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Original Article

Proposal of a non-invasive pelvic imobilization model: study in rats.

EDER JOÃO DE ARRUDA[1], CARLOS ALBERTO DA SILVA[2], RAFAELA FERREIRA[3], NIVALDO ANTONIO PARIZOTTO[4], MARCO AURÉLIO SERAFIM BONVINO[5].
[1]PhD Student in Physiotherapy – UFSCar (Federal University of São Carlos), BRASIL
[2]Professor of the Post-graduation Program in Physiotherapy – UNIMEP (Methodist University of Piracicaba)
[3]Master degree in Physiotherapy – Unimep (Methodist University of Piracicaba), BRASIL
[4]Professor of the Post-graduation Program in Physiotherapy – UFSCar (Federal University of São Carlos)
[5]PhD Student in Human Movement Sciences – UNIMEP (Methodist University of Piracicaba), BRASIL

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Abstract
The pelvic waist is a structure that holds great importance for locomotion. Interventions requiring pelvic immobilization deserve attention by the resulting functional loss. Thus, the study aimed to present a pelvic immobilization device for rats and evaluate the chemo-metabolic alterations on the pelvic musculature in question. Eighteen rats were divided into three groups (n = 6): control (C), pelvic immobilization for 7 days (IP) and remobilized for 7 days after 7 days of device use (RP7). Then analysis chemo-metabolic in pelvic muscles involved were done. The data were processed with the Kolmogorov-Smirnov test followed by ANOVA and Tukey test ($p < 0.05$ for all tests). The glycogen reserves reduced on average 68% in the IP group and 50% in the RP7 group. The glucose decay constant (KITT) had a delay of 10% in the IP group. The ratio of total protein / DNA showed that there was a reduction of 28 and 24% in the gluteo maximus and 32 and 16% in the iliopsoas from the IP and RP7 groups, respectively. It found an increase of cytokines (IL-6 and 10) in the IP and RP7 groups, and only the IP group showed an increased of TNF-α. So the model was effective in inducing the disuse of the muscles in question.
Key words: cytokines, imobilization, pelvic muscles, rats.

Introdution

The pelvis presents itself as an ostheomyoarticular structure holding great importance for locomotion, organ sustainment, thermoregulation and other functions related to the central nervous system.[1]

Emphasizing locomotion, the pelvis is a fundamental structure connecting the spine and lower limbs, and for motor performance of these structures it is necessary balance, supporting and coordination, in a way that any functional alteration on the pelvis might result in systemic functional loss.[2,3]

Recent data point out that pelvic ring injuries are strongly related to a high morbidity rate, resulting in short term complications and this might implicate significant costs for the functional recovery process. It is also stated that the occurrence of pelvic injury on big urban centers happens on a rate of about 23 for each 100,000 inhabitants and total mortality ranges from 4 to 23% between places.[4,5]

Since it is an anatomic structure that contribute for the mobility of the trunk and lower limbs, pelvic ring injuries commonly require immobilization, which is often employed from the acute to the outpatient stages of the treatment. [6] This way, immobilization as a therapeutic resource may trigger atrophy due to muscular disuse, which derives from a complex interaction of mechanisms that culminate with muscular catabolism. [7,8]

Regarding immobilization devices for studying muscular disuse, literature presents invasive and non-invasive models, which in its majority are developed for studying joint immobilization of the hind limb in rats [9-12] and invasive and non-invasive models of scoliosis induction and trunk restraint. [13-15]

Due to the absence in literature of an experimental model for immobilization and the significant issue resulting from the involvement to that structure, the present study aims to develop a model for pelvic immobilization for rats and evaluate the chemo-metabolic alterations on the pelvic musculature in question.

Methods

The present study got approval from the Ethics Committee for Animal Experimentation from the Methodist University of Piracicaba (UNIMEP) under the protocol no. 09/2018. The ethical principles established on the Guide for the Care and Use of Laboratory Animals were also strictly followed. [16]

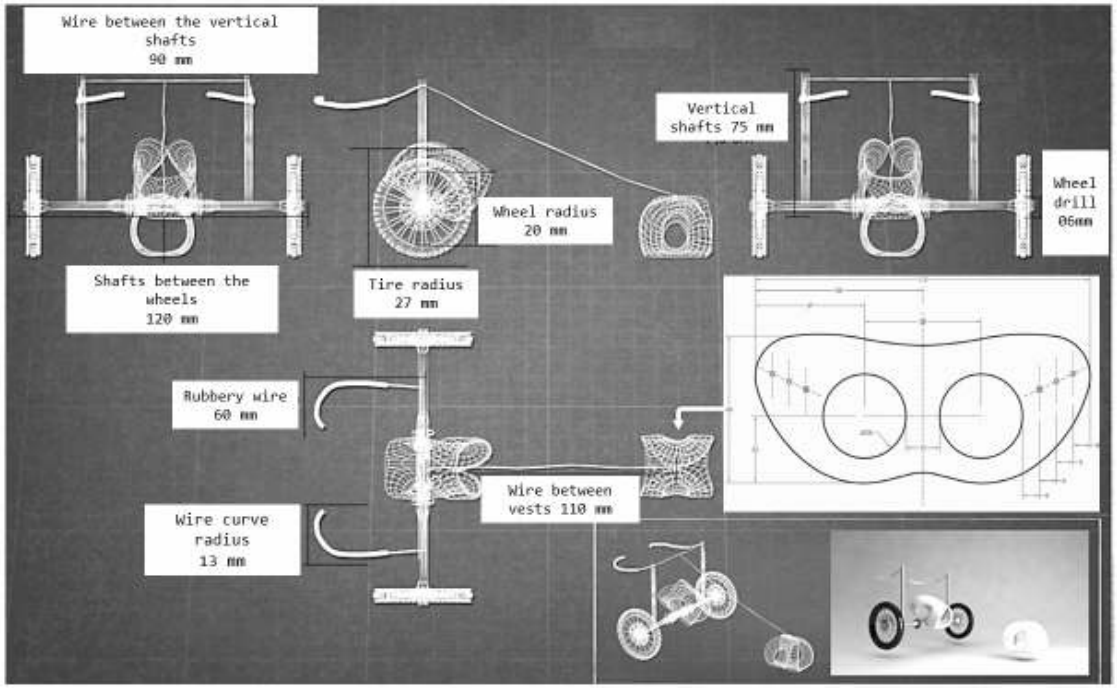
18 Wistar, male rats (*Rathus novergicus var, albinus, Rodentia, Mamalia*), 3 months old and weighting 250±20g were employed for this experimental procedure. The animals were sorted in three groups (n=6), of which: control (C), pelvic immobilization for 7 days (PI) and the re-mobilized group (RP7), that spent 7 days

Corresponding Author: MARCO AURÉLIO SERAFIM BONVINO, E-mail: marcoaureliofisio@yahoo.com.br

without the device, after the 7 days of immobilization. In order to increase the influence of the sample, it was opted for collecting bilateral muscular samples (n=12) from the studied groups.

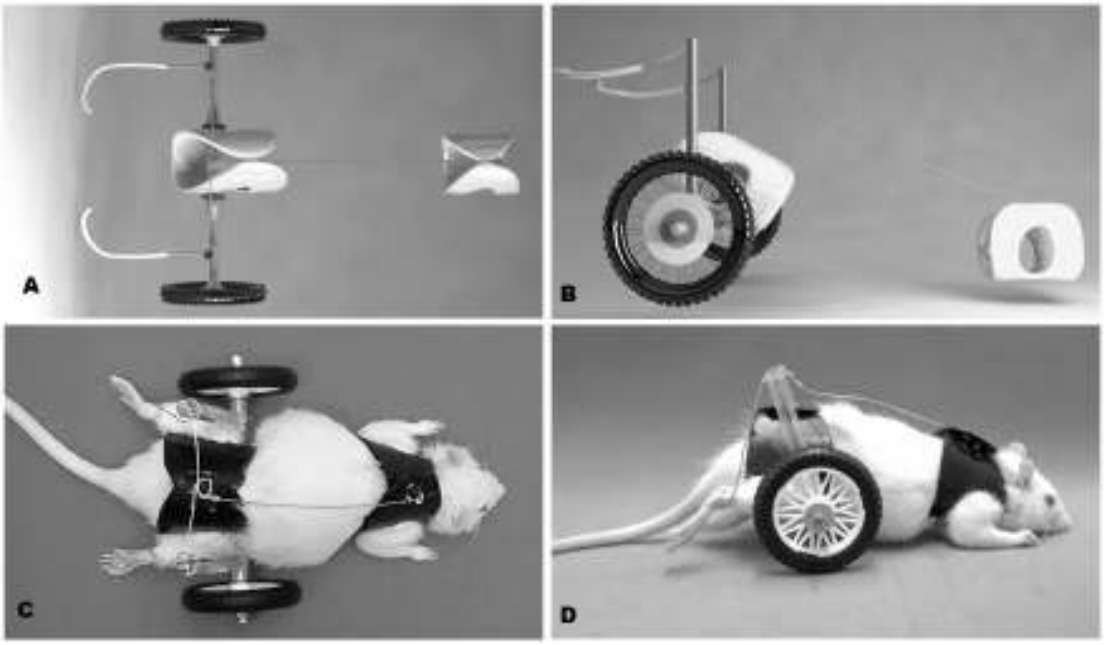
Immobilization Model

To develop the pelvic immobilization model, the current study was based on the work of Silva et al.[14], which presents a device composed of Polyvinyl Chloride (PVC) vests, appropriate for the induction of scoliosis in rats. In the present study, scapular and pelvic scoliosis induction vests were employed, followed by the suspension structure, which was developed using acrylic tubes, plastic wheels, thin wire, bolts and nuts. In the picture 1 it is presented the schematic representation of the model, designed in the Blueprint® software.



Picture 1: Graphical representation of the model applied to pelvic immobilization in 3 months rats. The measures are expressed in millimeters (mm).

The proposed model for pelvic immobilization consists in a system for suspending the (pelvic) posterior waist and hind paws (picture 2, A and C), in a way that the wheels (picture 2, B) favor mobility, while restricting the action of the involved musculature (picture 2D).



Picture 2: Pelvic immobilization device in top view - project (A) and fitted to the animal (C). Suspension system project in side view (B) and on the same plane (D) it's possible to observe the device fitted in the animal.

Evaluations

Evaluation of the muscular glycogen reserves (metabolic rate)

Samples from the following muscles were collected: rectus femoris (RF), adductor brevis (ADB), adductor longus (ADL), lower abdominal (LAB), upper abdominal (UAB), gluteus medius (GM Med), gluteus maximus (GM Max), Iliopsoas (ILP), and a cluster of the paravertebral muscles (PV) in the pelvic region. The acid hydrolysis in the presence of phenol method was employed.[17] The values are expressed in mg/100 mg of wet weight.

Concentration of Total Proteins and DNA (myonuclei rate):

Samples of the gluteus maximus and iliopsoas muscles were submitted to evaluation of the concentration of total proteins, through the PROTAL kit from Laborlab® and the samples of the DNA through the method proposed by Giles and Myers.[18]

Insulin Tolerance Test (ITT)

In order to conduct the ITT, on the 6th day the rats were anesthetized and after 10 minutes it was performed a cut on the animal tail where an aliquot of blood was collected and the glycaemia was assessed by a stripe used in glycogen testing, obtaining then the time zero. Followed up by the administration of insulin (2 U/Kg/ip - Biohulin), blood samples were collected in the 2.5 min, 5 min, 10 min, 15 min, 20 min, 25 min and 30 min timeframes, so the glycaemia could be assessed again to determine the Glucose Decay Constant (KITT). The group RP7 was evaluated 6 days after the removal of the immobilization model.

Serum level of Interleukin 6, 10 and TNF-α

The ELISA method was employed and the specifications corresponding to the Kit (BioSource International) were followed (NISHIYAMA et al., 2000).

After anesthesia of sodium pentobarbital (40 mg/Kg), the blood samples were collected through the renal vein, and then put on ice. The serum was isolated, packaged in an Eppendorf tube and then allocated in a freezer under -70º. The values are expressed in pg/mL.

Statistical Analysis

The data were submitted to an ANOVA Kolmogorov-Smirnov normality test and a Tukey test. In every calculation a critical level of $p<0.05$ was set.

Euthanasia

The animas were anesthetized with sodium pentobarbital (40 mg/Kg) and then submitted to the cervical dislocation technique. The group RP7 underwent euthanasia 7 days after the removal of the immobilization model.

Results

Evaluation of the glycogen reserves

It was observed that the musculature of the group that underwent the pelvic immobilization (PI) got severely compromised in comparison to the control group, in a way that the glycogen reserves reached critical reduction levels, as showed on table 1, that presents the percentage of reduction, as well as the values (table 2) of the glycogen concentration that express statistical difference ($p<0.05$).

Even though the group RP7 resumed walking for 7 days, it was observed that the glycogen reserves were not reestablished in comparison with the group C. On the other hand, when comparing with the group PI, there was an increase of the muscular glycogen reserves ($p<0.05^{\#}$) as pointed out in the tables 1 and 2.

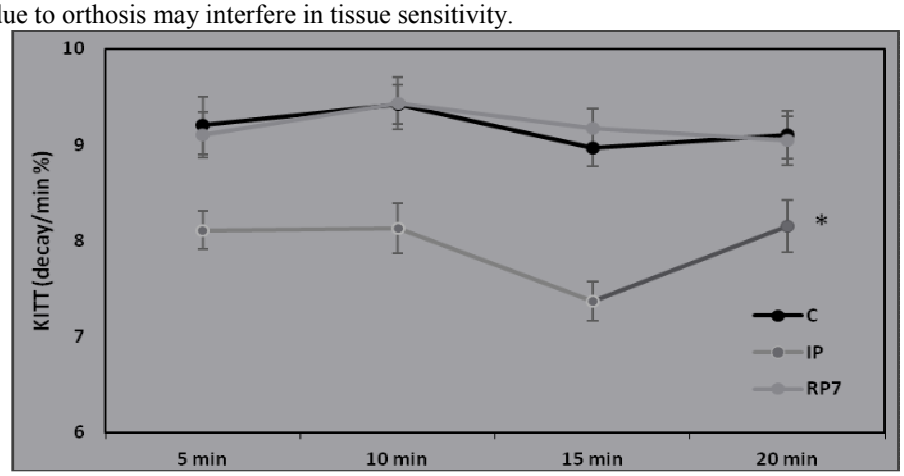
Table 1. Reduce percentage of energy reserves (glycogen) in the muscles involved in pelvic immobilization process of the groups IP and RP7 compared to Control ($p <0.05$ *) and increase percentage in the comparison between RP7 and IP ($p <0, \# 05$).

Muscles	↓ % Glycogen IP/C*	↓ % Glycogen RP7/C*	↑ % Glycogen RP7/IP#
Gluteus maximus	76 %	55 %	21 %
Gluteus médio	69 %	47 %	22 %
Paravertebral	64 %	43 %	21 %
Addcutor brevis	57 %	53 %	4 %
Addcutor longus	67 %	53 %	14 %
Rectus femoris	70 %	48 %	22 %
Iliopsoas	75 %	60 %	15 %
Upper Abdominal	66 %	40 %	26 %
Lower Abdominal	75 %	55 %	20 %

Table 2. Glycogen concentration (mg/100mg) of the groups: control (C), pelvic immobilization (IP) and remobilized after 7 days (RP7). Values are expressed in avarage±sem, n=12, p<0.05* compared to control and p<0.05# compared to IP.

Muscles	C	IP	RP7
Gluteus maximus	0,85±0,03	0,20±0,08*	0,38±0,03*,#
Gluteus médio	0,87±0,04	0,26±0,03*	0,46±0,05*,#
Paravertebral	0,81±0,05	0,29±0,03*	0,46±0,05*,#
Addcutor brevis	0,75±0,03	0,32±0,05*	0,35±0,03*,#
Addcutor longus	0,94±0,01	0,31±0,03*	0,44±0,02*,#
Rectus femoris	0,85±0,03	0,23±0,04*	0,41±0,03*,#
Iliopsoas	0,98±0,01	0,24±0,03*	0,40±0,04*,#
Upper Abdominal	0,88±0,03	0,30±0,07*	0,52±0,02*,#
Lower Abdominal	1,02±0,06	0,25±0,03*	0,46±0,01*,#

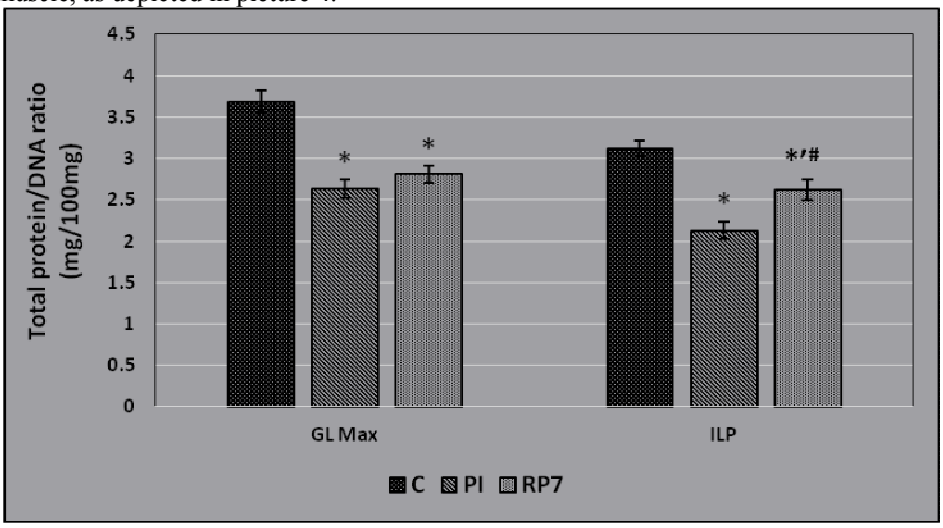
The set of data regarding insulin sensitivity revealed that there were no significant alterations between group C (9.10 ± 0.02) and group RP7 (9.13±0.02). On the other hand, the group PI presented a delay of 10% (8.24±0.03) in the decay constant in comparison to the group C (picture 3). That evaluation points out that the disuse due to orthosis may interfere in tissue sensitivity.



Picture 3: Glucose decay constant (KITT; % min) of the groups: control (C), pelvic immobilization (IP), remobilized after 7 days (RP7). Values are expressed in avarage±sem, n = 6, p <0.05 * compared to C.

Evaluation of the total protein/DNA ratio

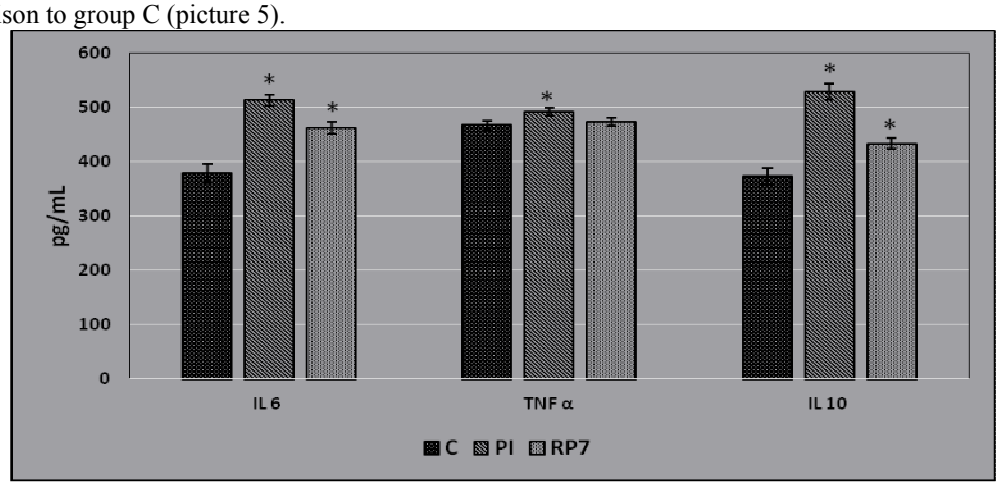
The total protein/DNA ratio of the gluteus maximus (GL Max) and Iliopsoas (ILP) muscles emphasized that the groups PI and RP7 presented reduction (p<0.05*) of 28 and 24% in GL Max and 32 and 16% in ILP, respectively, when compared to group C. On the other hand, group RP7, in comparison to group PI, demonstrated less compromise of the total protein/DNA ratio, highlighted by an increase of 19% (p<0.05*) in the ILP muscle, as depicted in picture 4.



Picture 4: Total protein/DNA ratio (mg/100mg) of the muscles gluteus maximus (GL Max) and Iliopsoas (ILP) of the control group (C), pelvic immobilization (IP) and remobilized after 7 days (RP7). Values are expressed in avarage±sem, n = 6, where * p <0.05 compared to control and #p <0.05 compared to the IP.

Evaluation of the Cytokines

In the evaluation of the serum level of interleukin 6, there was an increase of 27.3% on group PI and 19.2% on group RP7, when compared to group C. It was identified that interleukin 10 demonstrated an increase of 30.4% and 14.9% on groups PI and RP7, respectively. Finally, the tumor necrosis factor (TNF-α) presented a slight increase of 5% on group PI compared to group C, while group RP7 did not show any difference in comparison to group C (picture 5).



Picture 5: Serum level of interleukin 6 (IL6), interleukin 10 (IL10) and tumor necrosis factor (TNF-α) of the groups: control (C), pelvic immobilization (IP) remobilized after 7 days (RP7). Values are expressed in pg/ml and correspond to the avarage±sem, n = 6 p <0.05 * compared to C.

Discussion

The pelvic waist is a structure that holds great importance for locomotion, in a way that it can often be affected by conditions that demand therapeutic immobilization, which in an iatrogenic way results in disuse syndrome.

Studies point out that after 48 hours of disuse the muscular tissue suffer modifications of great magnitude, such as; alterations on the insulin signaling mechanisms, increase of inflammatory cytokines, decrease of the glycogenic reserves, as well as decrease of the protein content, besides alterations of histological and molecular aspects.[8,19] The effects of immobilization may show up right after a few hours, yet literature presents a consensus regarding immobilization time of 7 days.[9,10]

In the current study it was noted that both glycogenic reserves and decay constant (KITT) were compromised on the animals that had their pelvis immobilized, revealing compromise in glycogenesis, which can be resulting from insulin resistance. Although the animals that were re-mobilized after 7 days (RP7) did not recover their glycogenic reserves, it was observed that KITT was similar to the one from the control group, suggesting activation of the anabolic ways and catabolic inhibition.

These findings are supported by the mechanisms of regulation of the glycogen synthesis, once the insulin as an anabolic hormone stimulate the accumulation of glycogen through the increase of transportation of glucose in the muscle, while favoring the glycogen synthesis on the liver and on muscular tissue, process which happens through dephosphorylation of the glycogen synthase enzyme.[20]

The comprehension of the systemic repercussion can be based on the fact that right after the stimulus with insulin, the Akt phosphorylated and inactivated to GSK-3, which reduces the phosphorylation rate of the glycogen synthase, increasing its activity. It is also highlighted that the insulin also activates protein phosphatase 1, through a mechanism depending on PI 3-kinase, which directly dephosphorylates the glycogen synthase.[21]

The total protein/DNA ratio of the muscles GL Max and ILP was drastically reduced. As such, it is known that the control of the synthesis of proteins begins with the formation of ribonucleic acid (RNA) in the nucleus, under the control of the deoxyribonucleic acid (DNA). Thus, a reduction of the contractile activity interferes in fundamental mechanisms for gathering amino acids in muscular tissue, once the amino acid transportation rate is proportional to the contractile activity, regardless of insulin action.[8,22,23] Anatomical and biomechanical factors may have influenced so the ILP muscle presented an increased compromise.

To evaluate the immune response by applying the pelvic immobilization model, it was opted for analyzing the IL-6, 10 and TNF-α cytokines. During this analysis, it was observed that the proposed immobilization model activated pro-inflammatory (TNF-α and IL-6) and anti-inflammatory (IL-10 and IL1ra) mechanisms, with higher significance on group PI. It is also highlighted that the concentration increase of these cytokines aim to activate anabolic ways and minimize the activity of catabolic mechanisms in physical activity or disuse conditions.[24,25]

In the current study the IL-10, which is an anti-inflammatory cytokine demonstrated to be more elevated than the IL-6, which is pro-inflammatory. This indicates the pelvic immobilization triggered an increasingly intense inflammatory response, once that, when the muscular glycogen content is reduced there is

the activation of the transcription for the IL-6 gene.[24] It is worth pointing out that the expression of IL-10 was lower on group RP7 while the concentration of TNF-α did not suffer alteration on the same group. It is also important to bear in mind that the IL-10 is the main modulator anti-inflammatory cytokine in the production of TNF-α.[26]

Even though the group PI presented a slight increase of TNF-α, it is worth noting the production of TNF-α is not exclusively carried out by monocytes and macrophages, considering the gene and protein expression of TNF-α in skeletal muscle was characterized in human beings, in a study that demonstrated, for the first time, the presence of such cytokine in the absence of infiltrating monocytes or macrophages.[27] Such fact emphasizes that the muscular tissue can produce it by itself, acting so in autocrine and paracrine ways, in the exact amount to modulate a series of morphological and functional alterations on the tissue.[28]

It is noteworthy that the physiological alterations presented here are similar to the ones observed in another muscular disuse experimental models[9,10,15] which grants to this model a double utility since it favors the study of both physiological systems under simulated weightlessness and reduced muscular activity conditions.

Conclusion

The proposed pelvic immobilization model in experimental condition has proven to be effective in mimicking muscular disuse, and such fact is emphasized by the reduction of the energetic reserve on pelvic muscles, followed up also by the reduction of the total protein/DNA ratio. The proposed model also triggered a mechanism of attempt to reverse protein catabolism, highlighted by the increase of the serum level of interleukin 6 and 10. Finally, the methodological practicality and the low cost of the model deserve to be highlighted when fostering future studies that may look for early strategies of physiotherapeutic intervention in cases of pelvic immobilization.

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