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Original article

Plasma cytokine expression after lower-limb compression in rats☆

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ABSTRACT

Objectives: Muscle injury due to crushing (muscle compression injury) is associated with systemic manifestations known as crush syndrome. A systemic inflammatory reaction may also be triggered by isolated muscle injury. The aim of this study was to investigate the plasma levels of interleukins (IL) 1, 6 and 10 and tumor necrosis factor alpha (TNF-α), which are markers for possible systemic inflammatory reactions, after isolated muscle injury resulting from lower-limb compression in rats.
Methods: Male Wistar rats were subjected to 1 h of compression of their lower limbs by means of a rubber band. The plasma levels of IL 1, 6 and 10 and TNF-α were measured 1, 2 and 4 h after the rats were released from compression.
Results: The plasma levels of IL 10 decreased in relation to those of the other groups, with a statistically significant difference ($p < 0.05$). The method used did not detect the presence of IL 1, IL 6 or TNF-α.
Conclusion: Our results demonstrated that the changes in plasma levels of IL 10 that were found may have been a sign of the presence of circulating interleukins in this model of lower-limb compression in rats.

Expressão de citoquinas plasmáticas após compressão de membros inferiores de ratos

Palavras-chave:
Síndrome de esmagamento

RESUMO

Objetivos: A lesão muscular por esmagamento (lesão por compressão muscular) está associada a manifestações sistêmicas conhecidas como síndrome do esmagamento. A reação

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Modelos animais
Interleucinas
Fator de necrose tumoral α

inflamatória sistêmica pode também ser desencadeada pela lesão muscular isolada. O objetivo deste estudo foi investigar os níveis plasmáticos de interleucinas (IL) 1, 6, 10 e TNF-α, marcadores de uma possível reação inflamatória sistêmica, após a lesão muscular isolada resultante da compressão de membros inferiores de ratos.

Métodos: Ratos Wistar machos foram submetidos a uma hora de compressão dos membros inferiores por uma faixa de borracha. Os níveis plasmáticos de IL 1, 6, 10 e TNF-α foram medidos uma, duas e quatro horas após a liberação da compressão.

Resultados: Os níveis plasmáticos de IL 10 diminuíram quando comparados com outros grupos com diferença estatisticamente significante ($p < 0,05$). Não houve detecção, pelo método, da presença de IL 1, 6 e TNF-α.

Conclusão: Nossos resultados demonstraram que as alterações dos níveis plasmáticos de IL 10 encontradas podem ser um sinal da presença de interleucinas circulantes nesse modelo de compressão de membros inferiores de ratos.

Introduction

It has been well established that musculoskeletal trauma is associated with a significant inflammatory response mediated by a complex biological system of chemical mediators.[1] These mechanisms are present in muscle injuries.[2] In addition to the local inflammatory response, distant organs are subject to the effects of these mediators.[3] The systemic inflammatory response syndrome (SIRS) is a severe complication from this process.[1,4]

The crush syndrome is a systemic repercussion from muscle injuries, especially when the lower limbs are affected.[5] The type of muscle injury associated with crush syndrome occurs through compression, such as in the situation of victims under debris. Classically, there is extravasation of myoglobin in the circulation, with renal repercussions and in many cases, kidney failure.[6] Other mechanisms related to crush syndrome have been studied, with special interest in the inflammatory response.[7,8]

Cytokines are chemical mediators that are studies in relation to inflammation and SIRS. They act as chemotactic factors for inflammatory cells such as neutrophils and are capable of promoting systemic clinical responses such as pain and fever.[9] Production of anti-inflammatory cytokines occurs simultaneously during the process, and this diminishes or even eliminates the inflammation.[10] Today, several different cytokines are known. Among the ones that have been most studied, interleukin (IL) 1, IL 6 and TNF-α (tumor necrosis factor alpha) are characterized by being strongly associated with the inflammatory process. IL 10 is characterized by its antagonist function in the relation to the first three, i.e. its anti-inflammatory action. The proportions between production of pro and anti-inflammatory cytokines seem to determine the severity of the inflammatory response.[3]

We recently developed an experimental model for compression of the lower limbs of rats with the characteristics of a muscle injury due to crushing.[8] With the aim of contributing towards understanding the physiopathology of muscle compression at the cellular and molecular levels, we investigated in the present paper, whether plasma cytokines are present in rates subjected to lower-limb muscle compression, in an experimental model that simulates muscle injury due to crushing.

Materials and methods

After obtaining approval from the ethics committee for animal research, adult Wistar rats weighing 250–300 g ($n = 24$) were deprived of food but continued to have free access to water over a 12 h period before the experiments. The animals were then subjected to general anesthesia (pentobarbital intraperitoneally, 30 mg/kg; and 2% xylazine intramuscularly, 5 mg/kg) while spontaneous respiration was maintained for the start of the experiments. After these 24 animals' clinical condition had been stabilized, they were randomized into four groups ($n = 6$): three groups according to their lengths of survival after release from compression (1 h, 6 h and 4 h) and a control group ($n = 6$). A strip of elastic rubber (Esmarch bandage) of 10 cm in width and 80 cm in length was firmly applied around both hind limbs simultaneously, by the same researcher (Fig. 1). The pressure produced by the bandage was measured as 300 mmHg, using a variation of the Whitesides method, with the bandage still applied (Fig. 2).[11] After 1 h, the bandage was removed. The anesthesia was topped up when necessary. In accordance with the previous randomization, the rats were sacrificed 1, 2 or 4 h after removal of the bandage. The controls were kept under general anesthesia for 4 h, without compression or any other procedure. The rats were sacrificed using pentobarbital intraperitoneally, at a dose of 80 mg/kg and blood samples (2 mL) were collected by means of puncturing the heart using a needle and syringe. The blood samples that were taken were centrifuged immediately (4 °C) and the plasma was frozen (−80 °C) for subsequent analysis on the cytokines. The experimental design of this model was previously established and used by our research group.[8]

Expression of interleukins 1, 6 and 10 and TNF-α

Blood samples were collected (2 mL) by means of cardiac puncture immediately before the rats were sacrificed. The

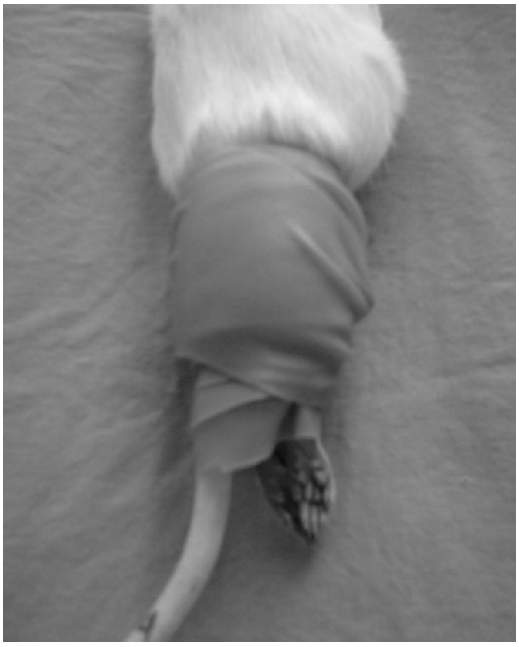

Fig. 1 – Esmarch bandage applied to the hind limbs, wrapped around them 10 times.

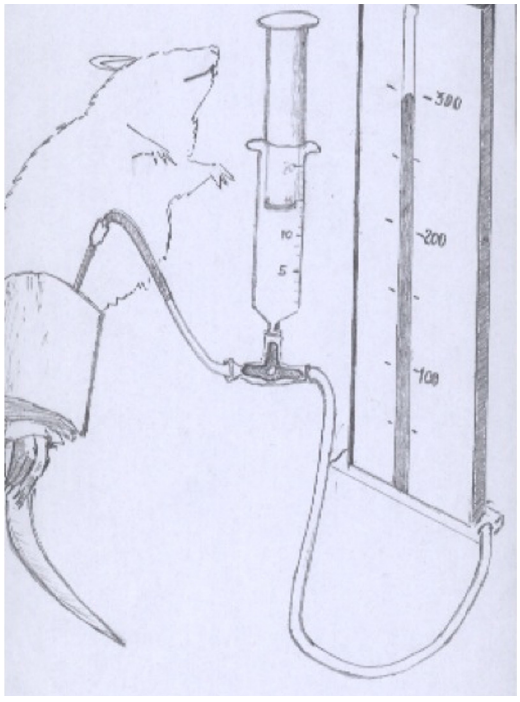

Fig. 2 – Modified Whitesides method: 15 mL syringe filled with air, with embolus under traction, connected to a three-way tap that led to one plastic tube containing air and another tube half-filled with physiological serum. One tube connected to a mercury sphygmomanometer and the other tube to a needle between the skin and the Esmarch bandage. With the standardized application of the Esmarch bandage (wrapped 10 times around the legs), the pressure needed to move the liquid meniscus was 300 mmHg. Diagram of the experiment (the scales do not correspond to reality in order make the method easier to understand).

samples were centrifuged at 1000 *g* (4 °C) for 10 min and the supernatant (approximately 1 mL of plasma) was placed in Eppendorf plastic tubes and stored at −80 °C for subsequent analysis. The interleukins 1, 6 and 10 and TNF-α were expressed in accordance with the manufacturer's instructions (PharMingen – OptEia®, BD Biosciences, Franklin Lakes, NJ, USA). Firstly, the specific antibodies were added to the wells of the reading plate: biotinylated anti-rat IL 1, anti-rat IL 6, anti-rat IL 10 or TNF-α monoclonal antibodies (PharMingen – OptEia®). The plates were incubated for 12 h (4 °C). The wells were washed five times with saline solution in phosphate buffer (PBS) with 0.05% Tween-20 (polyoxyethylene sorbitan monooleate, Sigma®, USA) (standard solution). Following this, 200 μL of the saline solution in phosphate buffer (pH = 7.0) with 10% fetal bovine serum was added to the plates, which were incubated at 4 °C for 30 min. The plates were then washed using the standard solution. The first column of the plate (eight wells) received 100 μL of the antigen solution (IL 1, IL 6, IL 10 or TNF-α) at increasing dilutions (PharMingen – OptEia®). Following this, 100 μL of each of the samples was added to the cells of the second column (the first column, as already described, was reserved for construction of the standard curve of each of the interleukins studied). Each sample was studied in two contiguous cells so that we would be able to use the mean from the values obtained. The incubation period for this phase was 2 h and this was again followed by washing the cells five times with the standard solution. Monoclonal antibodies bonded with biotin were added to the monoclonal plates (Biotinylated anti-rat; PharMingen – OptEia®). After incubation for 1 h, the wells were again washed using the standard solution. At this stage, 100 μL of horseradish peroxidase (HRP) conjugated with avidin was incorporated into each well. The plates were incubated for 30 min and were washed seven times with the standard solution. In the final stage, 100 μL of tetramethylbenzidine and hydrogen peroxide were added. The plates were incubated for 30 min. The final results were obtained using a spectrophotometer (wavelength of 570 nm for IL 1, IL 6 and IL 10; and wavelength of 450 nm for TNF-α).

Statistical analysis

The statistics were analyzed using the SigmaStat® software (SigmaStat for Windows, version 1.0, copyright 1992–1994, Jandel Corporation). The nonparametric Kruskal–Wallis test was used (ANOVA ranking test) and the results were described in terms of the median and interquartile range. Post-tests were then used to compare pairs in the groups. For this, the Student–Newman–Keuls *t*-test was used. To compare pairs after the Kruskal–Wallis test, the Dunn test was used.

Results

The animals' plasma was investigated for the presence of pro-inflammatory interleukins (IL 1, IL 6 and TNF-α) and anti-inflammatory interleukins (IL 10). Within the limits of the method used, no presence of pro-inflammatory interleukins was detected. On the other hand, IL 10 was found in all the

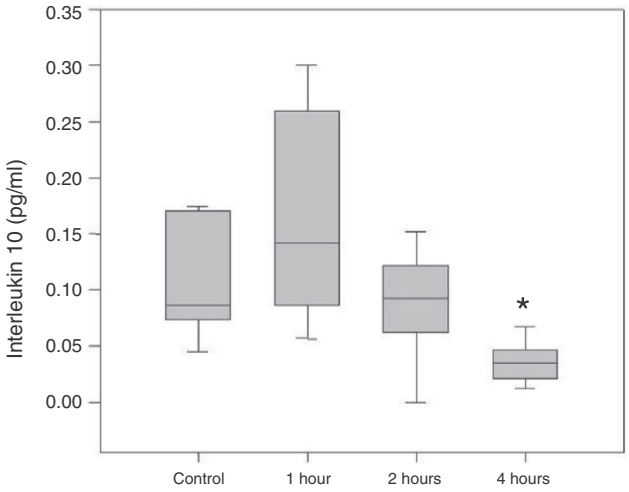

Fig. 3 – Variations in plasma levels of interleukin 10 found during the experiment. * Significant when the groups evaluated 1 and 4 h after release of the compression were compared ($p < 0.05$).

animals studied and, after an initial increase, it presented a progressive decline in plasma concentration (Fig. 1). The means obtained were 0.09 pg/mL (range: 0.073–0.17 pg/mL) for the control; 0.142 pg/mL (range: 0.085–0.259 pg/mL) for 1 h; 0.09 pg/mL (range: 0.061–0.121 pg/mL) for 2 h; and 0.03 pg/mL (range: 0.020–0.047 pg/mL) for 4 h (Kruskal–Wallis Analysis of Variance on Ranks). At the last study time (4 h after release of the muscle compression), this decrease acquired statistical significance in relation to the 1 h group (Dunn test; $p < 0.05$) (Fig. 3).

Discussion

There are several experimental models for studying injuries due to muscle compression and crush syndrome.[7,8,12–15] From our model, the results demonstrated significant alterations in IL 10 levels, 4 h after release of the compression.

Although the most reproducible results have been found through experimental models, clinical trials have correlated high plasma cytokine levels and severe conditions presented by patients with multiple traumatic injuries.[16] Recently, models for crush syndrome have focused not only on the classical findings of crush syndrome, but also on systemic alterations related to the inflammatory response.[7,8] However, among the studies in the literature that we reviewed, none of them correlated crush syndrome with the production of interleukins 1, 6 and 10 and TNF-α in the plasma, as seen in the present study.

Interleukins are polypeptides produced by inflammatory cells and they act at sites close to where they are produced.[17] Damaged muscle fibers may produce cytokines such as TNF-α, IL 1 β and IL 6 (2). When there is high production of interleukins, extravasation of these substances to the plasma occurs. Under these biological conditions, some of these cytokines serve as triggers for a systemic inflammatory reaction.[17]

Some interesting results have indicated that IL 10 has an important regulatory role in immunological and inflammatory responses because of its capacity to inhibit the production of pro-inflammatory cytokines by monocytes.[18] IL 10 reduces the levels of inflammation induced by TNF-α in endothelial cells, such as the production of reactive oxygen species and the adherence of leukocytes to the endothelium.[19] It is unclear whether IL 10 is connected with muscle injury, but there is some evidence that moderate to high levels of IL 10 inhibit the production of IL 1 and IL 6 under such conditions.[20] Our findings (increased IL 10 and non-identification of inflammatory cytokines) may be explained by these interactions between IL 10 and inflammatory cytokines.

In this model, pro-inflammatory cytokines were not found in the plasma after the injury caused by the isolated compression of the hind limbs of the rats. In reviewing the literature, we found data that may help in discussing why these substances were not detected. There is some evidence that IL 6 may only be produced in muscles that are contracted at the time of the trauma.[2] Because of the effect of the anesthesia, the muscles in the model proposed here would have been relaxed at the time of applying the compression, and this may corroborate the negative results found regarding inflammatory cytokine production. Furthermore, it is possible that subtle variations in the concentrations of IL 1, IL 6 and TNF-α may not have been detected in this model due to the limits of the methodology used or even because of the times that we chose for detection of cytokines (1, 2 and 4 h), since it is known that interleukins may have a half-life of only a few minutes. It is important to emphasize that IL 10 was present 1 h after releasing the compression and that there was a progressive decrease in its plasma concentrations over the course of the experiment. As stated earlier, studies have suggested that the final inflammatory reaction depends on the resultant between the pro- and anti-inflammatory mediators.[20] Indirectly, the decrease in the plasma concentration of IL 10 may be related to the presence of circulating IL 1, IL 6 and TNF-α. In conclusion, although IL 10 is an anti-inflammatory cytokine, the levels encountered may be an indirect sign of the presence of inflammatory interleukins in this model of muscle compression in rats.

Conclusions

Our experimental model for muscle injury due to compression (muscle injury due to crushing) demonstrated the presence and variation in levels of IL 10 in the plasma, with a peak 1 h after the compression was released and a decrease in the values found, 4 h after this release.

Conflicts of interest

The authors declare no conflicts of interest.

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