Blockage of intercellular adhesion molecule-1 (ICAM-1) in the prevention of reperfusion lesion in the skeletal musculature of EPM-1 Wistar rats[1]

Bloqueio das moléculas de adesão intercelular-1 (ICAM-1) na prevenção da lesão de reperfusão na musculatura esquelética de ratos Wistar EPM–1

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ABSTRACT

Purpose: Ischemia-reperfusion lesions are a form of acute inflammation in which leukocytes are considered to play a pivotal role. This study was made with the objective of determining whether the blockage of intracellular adhesion molecule-1, involved in the diapedesis of leukocytes, is efficacious in minimizing this lesions in the skeletal musculature of the posterior limbs of rats. **Methods:** The juxta-infrarenal aorta of three groups of six adult rats was clipped for six hours. After this, one group was sacrificed (control group) and the others underwent 24 hours of reperfusion, one with 0.9% physiological saline (reperfusion group) and the other with anti-ICAM-1 monoclonal antibodies (ICAM-1 group). A myeloperoxidase assay was utilized for estimating the infiltrate of neutrophils. Biopsies were obtained to make thin sections of hematoxylin-eosin and NADH. Blood samples were collected for making assays of biochemical parameters (creatinine; potassium; DHL; leukogram; venous pH; CK). **Results:** The myeloperoxidase levels were raised in the reperfusion ($p < 0.001$) and ICAM-1 ($p < 0.019$) groups in relation to the control group. The oxidative activity of the muscle fibers was significantly raised in the groups that underwent reperfusion. The other parameters did not present significant differences. **Conclusions:** The reperfusion lesion was bigger than the ischemic lesion. There was an increase in oxidative activity and inflammatory infiltrate with the reperfusion, without significant muscle necrosis being seen under the optical microscope. The blockage of ICAM-1 diminished the inflammatory infiltrate but not the rise in oxidative activity observed with the reperfusion.

Key words: Ischemia. Reperfusion. Intercellular Adhesion Molecule-1. Muscle, Skeletal. Myeloperoxidase. NADH. Diaphorase.

RESUMO

Objetivo: As lesões de isquemia-reperfusão (I/R) são uma forma de inflamação aguda na qual os leucócitos são considerados como tendo um papel fundamental. Este estudo foi feito com o objetivo de determinar se o bloqueio das Moléculas de Adesão Intercelular –1 (ICAM-1), envolvidas na diapedese dos leucócitos, é eficaz em minimizar estas lesões na musculatura esquelética dos membros posteriores de ratos. **Métodos:** A aorta infra-renal de três grupos de seis ratos adultos foi clampeada por seis horas. Logo após, um grupo foi sacrificado (grupo controle) e os outros foram submetidos a 24 horas de reperfusão, um com solução salina fisiológica 0,9% (grupo reperfusão) e outro com anticorpos monoclonais anti-ICAM-1 (grupo ICAM-1). A quantificação da enzima mieloperoxidase foi utilizada para estimar o infiltrado de leucócitos na musculatura. Biópsias foram obtidas e coradas com hematoxilina-eosina e NADH. Amostras de sangue foram obtidas e parâmetros bioquímicos foram analisados (creatinina; potássio; DHL; leucograma; pH venoso, CK). **Resultados:** Os níveis de mieloperoxidase foram aumentados nos grupos reperfusão ($p<0,001$) e ICAM-1 ($p<0,019$) em relação ao grupo controle. A atividade oxidativa das fibras musculares foi aumentada de maneira significativa nos grupos submetidos a reperfusão. Os outros parâmetros não apresentaram diferenças significativas. **Conclusões:** A lesão de reperfusão foi de maior magnitude que a lesão isquêmica. Houve aumento da atividade oxidativa e do infiltrado inflamatório com a reperfusão, não se observando necrose significativa da musculatura com o microscópio óptico. O bloqueio de ICAM-I diminuiu o infiltrado inflamatório mas não o aumento da atividade oxidativa observado com a reperfusão.

Descritores: Isquemia. Reperfusão. Molécula 1 de Adesão Intercelular. Músculo esquelético. Peroxidase. NADH. Desidrogenase.

Introduction

The restoration of blood flow to an extremity that has undergone acute ischemia initiates a series of events that may determine a significant deterioration of the lesion, multiple organ failure and death [1-3]. This paradox of the increase in the lesion with the restitution of oxygenation and vital nutrients to the cell is still not completely understood today and probably has a multifactorial etiology [4].

At the end of the 1980's, a large number of authors demonstrated beneficial effects on the skeletal musculature of animal models that underwent ischemia when the reperfusion was done using blood depleted in neutrophils [5-9]. Simultaneously, the identification of a congenital deficiency in a family of proteins present in leukocyte membranes which is responsible for the adherence of leukocytes to the endothelium iniciated the interest in the study of these molecules[10]. During this past decade, there has been great emphasis on the blockage of these adhesion molecules, which may be localized in leukocytes and/or endothelium, with the objective of minimizing I/R lesions [11-23]. Today, it is believed that signs are generated at the inflammation sites that activate the circulating phagocytes and the adjacent endothelium. As a consequence of the activation, one or both of the cell types become adhesive. The circulating leukocytes then rolling the endothelium, adhere firmly to it and migrate through the subendothelial matrix to reach the inflammation sites, where they release cytokines and lithic substances. This process, despite being essential to the organism's defense mechanisms, may sometimes have pathological consequences [24,25].

Intercellular Adhesion Molecule-1 (ICAM-1), initially described by Rothlein et al. [26], is one of these adhesion molecules, expressed in the endothelium [27] and responsible for the firm adhesion of leukocytes to it.

The objective of this work was to evaluate whether the blockage of ICAM-1, via the use of monoclonal antibodies administered at the outset of reperfusion, is efficacious in minimizing reperfusion lesions in the skeletal musculature of rats undergoing six hours of partial ischemia of the posterior limbs followed by 24 hours of reperfusion.

Methods

The study utilized 32 adult Wistar EPM-1 rats of UNIFESP-EPM animal colony. They were aged between 90 and 120 days, and weighed between 250 and 350 g. Ethical principles for animal experimentation as stated by the International Animal Protection Union and Law 6638 of May 1979, and revised in 1983 were strictly followed. A protocol was submitted to the Ethics in Research Committee of UNIFESP-EPM, and approved under registration n. 204/00.

Surgical technique - Eighteen animals received a preanesthetic dose of ethyl ether and were then completely anesthetized using chloral hydrate 10% (0.4 ml/Kg) by peritoneal route. After abdominal trichotomy and antisepsis using topical iodopovidine, a median 5 cm laparotomy was carried out, moving the bowel to the right. The juxta-infrarenal portion of the abdominal aorta was identified, isolated and ligated using 7-zero polypropylene thread. The whole procedure was performed with the assistance of a microscope viewer (DF Vasconcellos, bench model, series: 51314), at 25x magnification. Ligature efficacy was verified by the paleness of the hind limbs and the absence of pulse below the ligature, using the microscope viewer. Thereafter, the intestines were repositioned in the cavity and the abdominal wall was closed using 3-zero cotton thread in a continuous single-plane suture.

Randomization of the animals - Six hours later the animals were re-operated and a random selection was performed via sealed envelopes to divide the animals into three Groups of 6 rats.

Group I (group control): Without removing the ligature of the aorta, 3 ml of blood was collected from the animal's inferior vena cava. Next, two samples of the gastrocnemius muscle was withdrawn from the left hind limb. Euthanasia was performed by anesthetic overdose.

Group II (group reperfusion): The ligature of the animal's aorta was undone, allowing reperfusion of the hind limbs. The vena cava inferior was puncionated and 3 ml of 0,9% physiological saline was infused into the vena. The bowel was again positioned and the abdomen closed. Twenty-four hours later, the animals were again anesthetized, reopened and 3 ml blood were collected from the inferior vena cava. Two samples were removed from the gastrocnemius muscle of the left hind limb. Euthanasia was performed by anesthetic overdose.

Group III (group ICAM-1): Procedures were exactly the same as in Group II, up to the phase of the infusion of 1 ml of 0.9% physiological saline into the inferior vena cava, after the second laparotomy. In this Group, anti-ICAM-1 antibodies (ICN Biomedicals, Inc. – clone 1A29; cat no. 69-627; lot no. 75606; control R37) were added and diluted to a concentration of 2 ml/kg in 3 ml of 0.9% physiological saline. From this point on, the procedure was again identical to that of Group II.

Laboratory tests - The blood obtained from inferior vena cava was separated in three recipients. In a tube with anti coagulant (EDTA), one ml was added to leukogram and differential counting; 1½ ml were added in a dry tube to creatinephosphokinase (CK in unit/L), lactic dehydrogenase (LDH in unit/L), potassium (K in mEq/L) and creatinine (C in mg/dl) dosage; and 0,5 ml was added in a heparinized syringe to pH measurement.

The leukogram was performed in an automated laser flux cytometry analyzer (Cell Dyn 3700 or 4000) and the differential confirmed by reading of a microslide stained by giemsa. To access the pH, the sample was submitted to a selective ion technique on a gas analyzer-ABL5 Radiometer®. The reserved blood from the dry tube was centrifuged at 3000 rpm for 4 mi, and the plasma separated. Automatic and specific laboratory analyzer machines made all dosages. Potassium was accessed by selective ion method on a Hitachi 917- Roche®. Creatinine, CK and DHL were accessed by enzymatic method on a Hitachi 917- Roche®. All reagents were Roche®, specific to the reactions on these laboratory machines.

Organ samples - Two 1 mm samples from the gas-

trocnemius of the left hind limb were taken, weighed and frozen at –80 ºC for myeloperoxidase analysis.

Extraction of myeloperoxidase was taken from the tissue - The material was defrosted at room temperature and 0.5 ml of the cell membrane detergent HTBA (cetyldimethylethylammonium bromide; L-5335; lot 26H03542; Sigma) was added for 20 min. The samples were homogenized and incubated of 60º C for two hours to optimize the enzymatic extraction and inhibit proteases exogens. Thereafter, the material was centrifuged at 10,000 rpm for 20 min; the supernatant was separated and snap frozen at –80 ºC for later enzyme reading using a spectrophotometer.

Tissue Myeloperoxidase reading - The supernatant of the homogenized material was defrosted and 450 µl of TMB (3,4,5-trimethoxybenzoic acid, 8-diethylamine-octyl ester) - liquid substrate for Elisa (Dako) were added and then read on a 650-nm spectrophotometer. The results were plotted on a reference curve, on the myeloperoxidase axis, and the corresponding numbers of neutrophils were found on the other axis. The numbers found were then divided by the previously recorded weight of the material, thereby obtaining the concentration of neutrophils by weight for each tissue of each Group studied.

MPO reference curve - The total blood from 14 normal rats was collected in heparinized tubes. The polymorphonuclear neutrophils (PMNs) were separated into a 50ml plastic beaker containing a cell sedimentation filter layer made up of 8 volumes 2% methylcellulose (Sigma) and 5 volumes 34%sodium hypaque (Sanofi Winthrop). After 20 min at room temperature (RT), 50% of the plasma layer with a high amount of leukocytes and platelets was transferred to a plastic tube, which was centrifuged at 1000 rpm for 10 min at RT. The supernatant plasma, with a high amount of platelets, was discarded and the cell sediment was carefully resuspended in PBS pH 7.2 and centrifuged twice at 1000 rpm for 10 min. The supernatant was discarded and the cell sediment was mixed with 2 ml previously chilled hemolysis solution (NH_4CL: 8.29 g/l + $KHCO_3$: 1.0 g/L – Merck S.A.) and kept at 4 ºC for 5 min. The tube was again centrifuged at 1000 rpm for 10 min at RT, the supernatant was discarded and the cells were resuspended in PBS and centrifuged at 1000 rpm at RT. The cells were then resuspended in 1 ml PBS. The PMNs were counted in a Neubauer chamber, yielding a concentration of $7.5x10^6$ cells/ml. The technique was that described by Bradley [24] and Schierwagen [55] with few modifications.

Muscle biopsies: Fragments of the gastrocnemius were obtained after the sacrifice of the animals. They were placed in dry ice and frozen in liquid nitrogen. The period between its removal and freezing did not exceed fifteen minutes. Two thin sections were made from each biopsy: hematoxylin-eosin (HE) and NADH (B-nicotinamide adenine dinucleotide + nitroblue tetrazolium Grade III; Sigma).

Statistical analysis - ANOVA® method for the analysis of multiple variables was used. Values of $p \leq 0.05$, confidence interval of 95%, were considered statistically significant.

Results

MPO: For the construction of the calibration curve (Figure 1), we utilized the number of neutrophils counted in the Neubauer chamber at the different dilutions, and the respective optical density of the reading from the spectrophotometer.

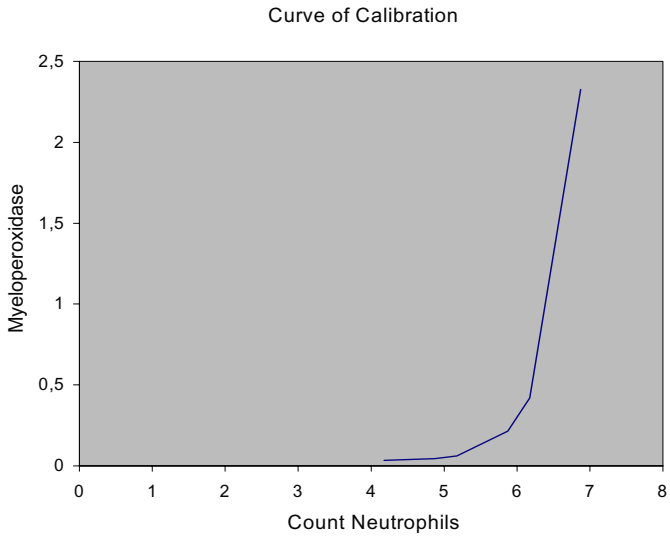

FIGURE 1 – Graph with the interpoled values on the calibration curve

The analysis of the levels of MPO enzyme contained in the gastrocnemius fragments obtained via biopsy demonstrated that there was a significant increase in MPO levels in the tissue that underwent reperfusion: control group versus reperfusion group ($p < 0.001$) and control group *versus* ICAM-1 group ($p < 0.019$). When we compared the ICAM-1 group with the reperfusion group, we observed a significant diminution in the levels of myeloperoxidase ($p < 0.001$) in the ICAM-1 group (Figure 2).

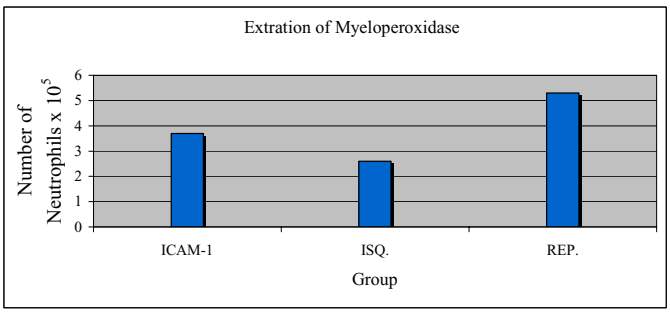

FIGURE 2 – No statistical difference between animals in the same Group was observed, however there was a significant increase in the readings from the animals of Group Reperfusion to the animals of Group Ischemia ($p<0,001$) and Group ICAM-1 ($p<0,001$). When comparing Groups Ischemia and ICAM-1, a greater concentration of defense cells in the latter was observed ($p<0,019$)

These results suggest that after ischemia the skeletal musculature of rats presents significant sequestration of neutrophils upon reperfusion which is partially, however significantly, diminished with the use of anti-ICAM-1 monoclonal antibodies for rats.

Muscle biopsies: The histological studies of the muscle biopsies in relation to the number of necrotic fibers (HE) were not significantly different between the three groups studied. The study of oxidative activity in the muscle cells (NADH) demonstrated a significant increase in activity in the groups that underwent reperfusion (Figures 3 and 4).

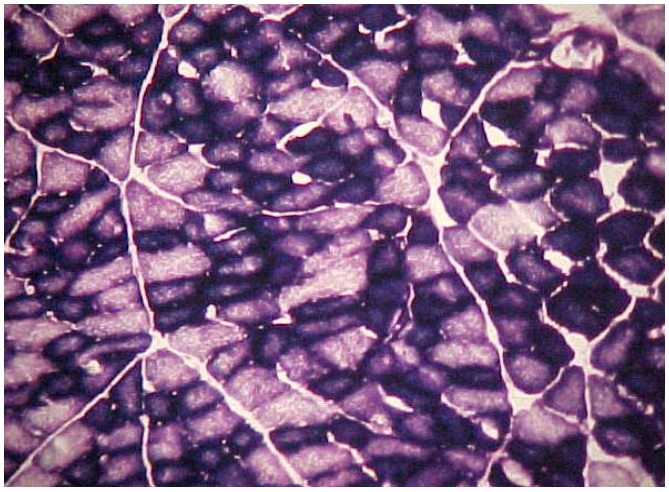

FIGURE 3 – Thin section from the ICAM-1 group, demonstrating an increase in oxidative activity (NADH x 125)

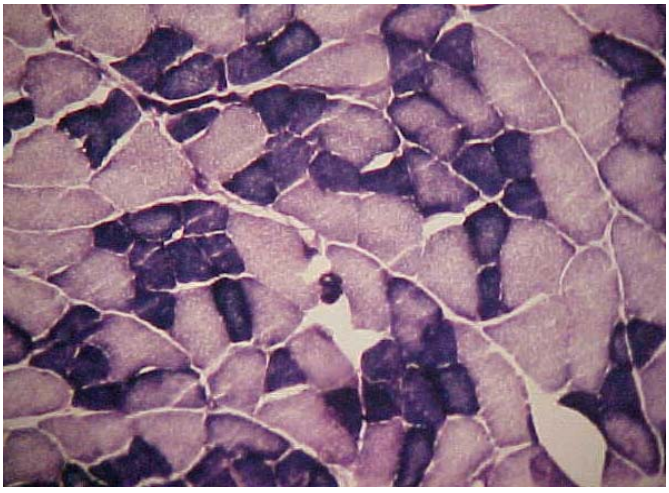

FIGURE 4 – Thin section from the ischemia group, demonstrating standart oxidative activity (whitin normality) (NADH x 125)

These results indicate that the six-hour duration of the partial ischemia was insufficient to cause significant necrosis and that the reperfusion lesion had a greater magnitude than the ischemic lesion, showing an increased oxidative activity, in the skeletal musculature of the rats posterior limbs. Electrolyte dosage: We did not observe any differences that was statistically significant in relation to electrolyte dosage, muscle lesion markers or renal function between the groups studied.

Discussion

Reperfusion of the skeletal musculature after ischemia of the lower limbs is associated with mortality that varies from 15 to 52% and an amputation rate from 12 to 22%, despite success in completing revascularization[2]. Undoubtedly reperfusion lesions contribute to these results and, the fact that such lesions initiate pathological reactions which can become exacerbated, enabling treatment[4,31].

For firm adhesion to occur, interaction between glycoproteins of specific membranes, presents in the leukocytes, and ICAM-1, presents in the endothelial cells, needs to occure. The glycoproteins are formed by the CD11/CD18 complex constituted by one beta subunit, CD18, and three distinct alpha subunits, CD11a, CD11b and CD11c, denominated integrins[25,34-36]. ICAM-1 is an immunoglobulin from the IgG protein superfamily [27].

ICAM-1 is expressed at low levels in the endothelium. In cultures of endothelial cells from the human umbilical cord, its expression increases when stimulated by agents like interleukin-1 and tumor necrosis factor alpha[37], in a process that requires synthesis proteins. The expression increases until it reaches a peak at 4 hours and, thereafter, it remains at high levels for more than 24 hours [38]. Other substances like Interferon, phorbol-esters and lipopolysaccharides may also induce or increase ICAM-1 expression. Even cells that normally do not express ICAM-1 may do so in the presence of stimuli [40].

In our study, the administration of anti-ICAM-1 monoclonal antibodies right at the start of reperfusion significantly diminished neutrophil infiltration into muscle tissue after ischemia. Our data showing that ICAM-1 blockage diminishes the inflammatory infiltrate in muscle tissue after ischemia is in agreement with the majority of studies in the literature [14,17,21-23]. Skejeldal et al. [41] mentioned not having obtained any beneficial effect from the use of anti-neutrophil serum, although they did not assay the MPO and they chilled the ischemic limb to 22º Celsius. This latter fact could in itself have been responsible for the results encountered, since chilling the ischemic limb has as influence as the duration of ischemia in determining the degree of muscle necrosis [42].

The fact that there was a significant increase in inflammatory infiltrate with the reperfusion, despite the use of antibodies, leads us to believe that other cell adhesion methods could have served as the migration route for the neutrophils. Kubes et al. [33] reported that the rolling (dependent of Selectin) and adhesion (dependent of ICAM-I) are not totally interdependent events. Diamond et al. [37] mentioned that the ligation sites for ICAM-1 with CD11a and CD11b are distinct and, depending on the clone of the anti-ICAM-1 antibodies, the ligation may be preferential or even specific to just one of these molecules. Issekutz et al. [43] reported that the joint blockage of CD11a and CD11b is more effective, since in the absence of one of these molecules, the other could acquire vicariousness. This data leads us to ask whether the fact that the significant increase in inflammatory infiltrate, was due to some specific process in the action of the antibodies.

Our data shows that partial ischemia for six hours did not determine significant muscle necrosis and are corroborated by numerous studies in literature [8,9,12,42,45,46]. Yokota et al. [8] mentioned that in cases where the ischemic injury does not determine significant muscle necrosis, the reperfusion lesion may have a greater magnitude than the ischemic lesion. In our work, we observed that the reperfusion lesion had a greater magnitude than the ischemic lesion, not only due to the inflammatory infiltrate but also due to the greater oxidative activity presented by the muscle cells.

Summers et al. [47] observed that changes in chemotactic activity of the neutrophils in I/R lesions can be observed even in the absence of any other evidence of lesions under the optical microscope. Weselcouch et al. [12] and Blebea et al. [44] reported than the first changes observed in skeletal muscles with reperfusion is an increase in microvascular permeability and edema, both phenomenas mediated by neutrophils, will occur without there being evidence of lesions under the optical microscope.

It is known that NADH is an indicator of oxygen consumption at the mitochondrial level. It has been used for measuring oxidation-reduction in different tissues, with its increase being related to tissue hypoxia and an increase in oxidative "stress" of the cells [48-51]. Horie et al. [52] reported that the increase in NADH staining in tissue undergoing I/R is associated with the accumulation of neutrophils in this tissue. In our work, we observed that the increase in oxidative activity in muscle cells with reperfusion was significant. However when we compared the ICAM-1 and reperfusion groups, for which the difference in MPO levels was significant ($p < 0.001$), we did not observe differences in the increase of oxidative activity.

The observation that reperfusion exacerbates the lesion, as demonstrated by the increase in oxidative activity and MPO levels, leads us to question whether the use of the antibodies was efficrat in minimizing the reperfusion lesions. The histological analysis of the HE thin sections did not demonstrate any beneficial effect, but perhaps if the duration of ischemia had been longer, until the time when muscle necrosis had become evident, we could have seen some difference. The levels of MPO enzyme were significantly reduced with the use of the antibodies ($p < 0.001$) in relation to the reperfusion group, with the increase, observed in the ICAM-1 group in relation to the control group being much less ($p < 0.019$), despite being significant. The analysis of the NADH thin sections demonstrated that there was a clear increase in oxidative activity with the reperfusion. Although there was not a tendency for this increase to be less in the ICAM-1 group, this data leads us to believe that the use of the antibodies was beneficial.

In summary, this study demonstrates that the blockage of ICAM-1 may diminish the sequestration of neutrophils in the skeletal musculature after ischemia, minimizing the reperfusion lesion. Despite the risk of infection inherent to this therapy [53,54],our data corroborate the impression that the study of adhesion molecules, a topic open for research, and the consequent possibility of interfering in the intimate action mechanisms of leukocytes, is a promising therapeutic objective that may have great usefulness in the future.

Conclusions

The reperfusion lesion was bigger than the ischemic lesion. There was an increase in oxidative activity and inflammatory infiltrate with the reperfusion, without significant muscle necrosis being seen under the optical microscope. The blockage of ICAM-1 diminished the inflammatory infiltrate but not the rise in oxidative activity observed with the reperfusion.

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