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ORIGINAL RESEARCH

Histomorphometric changes in the knee joint of Wistar rats after remobilization in a water enviroment

Alterações histomorfométricas na articulação do joelho de ratos Wistar após remobilização em meio aquático

Alteraciones histomorfométricas en la articulación de la rodilla de ratones Wistar después de removilización en medio acuático

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Abstract | The objective of this study was to investigate the effects of immobilization and analyze the influence of free remobilization or the association of therapeutic exercises in an aquatic environment on the morphology of the knee joint. Eighteen Wistar rats were used. They had had their right hind limb immobilized at the full extension of the knee for 15 days, and also divided into three groups: G1, which suffered no intervention; G2, which were subjected to free remobilization; and G3, which had remobilization through exercises (swimming and jumping) in an aquatic environment for 14 days. The right and left knee articulations were collected and embedded in paraffin for morphological analysis. The immobilization and remobilization altered the thickness of the articular cartilage and the number of chondrocytes. The articular cartilage and the synovial membrane suffer degenerative changes due to articular disuse during immobilization; and these changes were gradually reversed by remobilization in an aquatic environment. The combination of swimming and jumping in an aquatic environment proved to be an efficient alternative to articular remobilization and can be used as exercises in physiotherapy rehabilitation programs.

Keywords | Cartilage, Articular; Synovial Membrane; Knee Joint; Immobilization; Rehabilitation; Swimming; Physical Therapy Modalities; Rats, Wistar.

Resumo | O objetivo deste estudo foi verificar os efeitos da imobilização e analisar a influência da remobilização livre ou por associação de exercícios terapêuticos em meio aquático, sobre a morfologia da articulação do joelho. Foram utilizados 18 ratos Wistar que tiveram seu membro posterior direito imobilizado em extensão completa do joelho por 15 dias e que foram divididos igualmente em três grupos: G1 não sofreu nenhuma intervenção; G2 foi submetido à remobilização livre; e G3 teve remobilização por exercícios (natação e salto) em meio aquático por 14 dias. As articulações do joelho, direitas e esquerdas, foram coletadas e incluídas em parafina para análise morfológica. A imobilização e remobilização alteraram a espessura da cartilagem articular e o número de condrócitos. A cartilagem articular e a membrana sinovial sofreram mudanças degenerativas devido ao desuso articular na imobilização e estas mudanças foram progressivamente revertidas pela remobilização em meio aquático. A combinação de natação e salto em meio aquático se mostraram uma alternativa eficiente de remobilização articular, podendo ser utilizada como exercício em programa de reabilitação fisioterapêutica.

Descritores | Cartilagem Articular; Membrana Sinovial; Articulação do Joelho; Imobilização; Reabilitação; Natação, Modalidades de Fisioterapia; Ratos Wistar.

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Resumen | El objetivo de este estudio fue verificar los efectos de la inmovilización y analizar la influencia de la removilización libre o por asociación de ejercicios terapéuticos en medio acuático, sobre la morfología de la articulación de la rodilla. Se utilizó 18 ratones Wistar, que tuvieron sus miembros superiores derechos inmovilizados en extensión completa de la rodilla, por 15 días, y divididos igualmente en tres grupos: G1, no ha sufrido ninguna intervención; G2, fue sometido a removilización libre; y G3, removilización por ejercicios (natación y salto) en medio acuático por 14 días. Las articulaciones de la rodilla, derechas e izquierdas, fueron colectadas e incluidas en parafina para análisis morfológica. La inmovilización y removilización alteraron el espesor del cartílago articular y el número de condrocitos. El cartílago articular y la membrana sinovial sufrieron cambios degenerativos, debido al desuso articular en la inmovilización; estes cambios fueron progresivamente revertidos por la inmovilización en medio acuático. La combinación de natación y salto en medio acuático se mostró una alternativa eficiente de removilización articular, pudiendo ser utilizados como ejercicios en programas de rehabilitación fisioterapéutica.

Palabras clave | Cartílago Articular; Membrana Sinovial; Articulación de la Rodilla; Inmovilización; Rehabilitación; Natación; Modalidades de Fisioterapia; Ratas Wistar

INTRODUCTION

The knee is the largest synovial joint in the body, structurally designed to withstand heavy load[1], also one of the most complex due to the constant need to ally mobility and stability, opposing functions of a joint system[2]. For this reason, the knee is subject to injury, especially for people who practice sports. This occurs due to its anatomy, exposure to trauma, as well as imposed functional requirements[2].

After an injury and regardless of the method of treatment (surgical or conservative), associated lesions will be present, reflecting on the joint health and compromising the functionality of the knee[3]. Immobilization is a common option for treatment in orthopedic disorders, often indicated by sprains, or ligament, muscle and joint injuries, as well as fractures[4,5]. In addition to the beneficial effects, such as pain reduction, maintenance of bone alignment and life quality improvements[5,6], immobilization leads to movement restriction, which can negatively affect bone and muscle composition, blood supply of the fixed part and, in this case, joint structure and integrity[5,7,8].

The appearance of these multifactorial after-lesion injuries highlights the need to use a full rehabilitation protocol for individuals to reestablish their daily life activities and improve their functional capacity as quickly as possible[9]. In this sense, physiotherapy, consisting of different exercises and therapies, is frequently indicated for musculoskeletal injuries, with special attention to passive muscle stretching[10,11,12,13], treadmill running[14], floor jumping exercise[15], swimming[16,17] and a combination of aquatic exercises[18].

Among the available treatment options, aquatic physiotherapy can be considered one of the main interventions in the treatment of joint injuries. Water, through its physical properties, enables the performance of exercises that would be much more difficult on the floor, since it reduces body weight. Also, the greater range of motion and the higher temperature of the water increase joint mobility, speeding up the rehabilitation process[19,20].

Nevertheless, there is no consensus in the literature regarding the most appropriate time for the performance of exercises after an injury, or the intensity or type to be performed in an aquatic environment. Due to this, the objective of this study was to investigate the effects of immobilization and analyze the influence of free remobilization or by association of therapeutic aquatic exercises, started immediately after the immobilization is suspended, on the morphology of the knee joints of Wistar rats. With all this, we hypothesized that an aquatic remobilization program may be beneficial in the homeostasis of morphological constituents of the knee joint subjected to stress by the immobilization.

METHODOLOGY

Eighteen Wistar rats were used, all 10 weeks old, kept in light-dark photoperiods of 12 hours and at 23°C, with water and feed *ad libitum*. All procedures were approved by the Animal Ethics Committee of the Universidade Estadual do Oeste do Paraná, under protocol 03012.

After a week of acclimatization, all animals had their right hind limb immobilized for a period of 15 days. Immobilization was performed by means of plaster bandages, molded directly to the animal's body, keeping the knee in full extension and the ankle in complete plantar flexion, according to the model proposed by Carvalho, Shimano and Volpon[21]. After 15 days of immobilization, the animals were divided into three groups with six rats in each group:

G1 – euthanized immediately after the immobilization period;

G2 – free remobilization in the cage, and contact with water for about a minute during 14 days, so that they could receive daily water stimulus;

G3 – remobilized for 14 days through swimming and jumping in an aquatic environment, on alternate days. In the first six days of remobilization, swimming occurred for 20 minutes and jumping was performed in two sets of 10 jumps each, with 30 seconds of rest between sets. For the other eight days of remobilization, we performed a progression of periods and sets of exercises performed, with the swimming period of 40 minutes and jumping performed in four series of 10 jumps each with an interval of 1 minute between sets. The swimming exercise was performed without overcharging, while the aquatic jumping exercise was performed with 50% overload on body weight, adapted from Gaffuri et al.[22].

The animals of G1, immediately after the immobilization period, and the animals of G2 and G3, following the remobilization period were weighed and anesthetized with ketamine (50mg/kg) and xylazine chloridrate (10mg/kg). Under the effect of the anesthetic, the animals were decapitated using a guillotine and then dissected, extracting the joints of the right and left knees.

After fixation in 10% formaldehyde and decalcification in 5% trichloroacetic acid, the material followed the histological routine procedure for paraffin embedding. The sagittal sections of 7 µm were made onto glass slides and stained with hematoxylin and eosin[23] to enable general morphology analysis and safranin O-fast green staining for articular cartilage analysis[24].

The slides were examined under light microscope and photomicrographed using an *Olympus®* DP71 microscope (USA). The *Image Pro Plus 6.0* (USA) program was used, calibrated to the morphometric analysis of the thickness of the articular cartilage in the areas of contact, transitional and non-contact of the femur and the anterior, medial and posterior articular regions of the tibia[25]. Chondrocytes count was performed on an area of interest, a rectangle with 100 µm in width per 200 µm in length, which was superimposed with the same region analyzed of the articular cartilage thickness in both the femur and the tibia.

The results were presented with means ± standard deviations and analyzed by the *GraphPad Prism 6.0* (USA) program. The Student's t-test was used to compare the control immobilized/remobilized sides and Anova One Way with Tukey post-test was used for comparison between the groups. Statistical significance was established at $p<0.05$.

RESULTS

Articular cartilage thickness

There was a statistically significant increase in the thickness of the articular cartilage in the contact area of the femur in the immobilized/remobilized side when compared to the control side of all animals in G3 ($p=0.02$), as well as in the transitional area of the femur in animals of G1 ($p=0.01$). Comparing the groups, the articular cartilage left in the non-contact area appeared thicker only in animals immobilized, when compared to those remobilized by aquatic exercises ($p=0.03$) (Table 1).

As for the tibia, there was no significant difference in the thickness of the articular cartilage (Table 1).

Chondrocytes count

We found no differences in the number of chondrocytes between the control and immobilized/remobilized sides in any measurement points of the femur. Comparing the groups, the transitional area of the left articular cartilage showed the highest amount of chondrocytes in G3, when compared to G1 and G2 ($p=0.01$). Still, when comparing the groups regarding the right side, a lower cell density in non-contact in G1 was identified, when compared to G2 and G3 ($p=0.01$), as well as a chondrocytes reduction in the articular cartilage contact area of the femur only in the immobilized group, when compared to the remobilization group with aquatic exercises ($p=0.01$) (Table 2).

The posterior member submitted to immobilization showed lower chondrocytes count, compared to the control member in the posterior joint region (P3) of the tibia ($p=0.03$). When we compare the groups, an increased cell density was identified in the left anterior joint region (P1) in G3 compared to G1 ($p=0.04$). Regarding the immobilized/remobilized member, G1 showed a reduced number of chondrocytes in the posterior joint region (P3) compared to G2 and G3 ($p=0.01$) (Table 2).

Table 1. Articular cartilage thickness (μm) of the femur and the tibia

Cartilage thickness			Groups		
			G1	G2	G3
Femur	Non-contact area	Right	233.9±91.3	247.1±194.2	219.5±153.0
		Left	234.7±85.3[a]	180.4±25.6	148.8±21.0
	Contact area	Right	253.4±50.0	239.4±59.6	253.7±42.8*
		Left	250.5±55.3	242.6±52.9	193.6±36.9
	Transitional area	Right	333.5±104.7*	247.7±94.0	215.1±58.3
		Left	222.2±81.2	249.6±68.1	261.4±42.5
Tibia	P1[1]	Right	267.3±144.9	196.8±60.3	204.3±36.6
		Left	205.8±52.5	167.3±48.6	198.4±67.3
	P2	Right	287.4±69.2	231.3±43.2	306.5±101.1
		Left	274.7±129.9	252.4±99.9	255.7±73.1
	P3	Right	204.8±58.6	138.1±48.9	209.2±36.0
		Left	231.8±71.4	151.2±34.7	194.0±53.6

[1]P1 – anterior articular region; P2 – medial articular region; P3 – posterior articular region
* = significant statistical difference between the immobilized/remobilized (right) and control (left)
a = significant statistical difference between G1 and G3

Table 2. Number of chondrocytes in the anterior cartilage of the femur and the tibia

Number of chondrocyte			Groups		
			G1	G2	G3
Femur	Non-contact area	Right	19.8±4.4[a,b]	34.0±9.5	33.3±8.4
		Left	20.5±1.9	25.5±6.4	25.7±2.0
	Contact area	Right	18.5±3.9[a]	24.5±5.2	28.7±2.6
		Left	20.5±7.1	21.7±4.6	25.2±6.4
	Transitional area	Right	24.2±4.4	26.2±7.0	28.8±3.1
		Left	19.3±3.7[a]	21.3±9.0[c]	33.2±6.3
Tibia	P1[1]	Right	23.0±7.0	27.0±9.4	27.3±6.7
		Left	20.7±5.7[a]	27.3±6.7	32.0±8.5
	P2	Right	23.7±4.3	26.0±5.2	29.2±8.1
		Left	21.8±4.6	29.2±8.1	30.8±8.6
	P3	Right	18.8±4.4*[a,b]	26.3±5.6	30.2±2.6
		Left	25.3±6.3	30.2±2.6	26.7±5.0

[1]P1 – anterior articular region; P2 – medial articular region; P3 – posterior articular region
* = significant statistical difference between the immobilized/remobilized (right) and control (left)
a = significant statistical difference between G1 and G3
b = significant statistical difference between G1 and G2
c = significant statistical difference between G2 and G3

Morphological analysis

The joints of the left knee (control), of all groups, showed characteristic morphology with a smooth joint cartilage surface, organized in four normal cell layers. In the surface area, a greater cell density was visualized, with the chondrocytes arranged in horizontal clusters with flattened appearance. In the intermediate zone, cells took on a rounded appearance and stood-alone or in isogenous groups. Then, chondrocytes organized themselves into the gaps, corresponding to the deep zone, separate from the calcified zone by a basophilic line, called the tidemark (Figure 1A).

The synovial membrane also showed normal characteristics, i.e., two to three cell layers (synoviocytes type A and type B) in the synovial intima and subintima with a predominance of adipose cells (Figure 2A).

In the right knee joint of G1 (Figure 1B), we found that the immobilization caused morphological changes in cartilage of both the femur and the tibia. Flocculation areas were found, especially in the femur. A discontinuity of the tidemark in both the femur and the tibia was also found. The synovial membrane appeared moderately thickened with a disorganized intima, regarding the distribution of epithelioid synoviocytes and, in the subintima, the replacement of the type of connective tissue, from adipose to fibrous (Figure 2B).

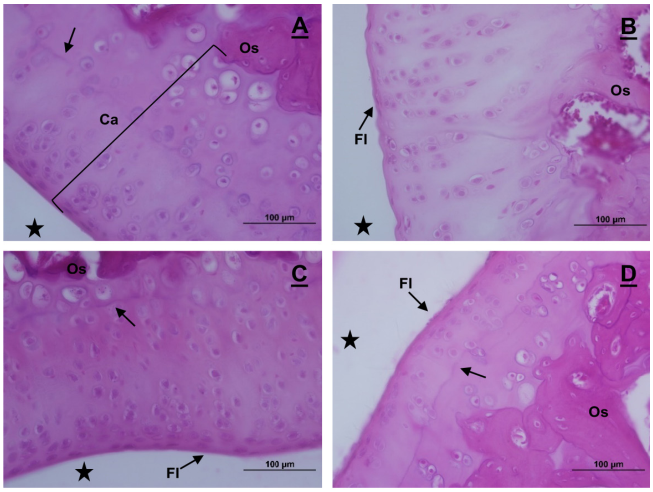

Figure 1. Articulate cartilage photomicrographs of the knees of Wistar rats in control (A), G1 (B), G2 (C) and G3 (D); sagittal section, hematoxylin and eosin coloration. In A, Details of the normal cellular organization of the articular cartilage (Ac), showing the presence of the *tidemark* (black arrow). In B, flocculation (Fl) on the surface of the cartilage and absence of the *tidemark*. In C, flocculation (Fl) less evident on the surface and discontinuity of the *tidemark* (black arrow). In D, light flocculation (Fl) on the surface and reorganization of the *tidemark*. Articular cavity (star), subchondral bone (Sb)

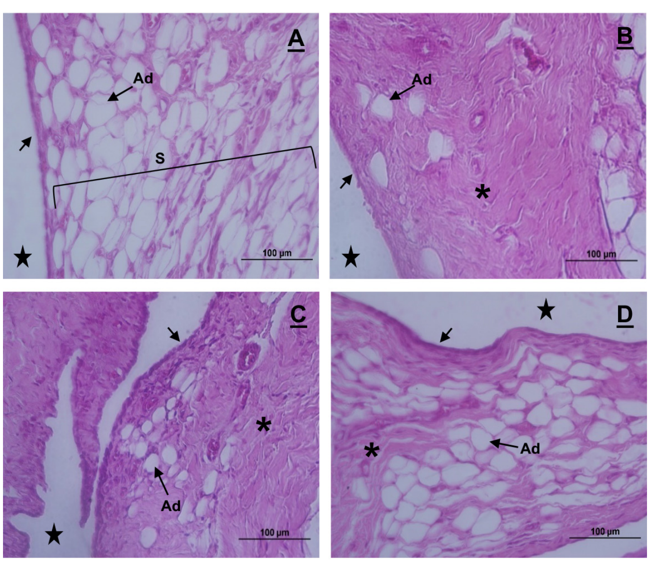

Figure 2. Synovial membrane photomicrographs of the knee articulation of Wistar rats in control (A), G1 (B), G2 (C) and G3 (D); sagittal section, hematoxylin and eosin coloration. In A, thin inner synovial membrane (black arrow) and a subintima membrane (S), with a predominance of adipocytes (Ad). In B, thickening of the synovial membrane, which presents itself as fibrous (asterisk), with few adipocytes and an intima with disorganized synoviocytes (black arrow). In C, moderate thickening of the subintima (asterisk) and the presence of adipocytes (Ad), with the beginnings of the intima reorganization (black arrow). In D, synovial membrane with a morphologically normal intima (black arrow), a less fibrous subintima (asterisk) and with a predominance of adipocytes cells (Ad). Articular cavity (star)

The animals of G2, freely remobilized, the cartilage still showed flakes in both the femur and the tibia, where there was also a subtle disruption of the tidemark (Figure 1C), with the presence of some cell clones. Regarding the synovial membrane, subintima remained slightly fibrous (Figure 2C); however, the intima was shown to be disorganized only in two of the animals.

In G3, remobilized using aquatic exercises, the cartilage remained slightly flocculated, although the organization of the tidemark was restored (Figure 1D). The synovial membrane showed areas of intima tissue reorganization, although the subintima continued to appear slightly fibrous (Figure 2D).

DISCUSSION

In this study, we verified that two weeks of fixed immobilization of the knee joint in full extension, to varying degrees, affect the thickness and the cell density of the articular cartilage of the femur and the tibia, and also cause degeneration in the cartilage and the synovial membrane. Forms of remobilization adopted in this study managed to recover the knee joint morphology, with the association of aquatic exercises producing more significant effects, indicating the reversibility of the changes induced by the immobilization model used.

The articular cartilage thickness of the tibia was not altered. As for the femur, it showed that immobilization in group G1 increased the thickness of the cartilage in the transitional area compared to the control. Hagiwara et al.[25], in their immobilization model with 150° of knee flexion, also found an increased thickness in the transitional area after 8 and 16 weeks. Maldonado et al.[26], on the other hand, observed a reduction in the thickness of the cartilage of the femur contact area, with the knee immobilized at 90° of flexion for 8 weeks. The authors postulate that the existing compressive force between the femur and the tibia in the contact area during the period of immobilization would cause a thickness reduction in this area and cartilage regeneration in adjacent areas, such as the transitional area[25,26].

There was also an increase in the thickness of the articular cartilage in the contact area in the immobilized/remobilized limb in animals of G3, compared to the control. According to Roos and Dahlberg[27], the relationship between physical activity and the amount of cartilage is the result of a complex mecanocelular transduction mechanism, in which the chondrocytes respond to the bearing weight with an increased proteoglycan content after an exercise, causing swelling of the cartilage. This could represent a compensatory mechanism to support additional loads after a period of immobilization, in this case, the aquatic exercise performed by G3 animals.

We verified no differences in the number of chondrocytes in the femur of the immobilized/ remobilized limb, compared to the control. As for the tibia, immobilization reduced the number of chondrocytes in the posterior joint region, compared to the control, resulting in changes in the articular biomechanics, caused by the extension immobilization, where contact between the posterior joint region of the tibia and the femur condyle was minimized. Hagiwara et al.[25], Ando et al.[24] and Maldonado et al.[26] observed a reduction in the number of chondrocytes in the different regions of the femur and the tibia, after periods ranging from 2 and 16 weeks of immobilization. The fixed angle of the knee flexion used in these studies may have been responsible for the differences in the results.

When comparing the groups, an increase in the number of cells in the transitional area, non-contact and contact of the femur was verified, as well as in the anterior and posterior articular regions of the tibia in remobilized animals in an aquatic environment, compared to animals only immobilized. Ando et al.[28], unlike this study, verified, in their 16-weeks free remobilization model, a reduction in cell density in the contact and transitional areas of the femur and tibia. According to the authors, the immobilization model used by them, 150° of flexion, caused progressive degeneration of the cartilage and the free remobilization used was unable to reverse these damages. The diffusion of synovial fluid inside the joint is required for the nutrition of the chondrocytes[28], and the remobilization that uses aquatic exercises seem to have improved this characteristic, leading to a higher cell density in these animals.

The fixed immobilization is a therapeutic measure indicated in some cases of musculoskeletal injuries, but that can lead to some changes in the immobilized body part. We found that 2 weeks immobilization caused flocculation at the surface of the articular cartilage and disorders in the cartilage matrix, as also verified in previous studies[24,26,29-31]. Immobilization may hinder the diffusion of synovial fluid in the joint cavity, reducing the supply of nutrients to chondrocytes[16,30], which in turn has its extracellular matrix and proteoglycans synthesis reduced when in disuse[32]. Changes verified in the synovial membrane of the G1 animals were also described by Melo et al.[29], Kojima et al.[30], Nagai et al.[31], Ando et al.[33] and Trudel et al.[34]. According to Del Carlo et al.[16], joint stiffness is the result of immobilization and caused a reduction of mobility, which would be responsible for the thickening of the synovial membrane.

Any animals freely remobilized, as well as those remobilized by exercise, showed articular cartilage with signs of recovery. In G2, the presence of cell clones was verified, which, according to Melo et al.[29], represent the hyperactivity of chondrocytes in an attempt to repair the tissue after a period of immobilization. In G3, cell clones were not visible and the tidemark had been reestablished, indicating that the exercise performed accelerated the recovery of the cartilage.

Similarly, the synovial membrane of both the G2 and G3 animals showed themselves as attempting to repair, with a reduction of fibrous tissue in the subintima, particularly in animals remobilized by exercises. According to Ando et al.[24], the restoration of movement reduces joint stiffness, improves the flow of synovial fluid in the joint cavity, promoting cartilage nutrition and its subsequent regeneration. The recovery of the synovial membrane after free remobilization and through swimming was also observed in Del Carlo et al.[16].

The two types of remobilization adopted in this study worked beneficially in restoring the morphological parameters of the cartilage and the synovial membrane. However, it appears that the exercises performed by the G3 animals were more efficient in the recovery of the joint due to a lower gravitational force in the movement execution done in water, which facilitates its implementation, and improves the distribution of synovial fluid, necessary in maintaining the homeostasis of the knee joint.

CONCLUSION

Full extension immobilization of the knee joint for 14 days alters its morphological parameters. The combination of swimming and aquatic jumping, when started immediately after the removal of the immobilization, allow for improvements in the joint morphology, which can be observed in the cartilage by the reduced flocculation, the reorganization of the tidemark, the presence of cell clones and in the synovial membrane. Thus, these exercises can be used as part of the physiotherapeutic rehabilitation program.

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