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HISTOMORFOMETRIC ANALYSIS OF THE ENDOCHONDRAL OSSIFICATION OF THE LIMBS OF RATS SUBMITTED TO USNIC ACID FROM *Cladonia substellata* (AHTI) ENCAPSULATED IN PLGA-MICROSPHERES

ANÁLISE HISTOMORFOMÉTRICA DA OSSIFICAÇÃO ENDOCONDRAL DOS MEMBROS DE RATOS SUBMETIDOS AO ÁCIDO ÚSNICO DE *Cladonia substellata* (AHTI) ENCAPSULADO EM MICROESFERAS DE PLGA

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ABSTRACT
The aim of this study was to evaluate the histology and morphometry of endochondral ossification in limbs of rats submitted to usnic acid from *Cladonia substellata* (AHTI) encapsulated in PLGA-microspheres. It was used 12 pregnant Wistar rats that were randomly distributed in the control groups (n = 6), which received 1.0 mL of saline solution; and treated (n = 6), that received 25 mg/kg/day of encapsulated usnic acid in PLGA microspheres by oral administration. It was analyzed six fetuses from each rat under study. At the 20th day of gestation were euthanized the females and their fetuses removed for histomorphometric analysis of the anterior and posterior limbs. The results showed the epiphyseal discs of the animals with cell alterations, as in the morphology and numbers of chondrocytes. It also showed a decrease in the amount of collagen type I collagen in bone tissue. However, this study showed low toxicity of the encapsulated usnic acid on bone development, when compared to its conventional dosage form. In this way, the toxicity of usnic acid can be reduced by encapsulation in PLGA-microspheres.

Keywords: Embryophetotoxicity, Encapsulation, Endochondral ossification, Usnic acid.

RESUMO
O objetivo deste estudo foi avaliar a histologia e morfometria da ossificação endocondral em membros de ratos submetidos ao ácido único de *Cladonia substellata* (AHTI) encapsulado em PLGA-microsferas. Foram utilizados 12 ratos Wistar grávidos que foram distribuídos aleatoriamente nos grupos de controle (n = 6), que receberam 1,0 mL de solução salina; e tratados (n = 6), que receberam 25 mg/kg/dia de ácido único encapsulado em microesferas de PLGA por administração oral. Foram analisados seis fetos de cada rato em estudo. No 20º dia de gestação, as fêmeas foram eutanizadas e seus fetos foram retirados para análise histomorfométrica dos membros anteriores e posteriores. Os resultados mostraram os discos epifisários dos animais com alterações celulares, como na morfologia e no número de condrócitos. Também mostrou uma

diminuição na quantidade de colágeno tipo I de colágeno no tecido ósseo. Entretanto, este estudo mostrou baixa toxicidade do ácido úsnico encapsulado sobre o desenvolvimento ósseo, quando comparado à sua forma de dosagem convencional. Desta forma, a toxicidade do ácido único pode ser reduzida pelo encapsulamento em PLGA-microsferas.

Palavras-chave: Embriophetotoxicidade, Encapsulação, Ossificação endocondral, Ácido Núcleo Usnico.

1 INTRODUCTION

The lichens are the result of the symbiotic association between fungi (mycobionte) and an algae or cyanobacteria (photobionte). They are one of the most important natural sources of biologically active compounds. Among these compounds, usnic acid is highlighted, which is extensively studied owing to its various biological activities (BENATTI; MARCELLI, 2007; OTÁROLA et al., 2010; NUNES et al., 2011).

Usnic acid [2,6-diacetyl-7,9-dihydroxy-8-9b-dimethyl-1,3 (2H, 9α / βH) dibenzofurandione; C18H16O7], is a secondary lichen metabolite, which has various pharmacological properties such as anti-tumor (BURLANDO et al., 2009), antiviral (SHTRO et al., 2014), anti-inflammatory (SU et al., 2014), antioxidant (SUWALSKY et al., 2015), and antifungal (NITHYANAND et al., 2015). However, there are some limitations that decrease its therapeutic efficacy, such as high hepatotoxicity, low water solubility and reproductive toxicity (SANCHEZ et al., 2006; SILVA et al., 2017; MARINHO et al., 2017; DA SILVA et al., 2020).

Recent research on the reproductive toxicity of free usnic acid administered during the gestational period of Wistar rats reports relevant signs of toxicity in the offspring of rats treated with the compound at a dose of 25 mg/kg/day, they present morphological malformations, alteration in the proliferation and differentiation of hyaline cartilage, decrease in the amount of type II collagen and delay in the process of endochondral ossification of animals. These studies demonstrate the ability of usnic acid to promote malformations in bone development (SILVA et al., 2017; DA SILVA et al., 2020).

Endochondral ossification depends on the proliferation and differentiation of hyaline cartilage for its formation. In this way, it becomes quite susceptible to interference, since it is a complex and controlled process. The extracellular matrix of this tissue consists mainly of collagen fibers type I that presents structural function giving strength and support to the tissue (VELOSA et al., 2003; PARCCELLI et al., 2015).

Some compounds when administered at sensitive stages of development, such as gestational age and infant age, may interact negatively with the body (Duewelhenke et al. 2006),

because most chemicals easily cross the placenta during gestation and may result in important effects on the development of passive organism (embryo and/or fetus) (WEBSTER; FREEMAN, 2001, LEMONICA et. al., 2008).

The therapeutic use of some compounds in the skeletal system, such as usnic acid in conventional pharmaceutical form, often becomes unfeasible, requiring alternatives that modify their physicochemical characteristics, minimize their toxic effects and improve their bioavailability (RIBEIRO- COSTA et al., 2004; DUEWELHENKE et al., 2006, DA SILVA SANTOS et al., 2006).

These limitations led to the development of innovative alternatives, such as encapsulation of usnic acid in controlled release systems, such as lactic and glycolic acid copolymer (PLGA) microspheres, which allow the toxic effects of the compound to be reduced in the body, while maintaining a stable its active principle, and enhancing its therapeutic application (RIBEIRO COSTA et al., 2004, SANTOS et al., 2006; SIQUEIRA-MOURA et al., 2009; GRUMEZESCU et al., 2014; MARTINELLI et al., 2014).

Given the reports of the toxic effect of usnic acid during pregnancy, and the relevance of research that develops biotechnological resources that enable its use in therapy, the objective was to evaluate histomorphometrically to endochondral ossification in limbs of Wistar rats submitted to PLGA-microspheres containing usnic acid from *C. substellata* at a dose of 25 mg/kg/day orally.

2 MATERIAL AND METHODS

2.1 LICHEN MATERIALS: EXTRACTION, PURIFICATION AND CHARACTERIZATION OF USNIC ACID

The lichen *C. substellata* (AHTI) was collected at the city of Mamanguape, Paraíba state, Brazil. The usnic acid (UA), the main substance of this study, was isolated, purified and characterized according to pre- established methodology at the laboratory of Natural Products on the Department of Biochemistry of the Federal University of Pernambuco (ASAHINA; SHIBAT, 1954). In order to obtain the Usnic Acid the lichen thallus of *C. substellata* was totally macerated and subsequent a Soxhlet extractor was used for a refined extraction, per 72 hours in chloroform. After that the extracted material was submitted to rotary evaporator at 60 ºC until partial evaporation of the solvent and later ending the total evaporation at room temperature.

As a result of that, a yellow powder was obtained with impurities, which it was submitted to the purification and crystallization processes with chloroform/ice-cold ethanol used as solvents (1:3 v/v). Moreover, after 48 hours, this solution underwent the vacum filtration process. Finally,

the material trapped in the filter was recrystallized from n-Hexane, thus obtaining crystals of unic acid. Furthermore, the formation of those crystals was confirmed by Thin- Layer Chromatography (TLC). The highlighted bands were identified by retention factor (Rf) and subsequent it was compared to standard reference compound.

2.2 PLGA-MICROSPHERES PREPARATION AND USNIC ACID ENCAPSULATION

The microspheres of PLGA were prepared by multiple emulsion solvent evaporation method, according to the methodology established by Ribeiro-Costa et al. (2004). All those procedures were performed in partnership with the Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco.

2.3 EXPERIMENTAL ANIMALS

It was used 12 Wistar rats provided by the vivarium of the Department of Nutrition of the Federal University of Pernambuco - UFPE, weighing about 250-300 g, virgin and 60 days old. It was analyzed six fetuses from each offspring studied, totaling 72 fetuses analyzed. The animals were kept in row cages in a photoperiod climate environment (12 hours light/12 hours dark) at a controlled temperature of 24 ºC ± 1 ºC with air exhaustion, and free access to food ad libitum and water during the experiments. The experimental protocols were approved by the Ethics Committee of the Federal University of Pernambuco (process No. 23076.029828 / 2013-94).

2.4 EXPERIMENTAL PROCEDURES

Initially, the females were submitted to the estrous cycle study in order to determine the fertile period. After ovulation was confirmed, females were paired with males (2: 1), overnigth, and mating confirmed the next day with the presence of the plug (whitish sperm mass at vaginal opening) or the presence of sperm on the vaginal smear.

The females were randomly distributed to the control group (n = 6) and treated (n = 6). Control group animals received 1.0 ml saline solution, while treated group animals received a solution of purified usnic acid at a dose of 25 mg/kg body weight diluted in 1.0 ml saline solution. Dose calculated based on LD50 study (Lethal dose that kills 50% of animals). The administration was from 6 to 15 days of gestation, orally (gavage), daily dose.

At the end of pregnancy the females were euthanized by intramuscular administration of ketamine (80 mg/kg) and xylazine (8 mg/kg) associated with thiopental (100 mg/kg). The cesarean section procedure was performed to open the uterus of each female and remove the fetuses. Then,

the fetuses were euthanized and their anterior and posterior limbs were collected for histomorphometric analysis.

2.5 HISTOPATHOLOGICAL AND HISTOMORPHOMETRIC ANALYSIS OF LIMBS OF THE FETUSES

The epiphyseal discs and the amount of type I collagen in the anterior (humerus) and posterior (femur) limbs of the fetuses of each offspring under study were analyzed. After euthanasia of the fetuses, the anterior and posterior limbs were collected and submitted to histological analysis. The material was kept in 10% formaldehyde neutral buffered (NBF) for 24 hours, processed, and submitted to routine histological technique for paraffin. The blocks obtained were cut into 4 μm in thickness, stained with Hematoxylin and Eosin (H&E) and Picrosirius Red. Then, the H&E stained slides were photographed and analyzed at 40X, 100X and 400X total magnification using the Motic® Images Plus program. 2.0 with a digital camera coupled to the computer and the optical microscope (Olympus BH-2, Japan). For as much as, Picrosirius Red stained slides were photographed and analyzed on the 40X objective using the ISCapture program (version 4.1) with a digital camera attached to the computer and the light microscope (Lumen-LM3100TLi). Ten photomicrographs were obtained from the histological preparations at each increase.

For morphometric analysis the photomicrographs were submitted to the appropriate measurements using the ImageJ software version 1:44 (Research Services Branch, US National Institutes of Health, Bethesda, MD, USA). To obtain the area of proliferation and hypertrophic zones, the total area of the transitional hyaline cartilage (endochondral ossification process) was measured, and then it was measured the proliferation and hypertrophic areas of the chondrocytes. The values of the related areas were divided by the value of the total area of hyaline cartilage getting a percentage of each area. In obtaining the amount of chondrocytes in the proliferation zone and in the hypertrophic zone, the chondrocytes present in each photomicrograph referring to each zone were quantified. In measuring the cell area of proliferating and hypertrophic chondrocytes, 1000 cells from each group were measured in square micrometers (μm2). In the amount of type I collagen present in bone tissue has been obtained this percentage of collagen in each photomicrograph. This collagen fibers were quantified by selecting birefringent red or orange shades, bands corresponding to collagen type I polarized (HILLS; JUNQUEIRA, 1991).

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2.6 STATISTIC ANALYSIS

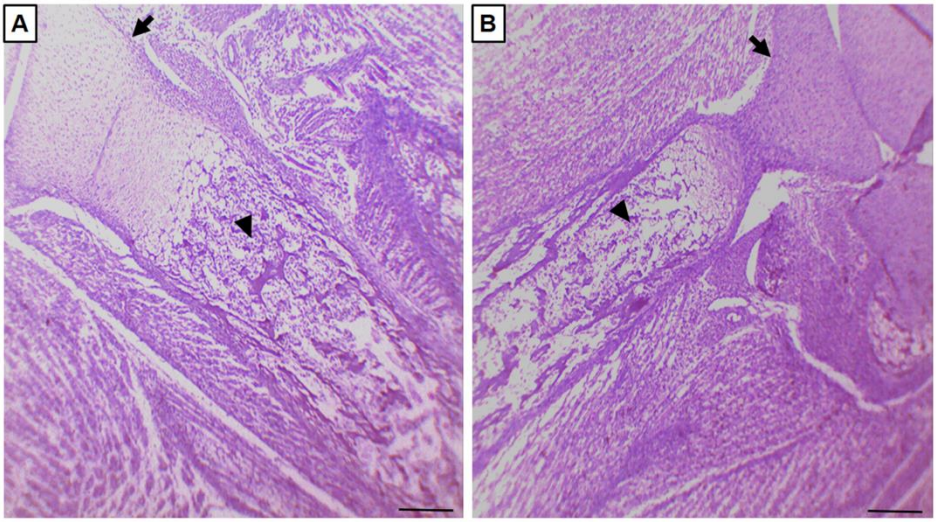
The results of the histomorphometric and histopathological parameters of the epiphyseal disc and collagen fibers were expressed as mean ± SEM (standard deviation) and the Kolmogorov-Smirnov statistical abnormality test was used, the data being evaluated using the Mann-Whitney U test (SPSS software, version 15.0), with significance $p < 0.05$.

3 RESULTS

3.1 HISTOLOGICAL ANALYSIS OF ENDOCHONDRAL OSSIFICATION

The figure 1 (A and B) demonstrates the development of endochondral ossification of the anterior and posterior limbs of the fetuses of group control (A) and treated with 25 mg/kg/day of encapsulated usnic acid in PLGA microspheres orally (B). In the histological observations of the anterior and posterior limbs, the secondary ossification centers (epiphyses) of the fetuses exhibited intact hyaline cartilage mold (arrows) in the treated group (Figure 1 B), compared to the control group (Figure 1 A). As also the primary ossification centers (diaphyses) revealed fully ossified (arrowhead) in both groups (Figure 1 A and B).

Figure 1. Photomicrographs of the femur of the fetuses of Wistar rats in the control group (A) and the group treated with PLGA-microspheres containing usnic acid at a dose of 25 mg/kg/day orally (B). Display of the hyaline cartilage in epiphysis mold (arrows) ossified diaphysis (arrowheads). Hematoxylin and Eosin Staining. Scale Bar: 100 μm.



3.2 HISTOMORPHOMETRIC ANALYSIS OF EPIPHESARY DISK ZONES

The results of the histological observations of the epiphyseal disc zones of the anterior and posterior limbs of Wistar rat offspring are shown in Figure 2 (A and B). In the epiphyseal plate was observed five zones: resting zone chondrocytes containing with rounded shape, situated in gaps involved in cartilage matrix, dispersed and without morphological change (asterisks);

proliferation zone with chondrocytes in mitotic multiplication showing a flat shape, forming longitudinal and parallel columns (smaller arrows); the hypertrophic zone with very large rounded chondrocytes, very clear nuclei and among them apoptotic cells (bars); calcified zone where we observed its beginning with the end of apoptosis and the deposition of minerals for bone tissue composition (larger arrows); and then the ossification zone consisting of cuboid shaped osteoblasts located at the periphery of the tissue, the flat shaped osteocytes situated in gaps within the matrix (arrow heads). In short, the epiphyseal plate of the areas of the anterior and posterior limbs of fetuses were not observed morphological change of the treated group compared to the control group.

Regarding the area of proliferation zones (smaller arrows) and hypertrophic zones (bars) of the epiphyseal disc represented in figure 2, no significant diferences ($p>0,05$) were observed between the groups analyzed. The table 1 expresses the quantitative values of the area of the respective zones.

Figure 2. Photomicrographs of the epiphyseal disc of the femur of the fetuses of Wistar rats in the control group (A) and the group treated with PLGA-microspheres containing usnic acid at a dose of 25 mg/kg/day orally (B). Display of resting zone (asterisk), chondrocyte proliferation zone (smaller arrows), hypertrophic chondrocyte zone (bars), calcified zone (larger arrows), ossification zone (arrowheads). Hematoxylin and Eosin Staining. Scale Bar: 100 µm.

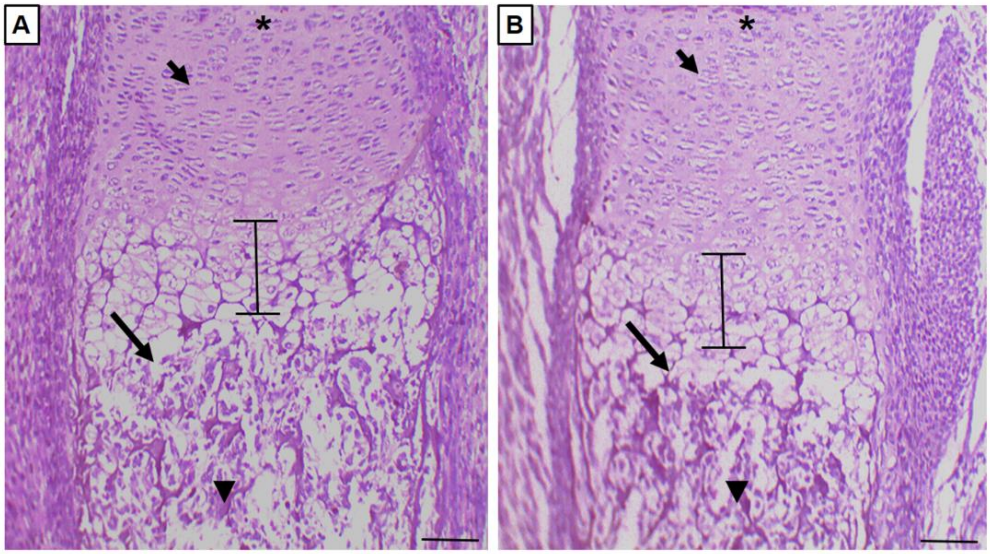


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Table 1. Histomorphometric analysis of the area (%) of the proliferation and hypertrophic zones of the epiphyseal disc of the anterior and posterior limbs of fetuses exposed to PLGA-microsphere containing usnic acid at a dose of 25 mg /kg /day orally (treated) and the control group. Data expressed as mean ± standard deviation (SD).

Limbs	Measured Zones (%)	Control Group (Média ± DP)	Treated Group (Média ± DP)	Value- p
Anterior	Proliferation	48,34 ± 8,34	49,30± 9,35	0,354
	Hypertrophic	51,65 ± 8,34	50,69 ± 9,35	0,357
Posterior	Proliferation	47,59 ± 8,20	49,14± 8.09	0,595
	Hypertrophic	52,40± 8,20	50,85 ± 8,09	0,595

3.3 HISTOMORPHOMETRIC ANALYSIS OF EPIPHESARY DISK CONDROCYTES

In the cell area results of proliferating (Figure 3 B) and hypertrophic (Figure 4 B) chondrocytes in the epiphyseal disc of the anterior and posterior limbs of the fetuses of the treated group revealed significant differences ($p<0,05$) in relation to the control group (Figure 3 A, 4 A). The table 2 expresses the quantitative cell area values of proliferating and hypertrophic chondrocytes.

In the evaluation of the number of hypertrophic chondrocytes in the epiphyseal disc of the anterior and posterior limbs of the fetuses in the treated group (Figure 4 B), it wasn't observed significant differences ($p>0,05$) in relation to the control group (Figure 4 A). However, there were significant differences ($p<0,05$) in the number of proliferating chondrocytes in the treated group (Figure 3 B) when compared to the control group (Figure 3 A). The respective values are described in table 3.

Figure 3. Photomicrographs of proliferating chondrocytes (arrows) of the femoral epiphyseal disc of the fetuses of Wistar rats in the control group (A) and the group treated with PLGA-microspheres containing usnic acid at a dose of 25 mg/kg/day orally (B). Hematoxylin and Eosin Staining. Scale Bar: 100 μm.

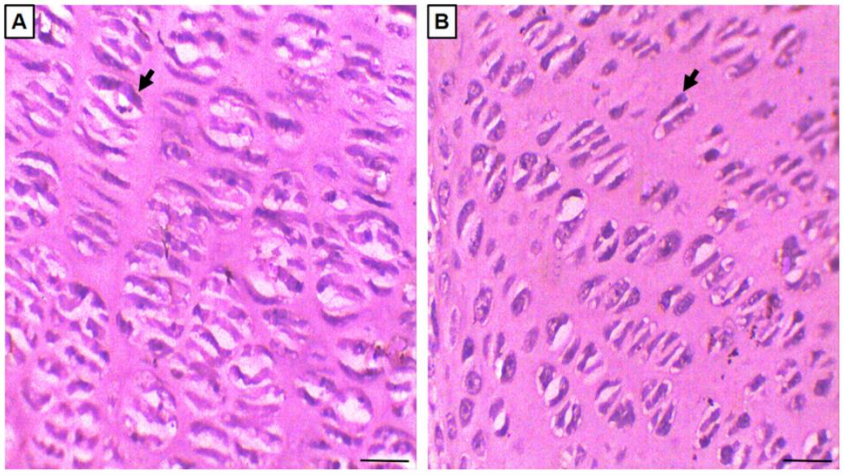

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Figure 4. Photomicrographs of hypertrophic chondrocytes (arrows) of the femoral epiphyseal disc of the fetuses of Wistar rats in the control group (A) and the group treated with PLGA-microspheres containing usnic acid at a dose of 25 mg/kg/day orally (B). Hematoxylin and Eosin Staining. Scale Bar: 100 µm.

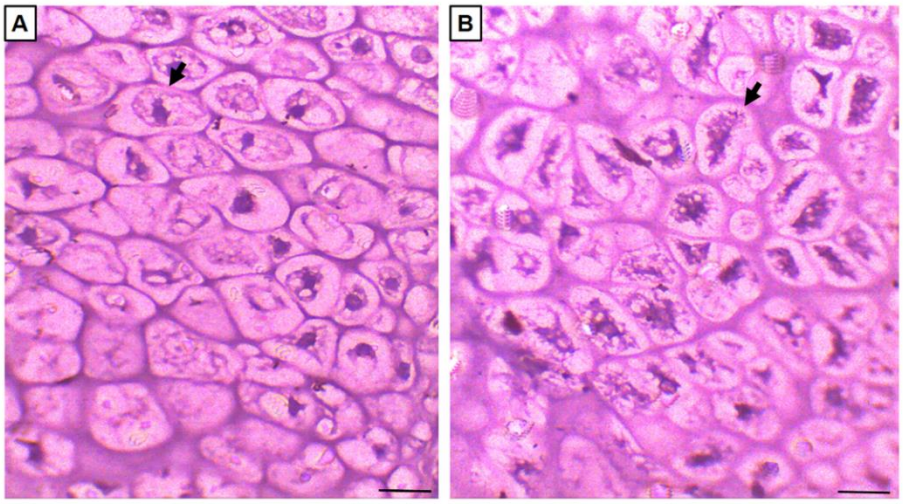

Table 2. Histomorphometric analysis of cellular area (µm^2) of the chondrocytes present in the proliferation and hypertrophic zones of the epiphyseal disc of the limbs of fetuses exposed to PLGA-microspheres containing usnic acid at a dose of 25 mg/kg/day orally (treated) and the control group. Data expressed as mean ± standard deviation (SD).

Limbs	Zonas de mensuração (µm^2)	Control Group (Média ± DP)	Treat Group (Média ± DP)	Value- p
Anterior	Proliferation	76,55 ± 21,24	60,22± 18,63	0,001
	Hypertrophic	401,66 ± 92,65	337,32 ± 68,25	0,001
Posterior	Proliferation	76,46 ± 20,43	66,18 ± 19,41	0,001
	Hypertrophic	385,44± 90,03	315,69 ±64,37	0,001

Table 3. Histomorphometric analysis of number of chondrocytes in the proliferation and hypertrophic zones of the epiphyseal disc of limbs of fetuses exposed to PLGA-microspheres containing usnic acid at a dose of 25 mg/kg/day orally (treated) and the control group. Data expressed as mean ± standard deviation (SD).

Limbs	Measured Zones	Control group (Média ± DP)	Treat Group (Média ± DP)	Value- p
Anteriores	Proliferation	81,75 ± 22,46	74,30 ± 19,65	0,001
	Hypertrophic	50,52 ± 10,61	49,03 ± 8,72	0,269
Posteriores	Proliferation	84,58 ± 18,92	76,40± 18,85	0,001
	Hypertrophic	52,66 ± 8,72	50,39 ± 8,26	0,213

3.4 HISTOMORPHOMETRIC ANALYSIS OF TYPE I COLLAGEN FIBERS IN BONE TISSUE

Figure 5 and 6 show histological analysis of of type I collagen fibers of the diaphyseal bone tissue of the anterior (humero) and posterior (femur) limbs of fetuses in treated and control groups. In the extracellular matrix of the diaphyseal bone tissue, it was observed the type I collagen fibers (arrows) extending all the way parallel to each other, with reddish birefringent shades in both the treated group (Figure 5 B and 6 B) and the control group (Figure 5 A and 6 A).

The type I collagen fibers present in the bone tissue of limbs of fetuses exposed to encapsulated uric acid in PLGA-microspheres at a dose of 25 mg/kg/day orally (treated) and the control group were randomly compared and are shown in table 4. The anterior limbs of the treated group (figure 5 B) showed no significant differences ($p>0,05$) when compared to the control (figure 5 A). On the other hand, in the hind limbs of the treated group (figure 6 B) showed significant diferences ($p<0,05$) in relation to the control group (figure 6 A).

Figure 5. Photomicrographs of type I collagen fibers (arrows) present in the bone tissue of the humerus of the fetuses of Wistar rats in the control group (A) and the group treated with PLGA-microspheres containing usnic acid at a dose of 25 mg/kg/day orally (B). Picrosirius Red Coloring. Scale Bar: 100 μm.

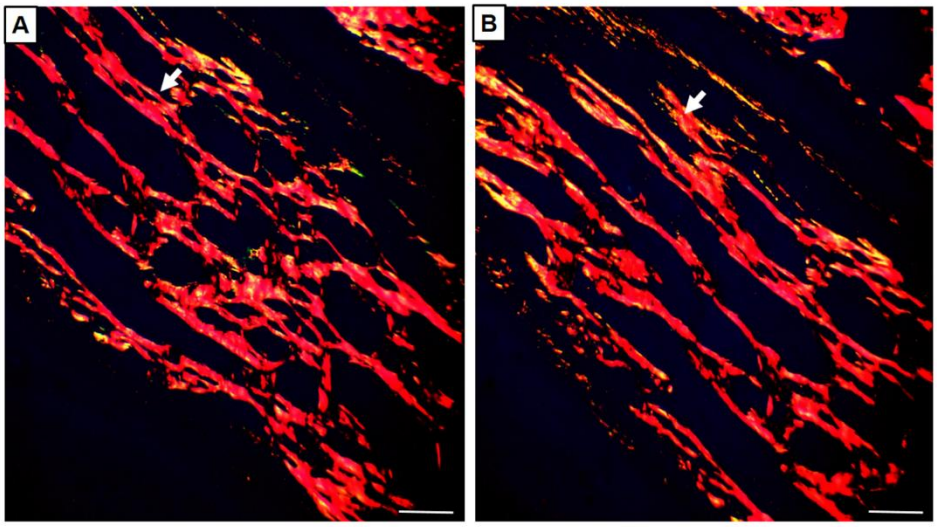

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Figure 6. Photomicrographs of type I collagen fibers (arrows) present in the femoral bone tissue of the fetuses of Wistar rats in the control group (A) and group treated with PLGA-microspheres containing usnic acid at a dose of 25 mg/kg/day orally (B). Picrosirius Red Coloring. Scale Bar: 100 μm.

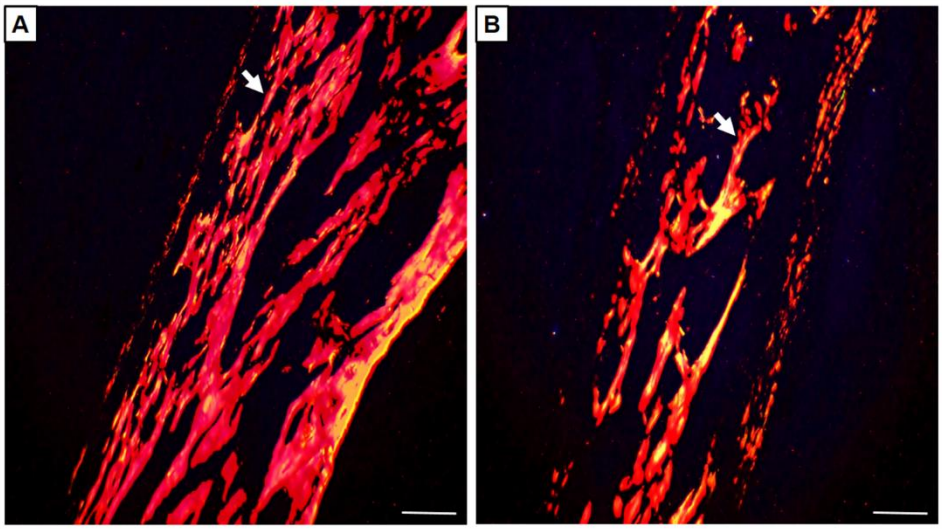

Table 4. Histomorphometric analysis of amount (%) of type I collagen fibers present in the bone tissue of limbs of fetuses exposed to PLGA-microspheres containing usnic acid at a dose of 25 mg/kg/day orally (treated) and the control group. Data expressed as mean ± standard deviation (SD).

Limbs	Controle Group (Média ± DP)	Treated Group (Média ± DP)	Value- p
Anterior	5,04 ± 3,22	4,49± 2,96	0,172
Posterior	4,71 ± 3,29	3,35 ± 2,35	0,001

4 DISCUSSION

Preliminary trials for reproductive toxicity of usnic acid during gestation report relevant signs of toxicity in offspring of pregnant rats treated with the compound in its free form at a dose of 25 mg/kg/day orally, such as the presence of limb malformations anterior and posterior of the fetuses. These studies demonstrate the ability of usnic acid to provide the appearance of abnormalities in the anatomical development of fetuses (SILVA et al., 2017; DA SILVA et al., 2020).

In the present study, the results of the histological observations of the endochondral ossification process of the anterior and posterior limbs of the fetuses exposed to encapsulated usnic acid presented the intact hyaline cartilage epiphyses and the diaphyses were completely ossified. Experiment carried out by Da Silva et al. (2020), with free-form usnic acid on the endochondral ossification process in the limbs of the fetuses, presented ossified tissue restricted to a small area in the center of the diaphysis indicating a delayed formation of endochondral ossification. Therefore, this study confirms the reduction of the toxicity of usnic acid through its encapsulation.

In the histomorphometric analyzes of the areas of proliferation and hypertrophic zones of the epiphyseal disc during the endochondral ossification process, there were no significant differences. While the administration of usnic acid in its free form showed a decrease of almost 50% in areas related areas (DA SILVA et al., 2020). Therefore, our results show a decreased toxicity of the encapsulated compound on endochondral ossification.

The cell area of chondrocytes present in lower average had analyzed areas compared to the control group. According to Pramyothin et al. (2004), usnic acid is capable of affecting the integrity of the cell plasma membrane and causing destruction of mitochondrial function, loss of control of cellular respiration and ATP synthesis and may cause changes in development, morphology and even cell death. Although these results show cellular encapsulated usnic acid toxicity, they indicate a reduction of their encapsulation toxicity in PLGA microspheres when compared to their free form.

While the lower number of chondrocytes in the treated group proliferation zones may be related to the interaction of extracellular matrix proteins with directly interacting cell surface receptors, initiating signal transduction pathways and inducing different processes such as cell proliferation. And in view of usnic acid acts on the integrity of the cell plasma membrane, it can affect protein interactions with cell surface receptors (PRAMYOTHIN et al., 2004; IRVING-RODGERS et al., 2010; DE SOUZA et al., 2011).

In the analysis of the amount of type I collagen present in the bone tissue of the treated fetus limbs, the anterior limbs did not present significant means in relation to the control group. On the other hand, the hind limbs showed a significant decrease from the mean compared to the control group, which was related to the embryonic development of the newborns' limbs, as the forelimbs develop first than the hind limbs. Once the administration of the compound in the present study was carried out from day 6 of gestation. And according to the literature, this embryonic development of mice, osteogenesis of the anterior limbs are already preset. Thus, administration of drugs during the period of development of the members can trigger different responses in both limbs (CARMEL et al., 2004; TIBONI et al., 2006).

According to Manela-Azulay et al. (2003), collagen synthesis may be influenced by exposure to toxic substances, which may inhibit the action of some enzymes, such as proline hydroxylase and lysine hydroxylase responsible for the hydroxylation process during collagen synthesis. Thus, the effect of uric acid may be related to inhibition of the action of these enzymes, and consequently, resulting in a smaller amount of collagen (GUSTAFSSON et al., 2003; SILVA;

PENNA, 2012). More detailed studies such as the molecular and biochemical studies are needed to assert this hypothesis more accurately.

Despite the oral 25 mg/kg/day encapsulated usnic acid presented toxicity in the development of endochondral ossification in neonates, we can say that the present study showed a low toxicity when compared with the administration of the free form of usnic acid at a dose of 25 mg/kg/day orally (SILVA et al., 2017; DA SILVA et al., 2020). Thus, it indicates that encapsulation by microspheres of PLGA is able to reduce the toxic effects on bone development caused by this compound.

Machida et al. (2000) evaluated CPT-11 encapsulated in PLGA microspheres on rat cancer cells. The results elucidated that encapsulation of the drug not only potentiated its antitumor activity, but also decreased its toxicity. Thus, this study confirms the effectiveness of PLGA microspheres in decreasing toxic effects.

In the followuing years, the antimetastatic activity and systemic toxicity of microsphere-encapsulated camptothecin and its free form in mice were analyzed. The data showed that the encapsulated drug showed antimetastatic activity similar to its free form, but its systemic toxicity such as weight loss, survival of animals and neutrophil count was much lower compared to the compound in its free form. This study confirms the reduction of toxic effects of drugs through innovative alternatives such as encapsulation (DORA et al., 2006).

Research into hepatic toxicity of 25 mg/kg/day PLGA microspheres containing usnic acid during gestation of Wistar rats reported toxicity of the encapsulated drug, but to a much lesser extent than that found for uric acid in its free form (MARINHO et al., 2017). Given these findings, it is concluded that the toxicity of the compound can be minimized by encapsulating it with PLGA microspheres.

These studies cited in the present study confirm that controlled release systems reduce side effects and to ensure a more bioavailable drugs. And when compared to conventional drug delivery systems, controlled release systems offer the great advantage of keeping the drug concentration constant over the therapeutic range for an extended period of time increasing therapeutic efficacy and significantly decreasing toxicity (HENRIQUE et al., 2006).

Therefore, the experimental model used in the present study demonstrated that the administration of usnic acid in its encapsulated form in oral 25 mg/kg/day PLGA microspheres presents a low toxicity on bone development. Evidencing that the toxicity of the compound can be minimized by its encapsulation in PLGA-microspheres.

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