Original article

Plasma cytokine expression after lower-limb compression in rats

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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.

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Expressão de citoquinas plasmáticas após compressão de membros inferiores de ratos

Resumo

Objetivos: A lesão muscular por esmagamento (lesão por compressão muscular) está associada a manifestações sistêmicas conhecidas como síndrome do esmagamento. A reação...
It has been well established that musculoskeletal trauma is a complex biological system composed of a sensitive system of chemical and biological factors which participate in the development of muscle injuries. In addition to the local inflammatory response, distant organs are subject to injury. The inflammatory response syndrome (SIRS) is a severe complication of this process.

The inflammatory response from muscle injury is characterized by cytokines, especially when the lower limbs are affected. The type of muscle injury associated with SIRS can be mechanical, systemic, and local processes.

Cytokines are chemokines that play a role in the inflammatory processes. IL-1 and TNFα are strongly associated with muscle injuries characterized by the onset of SIRS.

Materials and methods

After obtaining approval from the ethics committee for animal research, adult Wistar rats weighing 250-350 g (n = 24) were used. Anesthesia was induced with ketamine hydrochloride and xylazine (intramuscular injection of 5 mg/kg and 2 mg/kg, respectively). Then, the rats were subjected to aortic injury using a surgical blade. The rats were sacrificed 12 hours after the experiment. The duration of ischemia was determined by the length of survival after release.

The duration of compression was 30 minutes, and the muscle samples were collected 4 hours after release. The samples were collected immediately after the experiment and stored at -80°C for subsequent analysis on the cytokines.

Results

The expression of cytokines IL-1, IL-6, and TNFα was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) technique. The results were compared using ANOVA followed by Scheffe's test for multiple comparisons. The significance level was set at p < 0.05.

Discussion

The expression of cytokines IL-1, IL-6, and TNFα was significantly increased in the experimental group compared to the control group. These cytokines play a role in the inflammatory response and may contribute to the development of muscle injuries.

Conclusion

The results demonstrate the role of cytokines in the development of muscle injuries. Further studies are needed to elucidate the mechanisms and the role of cytokines in the inflammatory response.

Acknowledgments

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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 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
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. 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. 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. 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. 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.

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. 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. 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. 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. 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. 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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