison, differences were found between control x 24 hours (p= 0.028), control x 72 hours (p= 0.008), control x 7 days (p= 0.008), and control x 14 days (p= 0.008) time points (Figure 1).

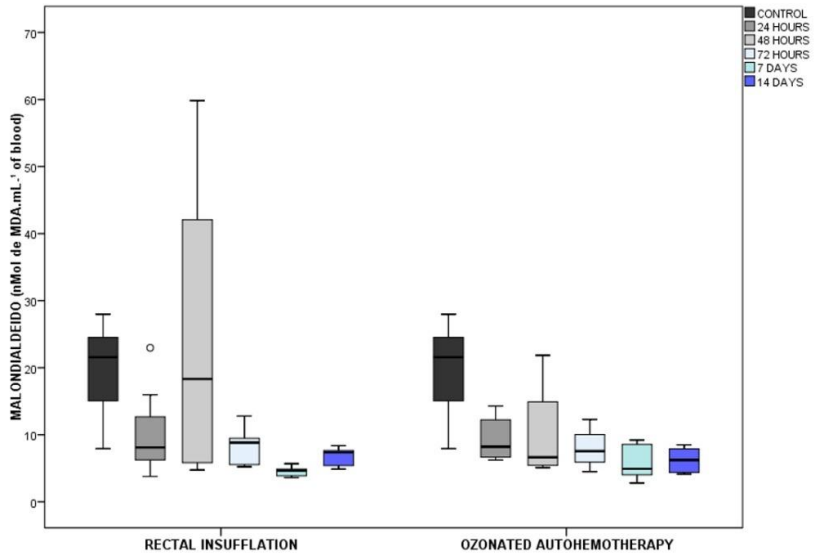

Figure 1. Quantitative data are given as box plots showing medians, means, and first and third quartiles for comparison between the RI and MOA groups for MDA values.

DISCUSSION

The two main administration routes of ozone therapy in athletes are major autohemotherapy and rectal insufflation. In the experimental conditions proposed in this study, no statistical difference was found between the results of the two therapies (p > 0.05), indicating that the two techniques are equally effective for applying ozone therapy to horses competing in cow jumping (*vaquejada*).

However, the RI route has practical advantages compared to MOA regarding its easy management, low cost, and less invasiveness, corroborating what was described by Moreira (2015). In addition, rectal application of O3 has systemic therapeutic effects, as mentioned by Viebahn-Hänsler *et al*. (2012).

It is common to observe changes in hematocrit values, total red blood cell count, and hemoglobin concentration in athletic horses

because of catecholamine release and, consequently, splenic contraction due to exercise (Orozco, 2007; Miranda *et al*., 2011; Pereira, 2015; Mattosinho, 2018).

It was observed in a simulation test in a work developed by Hunka *et al*. (2018) with *vaquejada* QM horses the elevation of hematological parameters during the simulation period and returning close to resting values within 15 min. Conversely, the animals in the present study showed increased concentrations in the combined RBC, hemoglobin, and hematocrit values that remained constant until the end of the experiment.

The results pointed out by this study are common in horses treated with ozone therapy, as shown by López (2007), who, using ozonated autohemotherapy in horses, observed a significant increase in RBC values. Likewise, Jaramillo *et al.* (2020) describe an increase in RBC, hemoglobin, and hematocrit values by administering ozone gas in horses via rectal insufflation compared to the control group. Thus, we can state that ozone therapy contributes to an increased number of blood cells in athletic horses.

The enzymes aspartate aminotransferase (AST) and creatine kinase (CK) are commonly used in athlete horses because they are markers of post-exercise muscle damage Hodgson *et al*., 2014; Thomassian *et al*., 2007). This study showed an increase with a statistically significant difference for AST, but it remained within the reference values (150-400 IU/L) proposed by Hodgson *et al*., 2014. On the other hand, although not statistically significant, CK values were above the reference value (0-270 IU/L) proposed by Hodgson *et al*. (2014).

Similar findings to our study were reported by Santos *et al*. (2019) and Sousa *et al*. (2018), where both evaluated quarter (QM) vaquejada racehorses and observed an increase in AST and CK enzyme concentration after 50 min and 120 min of physical activity, respectively. Similarly, Patelli *et al*. (2016) observed these alterations in QM horses practicing two high-performance, short-duration sports.
According to Chaves (2016), there is a correlation between physical conditioning, intensity, and exercise duration on the AST and CK serum activity. In high-intensity and short-duration sports such as vaquejada, it is common to find elevations in the values of these enzymes, as observed in the present study. In addition, Patelli *et al*. (2016) cite that increases in CK can reach 1000 IU/L without muscle damage. According to Thomassian *et al*. (2007) and Patelli *et al*. (2016), the elevation of these enzymes is associated with increased membrane permeability and slow clearance from the circulation, not associating these elevations with a muscle injury.

In equine sports medicine, blood lactate concentrations are important for providing information on the physical conditioning of athlete horses (Ferraz, 2006; Botteon, 2012; Masko *et al*., 2021). According to the study developed by Souza *et al*. (2017), it is common to observe an elevation in blood lactate concentrations after physical activity resulting from physical exhaustion promoted by the simulation of *vaquejada*. Similarly, other authors have reported similar findings of elevation in blood lactate concentrations as described by Lopes *et al*. (2009), who observed a 130% elevation of basal lactate level at the end of a *vaquejada* competition and by Santiago *et al*. (2014), who described an increase after simulating *vaquejada*, but which returned to basal values within 30min of rest.

Studies describing the use of ozone therapy in *vaquejada* horses are rare. However, our treatment study showed a significant change in blood lactate values in animals treated with two therapies. Although they do not have the reached reference values (10 – 16 mg/dL) as reached by Kaneko *et al*., 2008, the values reached have a reduction of 79.8% in relation to the basal value, after the treatments with ozone, continue the animals in a training routine.

Several authors mention supplementation use with antioxidant substances such as Coenzyme Q10, ascorbic acid (vitamin C), beta-carotene (vitamin A), and alpha-tocopherol (vitamin E) to control oxidative stress in horses and humans (Inal *et al*., 2011; Barbosa, 2012; Picchi, 2015; Svete *et al*., 2021). However, few studies analyze the effect of ozone therapy on the stimulation of antioxidant capacity and oxidative stress control in horses performing high-intensity, short-

duration exercise (Inal *et al*., 2011; Tsuzuki *et al*., 2015).

According to Smith *et al*. (2017), O3 can induce further differentiation of erythroblasts, leading to a progressive increase in the number of erythrocytes. This increase raises oxidative stress resistance due to efficient antioxidant mechanisms, as described by Fernandes *et al*. (2012). Thus, the ozone therapy used in the animals of this study provided a strengthening of hematological antioxidant defenses, making them more resistant to OS.

At the beginning of therapies, O3 induces a cascade of events producing transient and moderate oxidative stress (Smith *et al*., 2017). Ozone has a higher affinity to react with unsaturated fatty acids and antioxidant products (Travagli *et al*., 2010). Immediately, the body's antioxidant system is consumed to protect the macromolecules that make up the cell membrane, inactivating the ROS formed by the reaction (Bhatt *et al*., 2016; Di Mauro *et al*., 2019). In this context, these factors elucidate why MDA values were not elevated in the first moment (24h) after ozone therapies.

In a study by Antunes (2013), who assessed oxidative stress in endurance horses, MDA levels did not show significant values, correlating this fact to the good preparation of the animals for the proposed exercise. However, when evaluating horses of the same modality, Gondim *et al*. (2009) reported a significant increase in MDA concentrations compared to baseline values while remaining constant during the three days of the test. Sara *et al*. (2012) also proposed similar results using horses in a controlled environment test, where an elevation in MDA parameters occurred between 24h to 48h.

When investigating the effects of exercise on plasma antioxidant capacity, White *et al*. (2001) used plasma malondialdehyde measurement to demonstrate an increase in lipid peroxidation in Thoroughbred racehorses after exercise not treated with vitamin C, indicating that high-intensity exercise causes a significant increase in lipid peroxidation due to the increase in MDA concentrations caused by OS. Likewise, the present study showed that in *vaquejada* horses there was a decrease in MDA values after 24h of ozone treatments when compared to their baseline values. Thus, it can be said that animals that maintain reduced values of lipid peroxidation are less prone to the negative effects of EO.

CONCLUSION

This study demonstrates that there is no difference between the two therapies, indicating that the two techniques are equally effective for applying ozone therapy to horses competing *vaquejada*. Further research is necessary to identify other ways of using ozone therapy that contributes to reducing oxidative stress, which can improve the athletic performance of animals in different sports and promote better welfare to these animals.

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