ISSN 1413-3555
Rev Bras Fisioter, São Carlos, v. 12, n. 6, p. 488-94, nov./dec. 2008
Revista Brasileira de Fisioterapia©

Hematological changes produced by 1MHz continuous ultrasound, applied during the acute phase of iatrogenic muscle injury in rats

Alterações hematológicas provocadas pelo ultra-som de 1MHz na forma contínua aplicadas no tratamento da fase aguda de lesão muscular iatrogênica em ratos

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Abstract

Background: The literature shows the beneficial effects of low-intensity ultrasound therapy on the healing process of several biological tissues. **Objective:** To evaluate the effects of continuous ultrasound (CUS) on the hematological dynamics of an acute inflammatory process in iatrogenic muscle injuries. **Methods:** Sixteen Wistar rats (350 to 400g) were divided into a control group (CG=8) and an experimental group (G1=8). The rats were submitted to a surgical incision on the lateral aspect of the right hind limb, in which the biceps femoris muscle was transversally injured. CUS (1MHz) was applied to the injury site at an intensity of 0.4W/cm^2, for three minutes, one hour after injury and also eight and 24 hours after injury. At these times, blood was drawn by venipuncture of the retroorbital plexus, for analysis of red and white blood cells. **Results:** CUS reduced erythrocytes by 8% in the first blood collection (9.9±0.1 *versus* 7.8±0.1; x10^5/mm^3; p<0.001); it doubled the number of segmented neutrophils in the second collection (3,166.8±161.4 *versus* 6,426.2±306.0; x10^3/mm^3; p=0.008) and eosinophils in the third collection (2,883.6±99.0 *versus* 4,714.4±275.2; x10^3/mm^3; p=0.011), when compared to the CG. No differences between the groups were seen with regard to hematocrit, total leukocytes, rod neutrophils, monocytes or lymphocytes at the three times studied. **Conclusions:** Application of CUS for acute treatment of muscle injuries is contraindicated under this condition, because it promotes reductions in erythrocytes and increases in segmented neutrophils and eosinophils, thus favoring hemorrhage and increasing inflammatory process.

Key words: ultrasound therapy; rehabilitation; musculoskeletal system; wounds and injuries; inflammation; hematology.

Resumo

Contextualização: A literatura demonstra o efeito benéfico da terapia ultra-sônica de baixa intensidade sobre o processo de cicatrização de vários tecidos. **Objetivo:** Avaliar o efeito do ultra-som contínuo (USC) sobre a dinâmica hematológica do processo inflamatório agudo de lesão muscular iatrogênica. **Materiais e métodos:** Foram utilizados 16 ratos da raça Wistar (350 a 400g), divididos em grupo controle (GC=8) e grupo experimental (G1=8), submetidos à incisão cirúrgica na face lateral do membro posterior direito, onde o músculo bíceps femoral foi lesionado transversalmente. O USC (1MHz) foi aplicado sobre o local da lesão a uma intensidade de 0,4W/cm^2, durante três minutos, na 1^a, 8^a e 24^a hora após a lesão. Nestes períodos, foram realizadas as coletas de sangue por punção venosa do plexo retroorbital para as análises sangüíneas das séries brancas e vermelhas. **Resultados:** O USC diminui 8% dos eritrócitos na primeira coleta (9,9±0,1 *versus* 7,8±0,1; x10^5/mm^3, p<0,001); dobrou os neutrófilos segmentados na segunda coleta (3.166,8±161,4 *versus* 6.426,2±306,0; x10^3/mm^3 p=0,008) e os eosinófilos na terceira coleta (2.883,6±99,0 *versus* 4.714,4±275,2; x10^3/mm^3 p=0,011) em relação ao GC. Não se observaram diferenças entre os grupos no hematócrito, leucócitos totais, neutrófilos bastonetes, monócitos e linfócitos, nos três momentos estudados. **Conclusões:** A aplicação do USC no tratamento agudo de lesão muscular é contra-indicada nesta condição, pois promove a redução dos eritrócitos, aumento dos neutrófilos segmentados e dos eosinófilos, favorecendo a hemorragia e o aumento do processo inflamatório.

Palavras-chave: terapia por ultra-som; reabilitação; sistema musculosquelético; ferimentos e lesões; inflamação; hematologia.

Received: 21/04/2008 – **Revised:** 22/07/2008 – **Accepted:** 29/09/2008

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Introduction

Muscle injuries are common during physical and sports activities[1]; these injuries occur due to several mechanisms, including direct trauma (lacerations, contusions and tensions) and indirect causes (ischemia and neurological impairments)[2,3]. Therapeutic ultrasound is commonly recommended for treatment of musculoskeletal injuries, however scientific evidence of its effectiveness is still controversial[4-7].

Low-intensity ultrasound (US) is used during Physical Therapy practice. Traditionally, US varies in frequency (1 to 3MHz), intensity and dosage (0.1 to 3W/cm^2), application time and type of wave (continuous and pulsed)[8,9]. The biophysical effects of ultrasound are traditionally divided into thermal and mechanical (non-thermal). Baker, Robertson and Duck[8] claim it is inadequate to assume that the thermal effects correspond to exposure to the continuous wave and the mechanical effects to the pulsed wave because these effects occur simultaneously. However, the thermal and/or mechanical therapeutic effects are optimized according to the type of wave[8]. They also depend on the other parameters used and on the interaction of these parameters with the different biological tissues[5,9,10].

US has the peculiarity of interacting with the circulatory system, causing coagulation changes due to fibrinolysis[11], thrombolysis[12], change in vasomotor activity due to the release of nitric oxide[13] and angiogenic stimulus[14]. However, these answers have been described in specific and controlled situations.

Muscle repair and remodeling occur in four interrelated and time-dependent stages: degeneration, inflammation, regeneration and fibrosis[1]. Immediately after the musculoskeletal injury, exudates are formed in the space between the muscle fibers, where fibroblasts and macrophages are activated to produce additional chemotactic signals (growth factor, cytokines, and chemokines) for inflammatory cell circulation[1] and satellite cell activation[15]. The injured myofibrils suffer necrosis and self-digestion[16]. The fast degeneration of these myofibrils activates the inflammation phase and contributes to tissue remodeling[15]. Inflammation is the most important phase in the muscle remodeling process, when therapeutic interventions should limit the area affected by the hematoma and excessive inflammation[17]. The functional damage is associated with the spatial and temporal distribution of the inflammatory cells, as well as to the type and magnitude of the response[18].

Low-intensity US (<1W/cm^2) is commonly used to accelerate the tissue remodeling process after muscle injury[19]. This therapy is indicated because it decreases the size of the damaged area, and increases collagen deposition and elastic resistance[20]. However, the biological mechanisms of its effects have yet to be fully understood[19]. It is known that the damaged area becomes a source of physical and chemical signals which modify the hematological concentrations of the white blood cells (leukocytes) and red blood cells (erythrocytes)[1,15]. Recent data of this present research group suggest that pulsed US within 24 hours of the muscle injury promotes a reduction in total leukocytes as well as in segmented neutrophils and monocyte cells. This data suggests that pulsed US promotes inhibition of white blood cell proliferation[21] therefore more specific studies are required to better understand this interaction.

To date, there have been no experimental studies that evaluate the effect of continuous ultrasound (CUS) on hematological dynamics of iatrogenic acute muscle injuries. The aim of this study was to evaluate the effect of CUS on the hematological dynamics of different types of leukocytes and erythrocytes in this condition.

Methods

Animals

Animal manipulation was according to the animal testing guide, and this study was approved by the Research Ethics Committee of Universidade de Cruz Alta (Unicruz), protocol number 002/2008. All of the animals were maintained on a 12-hour dark/light cycle at 20 to 24ºC and relative humidity of approximately 50%. Food and water were *ad libitum* during the entire experimental protocol. The animals' maturation time was 29 weeks. Sixteen mature Wistar rats (weighing 350 to 400g) were used in this study. The rats were randomized to the control group (submitted to the injury protocol and the therapeutic procedure with the ultrasound equipment turned off; CG=8) and the experimental group (submitted to the CUS therapeutic protocol; G1=8). The groups were submitted to a surgical incision on the lateral aspect of the right hind limb according to the injury protocol.

Injury protocol

The animals were anesthetized with a combination of xylazine (7mg/kg) and ketamine (70mg/kg) administered intraperitoneally. A longitudinal surgical incision was made on the skin of the right hind limb to facilitate the subcutaneous tissue rupture and to provide easy access to the medium portion of the biceps femoris muscle. Its fibers were transversally incised in approximately 50% of the volume. Later, the skin lesion was closed by surgical suture. This muscle was chosen due to its easy access in rats and adequate distance from bone structures which could indirectly interfere in the therapeutic US stimulus.

Ultrasound treatment

After the surgery, the rats were treated with CUS which was applied directly to the injured area. The ultrasound equipment was the AVATAR V (model 9075 Biosistemas Equipamentos Eletrônicos Ltda, Amparo, São Paulo, Brazil), calibrated by the manufacturer before the study, through the radiant force method. In this method the ultrasound energy emanated from the transducer is applied to a submerged cone in water (target) and the mechanical energy (ultrasound) is 'weighted', and then converted into its thermal equivalent (Watts). The US was applied continuously for three minutes at a frequency of 1MHz and intensity of 0.4W/cm^2, using a 3cm-diameter head (number TR3CCE02) with an effective radiating area (ERA) of 5cm^2. The treatment head was moved over the injured area in a circular motion corresponding to 1/3 of its radius[22]. The procedure took place immediately after the surgery, on the 8th and on the 24th hour after the injury protocol. The animals of the CG were manipulated in the same way, but with the equipment switched off.

Hematological preparation and measures

Blood samples were collected through venipuncture of the right retroorbital plexus, with the aid of a microhematocrit capillary tube, previously heparinized, and conditioned in ependorff tubes with anticoagulants[23]. The samples were collected one hour, eight hours and 24 hours after the injury.

A Neubauer chamber and a macrodilution technique were used to determine the number of leukocytes per milliliter (mL) of blood. To achieve that, 20 μL of blood were diluted in 4mL of Türk liquid and the number of leukocytes in the four wide angle squares was counted. This number was then multiplied by 50, and the results were given in μL. Before the cell count, the Neubauer chamber was placed for five minutes inside an inverted Petri dish containing a moist cotton ball to allow cell sedimentation. To observe the morphology and do the differential count of the leukocytes, for which the examiners were blind, a smear of blood was made on a slide and received a Romanowsky stain. After being washed and dried at room temperature, the slide was examined under an optical microscope. One hundred cells were counted according to the Shilling zigzag technique, and the values were expressed in x10^3/mm^3.

A Neubauer chamber and a macrodilution technique were also used to determine the number of erythrocytes per milliliter (mL) of blood. Marcano liquid was used as diluent for the erythrocyte counting. Four mL of the diluent to 20 μL of blood were used, and the erythrocytes in the five middle squares of the central square were counted. Then, this number was multiplied by 10,000 and the values were expressed in x10^5/mm^3. In the hematocrit determination, the microhematocrit tube was filled with blood up to approximately 3/4 of its capacity and one of its ends was sealed with the aid of a Bunsen burner. Then, the capillary tube was placed in a microcentrifuge for five minutes at 3,000 rpm, and the reading was carried out on the appropriate card.

Statistical analyses

The data are shown as mean and standard deviation. Two-way repeated measures analyses of variance (ANOVA), followed by Bonferroni *post hoc* tests were used to compare the hematological changes between groups. The α level considered for analyses was set at 0.05.

Results

The hematocrit demonstrated a progressive reduction ($p<0.001$). For G1 this reduction was only observed in the last collection, and for CG this reduction happened on the eighth and on the 24th hour. There were no differences between groups ($p=0.076$) or in the interaction between them ($p=0.077$), according to Table 1.

The erythrocytes showed a reduction of approximately 8% in the first blood collection for G1 (CUS *versus* controls; $p<0.001$). CG showed reduction compared to the first hour, on the second and third collections of approximately 27% ($p=0.011$), and, in G1, this variable did not change over time (Table 1). The modification in the interaction between groups ($p<0.001$) represents the maintenance of erythrocyte concentrations in G1 and the reduction in CG (Figure 1).

The total leukocytes did not change in both groups during the experimental protocol (Table 1). G1 increased the blood concentrations of segmented neutrophils in the eighth hour (CUS *versus* controls; $p=0.008$) and remained unaltered over time. Conversely, CG values changed over time ($p=0.045$), and in the last collection, these values represented approximately half of the first hour (Figure 2). The young neutrophils (rods) and the monocytes ($p=0.014$, unconfirmed by the Bonferroni test) did not change during the experimental protocol (Table 1).

The eosinophils in G1, in the last collection, increased three times when compared to CG (CUS *versus* controls; $p=0.011$). Regarding the first and the second collections (time $p=0.015$), this concentration increased 225 and 360% respectively (Table 1). The changes in the group interaction

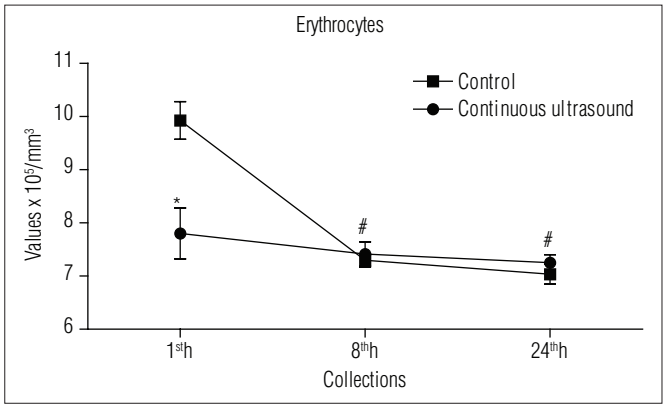

Figure 1. Behavior of erythrocytes during experimental protocol. Values are presented as mean ± standard error (x10^5/mm^3). For the comparisons among the groups using the two-way ANOVA with repeated-measures (group p<0.001; time p=0.011; interaction p<0.001) followed by the Bonferroni *post hoc* test. *p<0.05 variation among the groups; #p<0.05 variation over time *versus* 1st hour; CUS: Continuous ultrasound; Control: group submitted to the procedure with US equipment switched off.

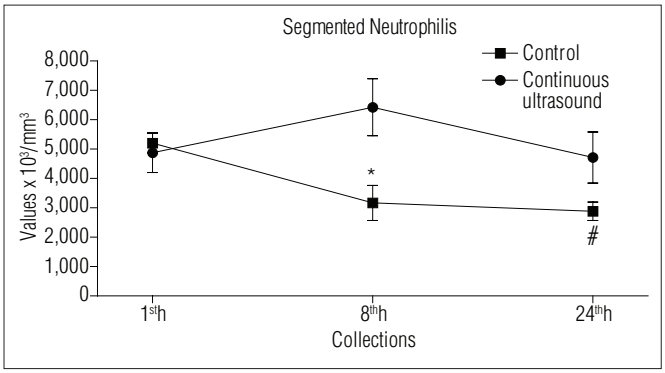

Figure 2. Behavior of segmented neutrophils during experimental protocol. Values are presented as mean and standard error (x10^3/mm^3). For the comparisons among the groups using two-way ANOVA with repeated-measures (group p<0.001; time p=0.011; interaction p<0.001) followed by the Bonferroni *post hoc* test. *p<0.05 variation among the groups; #p<0.05 variation over time *versus* 1st hour; CUS=continuous ultrasound; Control=group submitted to the procedure with US equipment switched off.

Table 1. Hematological variables after muscle lesion and application of continuous ultrasound.

Hematological variable	Unit	Group	Collections			ANOVA p Value		
		n (9)	1st hour	8th hour	24th hour	Group	Time	Interaction
Hematocrit	%	Control	48.8±0.4	43.9±0.4#	37.5±0.3#†	0.076	<0.001	0.077
		CUS	48.0±0.2	46.8±0.4	41.7±0.3#†			
Erythrocytes	x10^5/mm^3	Control	9.9±0.1	7.3±0.04#	7.0±0.06#	<0.001	0.011	<0.001
		CUS	7.8±0.1*	7.4±0.07	7.2±0.05			
Leukocytes	x10^3/mm^3	Control	9075.5±137.4	8642.1±161.4	8133.1±185.1	0.425	0.602	0.638
		CUS	9840.0±487.9	8837.4±330.3	9690.9±337.0			
Segmented Neutrophils	x10^3/mm^3	Control	5204.5±105.3	3166.8±161.4	2883.6±99.0#	0.008	0.045	0.157
		CUS	4879.0±214.4	6426.2±306.0*	4714.4±275.2			
Young Neutrophils (Rods)	x10^3/mm^3	Control	37.6±6.6	32.2±4.8	68.6±8.5	0.093	0.328	0.042
		CUS	81.8±5.9	160.2±19.2	45.5±4.9			
Monocytes	x10^3/mm^3	Control	464.1±30.7	822.5±35.7	864.8±35.2	0.144	0.014	0.840
		CUS	287.9±19.3	704.7±77.8	526.0±26.4			
Eosinophils	x10^3/mm^3	Control	188.8±9.6	144.9±13.2	134.3±6.6	0.011	0.015	0.015
		CUS	183.4±21.7	112.4±11.3	413.3±24.1*#†			
Lymphocytes	x10^3/mm^3	Control	3259.8±87.8	4471.9±158.7	4158.0±115.6	0.187	0.072	<0.001
		CUS	4409.5±259.4	1673.7±50.0#	3958.8±138.6†			

Values are presented as mean±standard error. CUS=continuous ultrasound; Control=group submitted to the procedure with the US equipment switched off. p=evaluation of the comparisons among the groups using two-way ANOVA with repeated measures followed by the Bonferroni *post hoc* test. *p<0.05 variation among the groups; #p<0.05 variation over time *versus* 1st hour; †p<0.05 variation over time versus 8th hour.

(p=0.011) demonstrate that, while CG data remained constant in the last collection, the values increased in G1 (Figure 3).

The lymphocytes showed no difference between groups or over the course of the experimental protocol (time). However, the interaction (p<0.001) changed, with a decrease in G1 values in the second collection compared to the other times (Table 1).

Discussion

The main result of this study was the demonstration, for the first time in the literature, that CUS treatment, applied to an acute inflammatory process in iatrogenic muscle injuries, promotes: erythrocyte reduction in the first hour; increase in parts of the leukocytes represented by the segmented neutrophils in the eighth hour and increase in parts

of the leukocytes represented by the eosinophils in the 24th hour.

CUS produced greater erythrocyte reduction in the first hour after muscle injury, possibly due to its thermal effect[10], which stimulates more extensive hemorrhage. Because erythrocytes are the most abundant cells in the blood, this hemorrhage causes erythrocyte reduction. The endothelial injury sets off a sequence of events, starting with the platelet deposition which leads to white thrombus formation and temporarily obstructs the endothelial injury[24]. This thrombus is quickly infiltrated by fibrin, where the erythrocytes are captured, and the red thrombus is formed. The red thrombus is the main reason for the occlusion of the ruptured blood vessel[25], a mechanism that was probably present in this study.

Another aspect to be considered is that the erythrocytes decreased over time only in CG (in the eighth and in the 24th hour), which might have been induced by the successive blood collections. This reinforces the fact that the hemorrhage induced by CUS represented the difference in the first hour. The hematocrit decreased over time in both groups which reinforces the hypothesis that successive collections and hemorrhage combine to reduce erythrocytes.

In addition to the thermal effects, other mechanisms by which CUS may have induced the hemorrhage are described in the literature, such as nitric oxide release which induces dependent endothelial vasodilation[13], fibrinolysis[11] and thrombolysis[12]. These phenomena may also be involved in this answer.

Because neutrophils are the most abundant of the white blood cells, a significant number of them is passively collected by the temporary thrombus when a vessel is ruptured[26,27]. In the early phase after the musculoskeletal tissue injury, polymorphonuclear leukocytes (neutrophils) are the most abundant cells in the injured area[1], and one day after the injury, those cells will constitute 50% of the cells that migrated to that area[27]. This phenomenon can explain the segmented neutrophil reduction in CG during the course of the experiment.

After this passive overflowing, neutrophils migrate to the surface of the injury to produce a barrier against the invasion of microorganisms and to promote active recruitment of more neutrophils from adjacent uninjured vessels[26,27]. CUS changed this response, and in the eighth hour, the systemic concentration of segmented neutrophils was higher than in CG.

Later, the beginning of the inflammatory reaction is intensified with the satellite cells and the necrotic tissues of the muscle fibers. They are stimulated by the local release of cytokines (IL-6; IL-1β) and cellular growth factors (TNF-α; FGF; IGF) which act on the chemotaxis, increasing the response and overflow of inflammatory cells[1]. This mechanism is optimized

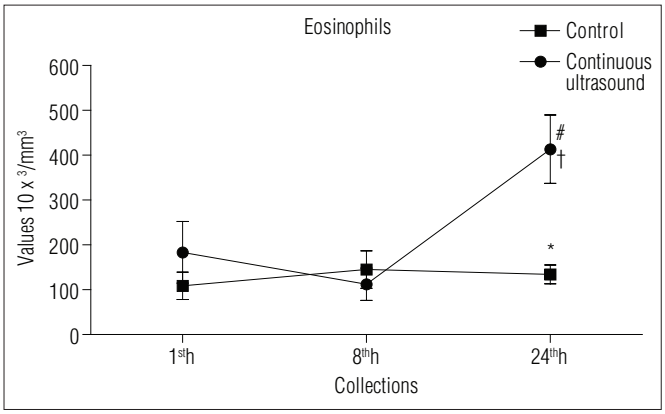

Figure 3. Behavior of eosinophils during experimental protocol. Values are presented as mean and standard error (x10³/mm³). For the comparisons among the groups using two-way ANOVA with repeated-measures (group p<0.001; time p=0.011; interaction p<0.001) followed by the Bonferroni *post hoc* test. *p<0.05 variation among the groups; #p<0.05 variation over time *versus* 1st hour; †p<0.05 variation in the time *versus* 8th hour. CUS=continuous ultrasound; Control=group submitted to the procedure with the equipment switched off.

by the thermal effect of CUS. Another aspect to be considered is that there was no segmented neutrophil reduction in the first hour in the experimental group. The increase in systemic concentration of segmented neutrophils in the eighth hour and its slower reduction in the 24th hour suggest a pro-inflammatory systemic response of this therapy.

The polymorphonuclear leukocytes (or neutrophils) are progressively replaced by monocytes[1], which are very abundant in the injured area between the second and the fifth day[28]. Therefore, their blood concentrations must change before this period. The results observed in the present study suggest that there were no changes in the systemic concentrations of these cells during the experimental protocol, however the 24-hour time frame may not have been enough to alter this variable.

According to the basic inflammation principles, the monocytes are transformed into macrophagocytes which, then, actively begin the proteolysis and phagocytosis of the necrotic material by releasing lysosomic enzymes[29]. The phagocytosis of macrophagocytes is a process remarkably specific to necrotic material, such as the preserved cylinders of the basal membrane which surrounds the necrosis area of the injured myofibrils that survived the macrophage attack. They serve as scaffolding in which viable satellite cells begin to form new myofibrils[1].

The monocytes not only help the neutrophils eliminate microorganisms through phagocytosis but also present their peptides through the major histocompatibility complex to the auxiliary T cells. Thus, the phagocytosis of these cells acts as a link between the innate and the adaptive immune systems[28]. However, the lymphocytes, at the end of the repair phase,

constitute the most abundant subsystem[27]; they are attracted to the injured area in equal number to the monocytes and, from the 14th day, the leukocytes predominate the area[30]. The changes in lymphocyte interaction for G1 represent natural oscillations in the counting of these cells in repeated evaluations, because they are not accompanied by differences between groups or differences in the time.

Inflammatory mediators make the uninjured capillary vessels dilate which slows down blood circulation and allows leukocyte margination and connection to adhesion molecules expressed in the endothelial cells. The eosinophils appear in the final phases of repair and may be related to the production of growth factors[31]. The results of the present study suggest that after 24 hours of muscle injury treated with CUS, there was a nearly three-fold increase in the cellular concentration of eosinophils, however these values are still within physiological parameters.

The results of this research regarding CUS application differ from the pulsed US study[21] that suggested that this form of application decreases white cells (total leukocytes, segmented neutrophils and monocytes). In this study there were an increase in white blood cells and a decrease in red blood cells. A combination of factors, including the type of examined tissue and injury, and the US application (continuous or pulsed), intensity, and frequency of treatment, can explain the different results from other studies[21,32].

The limitations of this research reside in the absence of histological and histochemical tissue analyses, which would have allowed the comparison between the systemic hematological data and the tissue data, as well as the quantification of the injured area through ultrasound.

Conclusions

The present experimental study demonstrates that CUS use (on the 1st, 8th and 24th hour, with a three minutes applications and 0,4W/cm^2 intensity) in the acute phase of iatrogenic muscle injury promotes changes in hematological dynamics. These changes are characterized by erythrocyte reduction and an increase in segmented neutrophils and eosinophils. These modifications suggest increased hemorrhage and an amplified inflammatory response of the muscle and confirm the contraindication of CUS application on acute muscle injury.

Acknowledgments

To Adão Saurin, undergraduate Physical Therapy student, and to Danielli Maria Donadel, undergraduate veterinary student, who collaborated in the data collections; to laboratory technician Jéssica Arsand of the Veterinary Medicine course, who helped to process the hematological analyses; and to the employees of the Unicruz vivarium, Roberto Machado Moraes and Giovane Lopes Seccon.

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