Effects of Underwater Lipus on the Morphohistology of the CF-1 Mice Growth Plate in Late Lactation Stage

Efectos del Ultrasonido Subacuático Sobre la Morfohistología de la Placa de Crecimiento de Ratones CF-1 en Etapa de Lactancia Tardía

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SUMMARY: Ultrasound is one of the most used tools in physiotherapy, its LIPUS modality allows to treat musculoskeletal injuries, but like the conventional ultrasound it is contraindicated its application in areas close to growth cartilage by a possible closure of this one. In the literature there is no conclusive evidence of this contraindication, so the objective of the present study is to evaluate the effects of doses equivalent to underwater LIPUS on the histomorphometry of the proximal tibial epiphysis growth plate in an animal model. LIPUS was applied in underwater mode in the right pelvic limb to 8 mice in p 15 for 4 days, 2 times a day. On completion of 8 sessions the histology of the stimulated limb growth plate was sacrificed and the histology. When evaluating the histomorphometry of the plaque, a significant increase in the thickness and area of the proliferative zone stimulated with LIPUS (p = 0.048) was observed. In the hypertrophic area were observed no significant differences in thickness or areas, but in the hypertrophic area there were significant changes in the histological organization, observing a significant increase of the entire columns in the stimulated plaque (p = 0.04). LIPUS in underwater modality modifies the histomorphometry of the proximal epiphyseal PC of the tibia of rodents in the late postnatal stage, promoting proliferation and columnar organization in the proliferative and hypertrophic zone, respectively.

KEY WORDS: **Low-intensity pulsed ultrasound; CF-1 mice; Late lactation; Growth Plate.**

INTRODUCTION

Ultrasound (US) is a type of mechanical wave characterized by a frequency higher than 20 kHz (Cameron, 2009). For decades the US has been used as a tool for physiotherapy that promotes tissue repair in musculoskeletal injuries. (Padilla *et al.*, 2014). The modification of the parameters of the US wave allows to focus the treatment to different tissues, different depths and generate varied effects, covering a wide spectrum of therapeutic objectives norms according to the FDA (Cameron).

One of the US modalities presenting the most clinical evidence is the low-intensity pulsed ultrasound (LIPUS), Which corresponds to a pulsating application characterized by the use of final intensities less than $0.1 \, W/cm^2$ (Cameron). The athermic effects of LIPUS have proven to be beneficial in the repair of unbound fractures (Cameron; Padilla *et al.*).

As for the contraindications for the use of the US, in a review conducted by Batavia (2004), it was determined that the application of US in areas close to the growth plate (PC) is the second most frequently reported contraindication in the specialized literature (Batavia). This contraindication has its origin in a publication by DeForest *et al.* (1953), who observed the closure of the PC after the application of high doses of US. Based on this background, it is proposed as a contraindication to apply therapeutic US in pediatric patients in areas close to the PC, since it could cause a premature closure of the plaque or alterations in the chondrocyte maturation (Cameron).

The PC is a characteristic structure of the endochondral ossification processes, it is composed of highly organized cartilaginous tissue, it is located between the epiphysis and the diaphysis of the long bones and whose main function is the modulation of the longitudinal growth

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of the bone (van der Eerden *et al.*, 2003). The PC is divided into three zones, each with distinctive morphological characteristics (Mackie *et al.*, 2008), From epiphysis to diaphysis, we will find:

The reserve zone (ZR) is composed of progenitor chondrocytes. These cells are crucial for the orientation of the chondrocyte columns of the contiguous zones, and therefore, fundamental in the orientation of bone growth, probably secreting a PC orientation factor (Mackie *et al.*). These chondrocytes upon receiving various stimuli are able to begin to proliferate, giving rise to the proliferative zone (van der Eerden *et al.*).

In the proliferative zone (ZP), the chondrocytes adopt a flattened appearance, begin to divide, are organized in columns and initiate the synthesis of substantial amounts of extracellular matrix of collagen (MEC) (van der Eerden *et al.*).

In the hypertrophic zone (ZH), chondrocytes have a rounded appearance due to cytoplasmic hypertrophy, accompanied by a marked intracellular increase in calcium concentration, and an increase in the synthesis and secretion of X-type collagen, in addition to other proteins Of the MEC (van der Eerden *et al.*).

In the proximity of the diaphysis the hypertrophic chondrocytes (CH) enter into apoptosis, left an ECM scaffold for the bone tissue in formation (van der Eerden *et al.*). After the death of HC, there will be empty lagoons, spaces that will later be occupied and invaded by blood vessels and osteoprogenitor cells, osteoclasts, osteoblasts and bone marrow cells (Mackie *et al.*).

The process of differentiation and passage from the progenitor chondrocyte to the CH is regulated by internal factors and factors external to the PC.

Within the external factors it is possible to emphasize the mechanical loads. The bony tissue exposed to mechanical loads responds with diverse adaptations both elastic and plastic, described by two laws; The Law of Wolf, which finally expresses the activity of bone formation and resorption (Villemure & Stokes, 2009), And the Hueter-Volkmann Act, which refers to mechanical waves (expressed in external stresses) exerted on the PC modulating the length and bone geometry of a developing bone (Villemure & Stokes).

There is evidence that US application of bone tissue in fractures accelerates metabolism and promotes cell differentiation (Padilla *et al.*). Since the fracture repair process replicates many ossification phases of bone growth,

it is assumed that the acceleration effects in the consolidation of traditional US fractures and LIPUS doses are extrapolated to the ossification process (Cameron). Based on this background, Korstjens *et al.* (2008) *in vitro* cultures of chondrocytes obtained from patients with osteoarthritis (*in vitro*) after the application of classical LIPUS for 6 days, observed an increase in the rate of proliferation. Nolte *et al.* (2001) performed a morphohistological analysis on *in vitro* cultures of metatarsals after the application of LIPUS for 6 days, observing changes in the rate of bone growth expressed in an increase in the length of the diaphysial ossification center. It was hypothesized that this result could be explained by the activation of the PI3K / Akt pathway as the trigger for proliferation of chondrocytes mediated by LIPUS (Takeuchi *et al.*, 2008).

In *in vivo* studies, Oyanarte *et al.* (2009) when applying LIPUS for 26 days in mandibular condyles of male rats, found an increase in ECM secretion in ZH, increased chondrocyte hypertrophy in ZH and alterations in the cellular order of the treated group. After the application of LIPUS to the temporomandibular joint of rabbits for 4 weeks, El-Bialy *et al.* (2003) found an increase in chondrocyte hypertrophy of the chondrogenic layer, increased endochondral ossification and a marked increase in Metabolic activity in the group treated with LIPUS. On the other hand, Lyon *et al.* (2003) and Barreto *et al.* (2011) found alterations in thickness and number of chondrocytes of the layers after US application with high intensities (greater than 1 [W / cm2]), Attributing these results to the thermal effects of US, since they did not find changes with doses with athermic effects, as they are the ones inferior to 0.5 [W / cm^2].

In summary, from this background we could deduce that the *in vivo* evidence would not consistently describe morphological alterations of the PC product of the application of LIPUS and that the alterations observed could be related to thermal effects of the highest intensity of US.

In clinical practice, air is not an adequate medium for the propagation of the US, the main means of propagation must be a coupling material between the target tissue and the US head, some of the most commonly used, are gel or water (underwater US) (Cameron). Underwater US is a method which involves steeping the tissue to be treated and the head US. Although there is very little research regarding this modality, the evidence maintains that it facilitates the coupling of irregular surfaces with a lower heat transfer, reducing the thermal effects (Poliachik *et al.*, 2014) And increasing the area of effective irradiation of the head, improving the safety of the therapeutic application (Cameron).

There is evidence that in *in vitro* models LIPUS increases the proliferation of chondrocytes, but these effects are not clearly replicated in the PC morphology of *in vivo* models. There is only evidence that the morphological alterations would be related to thermal effects of high intensity US. In this context, one of the modalities of US application that diminish the thermal effects is the underwater application, the effects of LIPUS applications in underwater mode on the morphology and histology of the PC. For this reason the objective of the present study is to evaluate the effects of doses equivalent to underwater LIPUS on the histomorphometry of the growth plate of the proximal tibia epiphysis in an animal model.

MATERIAL AND METHOD

Samples and US Application Protocol. Eight male mice of the 15-day-old CF-1 strain (p15) were housed in plexiglass cages under controlled laboratory conditions with reverse light-dark cycle at 20 ± 2 °C , With water and food *ad libitum*. All care and procedures were developed in accordance with the Canadian Manual of Care and Use of Experimental Animals (Olfert *et al*., 1993).

At 15 days the mice were treated twice daily for 4 consecutive days with an underwater LIPUS application protocol, which consisted of sedation with chloroform and immersion of the limbs in water at a temperature of 26 ± 1 ° C. The US was applied in an underwater mode in a pool of Plexiglass with Enraf Sonopuls 491® equipment aimed at the right pelvic limb that was held, while the lower left limb was subject in the same conditions as its contralateral, but isolated from the waves Ultrasonic by a double wall with air interface. The left pelvic limb was used as a control sample.

The parameters used were for SATA similar to LIPUS for bone repair, with 1 [MHz] being the pulsatile base frequency with a SATA of 0.03 [W/cm^2] for 3 minutes. It is important to note that these parameters do not produce significant increases in the local temperature (Poliachik *et al*.). The US team was calibrated and the distance from the near field underwater at the same temperature of the procedure was evaluated by technicians of the responsible brand. According to the report of the technicians the near field was described up to a distance of 110 mm, taking into account the low dispersion and high homogeneity of the wave in the near field, it was decided to place the limbs at 80 mm of the transducer.

Once the 8 sessions of LIPUS were fulfilled, a day for the sacrifice was waited in order to allow the effect of the US. At day p20 the mice were sacrificed by intraperitoneal injections of thiopental. It is important to emphasize that all the handling, care and sacrifice of animals was in accordance with the Canadian Guide on Care and Use of Experimental Animals (Olfert *et al*.).

Preparation, photographing and analysis of histological samples. The histological samples were prepared in the Laboratory of Histological Techniques of the Pontificia Universidad Católica de Valparaíso, Chile.

The pups were fixed immediately postmortem in Bouin for 72 hours, and were dissected at the hip level, however, for the analysis only the tibia was used. After extraction, the samples were decalcified in 10 % w/v ethylidiaminetetracetic acid (EDTA, pH 7.4) for 3 days, and then dehydrated in a battery of alcohols (70 % to 100 %) to be included in cubes Paraffin (Paraplast®).

At the end of these processes, coronal sections were extracted from each tibia (5 - 7 µm thick). The cuts of greater bone length were selected and rehydrated in a battery of alcohols at descending concentrations. Hematoxylin - eosin sections were stained to finally mount the sample on a slide.

Only histological images of the proximal tibial PC were used. The photomicrographs were taken with a Hearter BA-410® light microscope and a Canon® EOS 5D Mark digital camera, under controlled lighting conditions and at a 5x magnification. The images were imported in digital format and measurements were performed with the Image Pro Plus® program.

Morphometric analysis of the PC. In order to delimit the PC areas, the criteria described by Miralles-Flores & Delgado-Baeza (1992). Specifically, ZP was defined as the zone extending from the first flattened chondrocyte proximal to the epiphysis, to the first CH proximal to the diaphysis. The ZH was delimited from the first CH proximal to the epiphysis and the last transverse septum proximal to the diaphysis, or to the last rounded CH.

The thickness and area of ZP and ZH were evaluated. To measure the thickness of the CP zones, the criteria described by Snow *et al*. (2007) were used. The thickness of each zone was determined from the arithmetic average of five measurements per zone, performed parallel to the axial axis of the bone, between the limits defined by zones and taken at equidistant intervals throughout the thickness of the corresponding zone. The thickness of the PC was estimated as the sum of the mean lengths of ZP and ZH (Snow *et al*.). The area of each zone was evaluated using the Image Pro Plus® area polygon tool.

The ZR was not evaluated because its magnitude is greatly diminished by the presence of secondary ossification points at this stage of growth.

Histological analysis of the PC. The columns of the ZH and ZP were analyzed, where the number of total columns (CT) of the ZP and ZH were observed; and the number of cells in the ZH (nZH) per column. When estimating the CT of the ZH, two types of columns were also considered: complete columns (CC), whose length is equal to the thickness of the corresponding zone, and incomplete columns (IC), where the length of the column is smaller than the thickness of the corresponding area (Miralles-Flores & Delgado-Baeza). Subsequently all the data were tabulated in the program Microsoft Excel 2010® for statistical analysis.

Statistical Methods. The data were analyzed by means of descriptive statistics, expressing the mean, standard deviation, standard error and coefficients of variation of each parameter. The distribution was also evaluated by the Shapiro-Wilk test for the treated samples and control samples.

Inferential statistics were evaluated using Student's T for non-parametric data (Mann-Whitney Test U), using a $p \leq 0.05$ as a significant value.

RESULTS

The weight of the mice was evaluated every day, from day 1 of treatment (day 15 postnatal) (Fig. 1), there were no significant changes in the weight of the pups during the days of treatment.

As for total PC thickness (Fig. 2A), measured as the sum of ZP and ZH, no significant differences were found between Control (256.7 ± 36 µm) and LIPUS (295.3 ± 42 µm) p = 0.242.
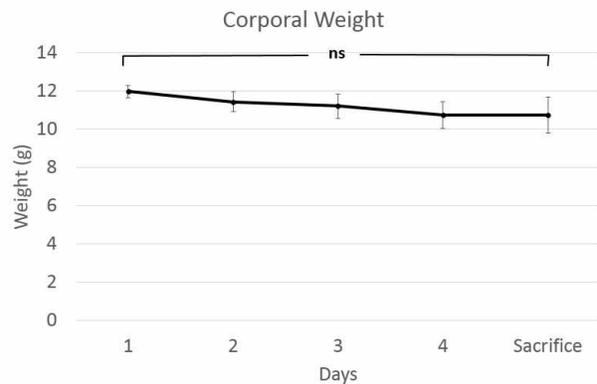

Fig. 1. Body weight gains in the day of treatments; Day 1 of treatment is the 15 postnatal day; n=8; ns=p>0,05.
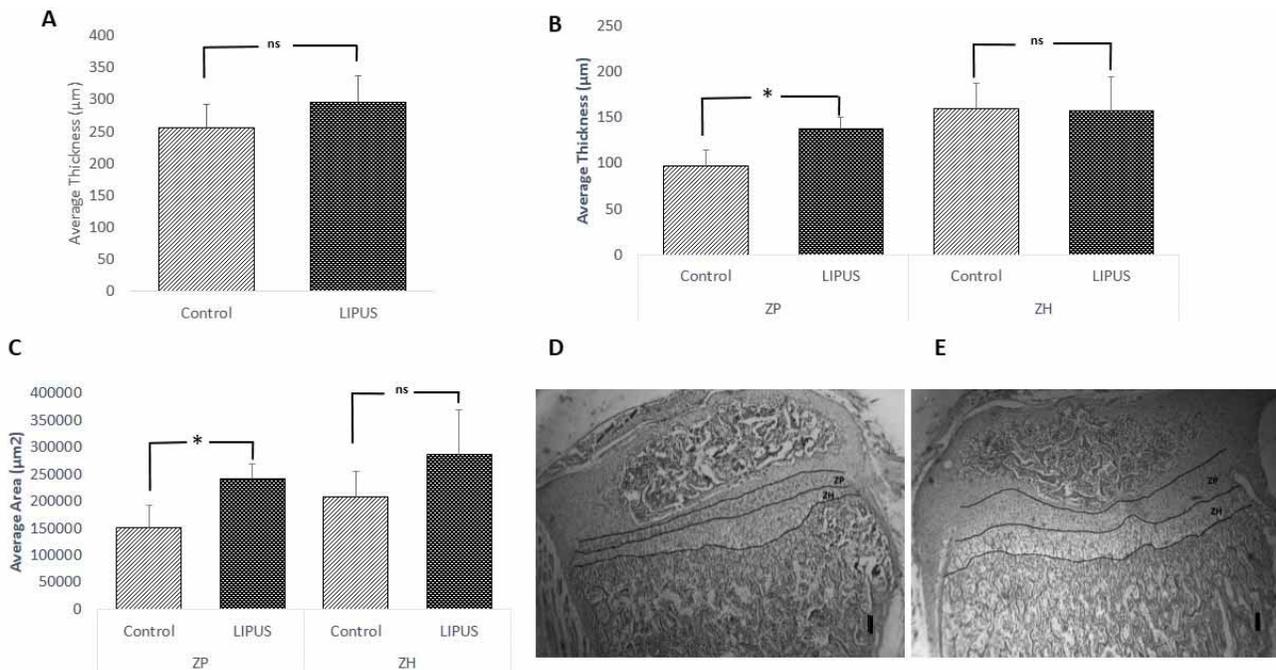

Fig. 2. Morphometric effects of LIPUS application on total PC thickness (A); Effect of LIPUS on the thickness of the proliferative zone (ZP) and the hypertrophic zone (ZH) (B); Effect of LIPUS on the area of the proliferative zone (ZP) and the hypertrophic zone (ZH) (C); Representative photograph of the control growth plate (PC)(D); Representative photograph of the LIPUS growth plate(PC) (E). ns=p>0,05; *=p<0,05; Bars: 100 µm

Analyzing the thicknesses of the layers separately, a significant increase in the ZP thickness of the LIPUS samples (137.71 ± 12.78 µm) was observed in comparison to the Control samples (97.11 ± 17.49 µm, p = 0.048), without significant changes in ZH (control 159.6 ± 27.97 µm, LIPUS 157.6 ± 37.52 µm, p = 0.34) (Figs. 2B, 2D & 2E).

Similar results were observed when analyzing the areas of each of the zones, the area of ZP was significantly higher in the group exposed to LIPUScompared to the control samples (Control 151394.86 ± 41082.29 µm^2, LIPUS $241221.56 \pm$ (Control: 208424 ± 47202 µm^2, LIPUS 286108 ± 83227 µm^2, p = 0.34) (Figs. 2C, 2D & 2E).

When evaluating the histology of the areas described, no significant differences were observed in the number of total columns in the evaluated zones (Fig. 3A) (ZP: control 114.5 ± 30.74, LIPUS 146.5 ± 30.09, p = 0.5), (ZH: control 140.3 ± 29.460, LIPUS 133.8 ± 41.850, p = 0.44).

As for the type of columns observed, there was a significant increase in complete columns (Figs. 3B, 3D and 3E) in the samples exposed to LIPUS ($14 \pm 4,813$), compared to the control samples ($5 \pm 2,380$), p = 0.048.

In ZH the average cell number of each column was evaluated by observing a significant increase of cells per column in the samples exposed to LIPUS (5.16 ± 0.582 cells / col) relative to the control samples (3.75 ± 0.023 cells / Col) (Figs. 3C, 3D and 3E), p = 0.04.

DISCUSSION

The aim of the present study is to evaluate the effects of dose equivalent to underwater LIPUS on the histomorphometric development of CP in male CF-1 mice in post-lactation phase p20.

The application of US with doses similar to LIPUS in underwater mode at a distance of 80 mm seeks to reduce the thermal effects, with a uniform and constant beam of ultrasound. To achieve this, as mentioned in the methodology, the equipment was calibrated to empirically determine the distance range of the near field. Poliachik *et al.* showed that with intensities lower than 100 mW/cm^2, in underwater mode the thermal effects are minimized. With respect to beam uniformity, Shaw & Hodnett (2008) argues theoretically that
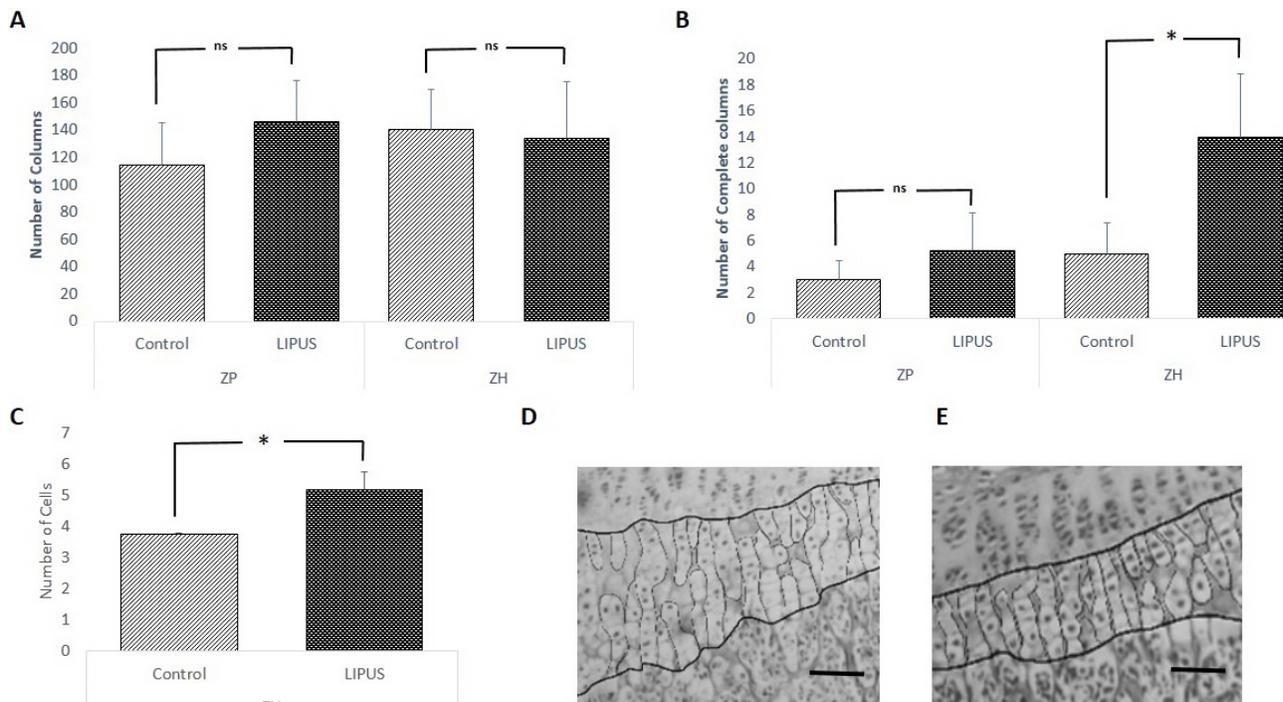

Fig. 3. Histological effects of LIPUS application on number of total columns in the proliferative zone (ZP) and the hypertrophic zone (ZH) (A); Effect of LIPUS on the complete columns of the proliferative zone (ZP) and the hypertrophic zone (ZH) (B); Effect of LIPUS on the number of cells per column in the hypertrophic zone (ZH) (C); Representative photograph of the control growth plate (PC)(D); Representative photograph of the LIPUS growth plate(PC) (E). ns=p>0,05; *=p<0,05; Bars: 100µm

for a head of 5 cm^2 the boundary between the near-distant fields is 100 mm. Therefore, according to the literature and the empirical measurement performed during the calibration of the equipment, the distance of 80 mm is located in the near field, in which the peak of intensities (area of beam uniformity) is prevented and The attenuation of the wave, and the thermal effects are minimized.

When analyzing the weight of rodents during LIPUS treatment, no significant changes in body weight were observed. Bustamante *et al*. (2013) describe that in the early postnatal stage of CF-1 strain rodents, significant increases in body weight can be observed. In the case of the present study this significant increase was not observed, showing that the treatment of underwater LIPUS in sessions of 2 times a day for 4 days, has systemic effects on the development of the rodent. In the present study the same rodent was used to obtain the stimulated sample and the control sample, thanks to this strategy we can expect that the systemic effects of the underwater treatment affected the control samples and the stimulated samples in the same way. Therefore, the differences observed in the histomorphology of CP can be directly attributed to exposure to the ultrasonic beam and should not be attributed to the treatment modality and its systemic effects.

It is important to note that this is the first study to analyze the morphometric and histological changes on the PC after the application of US with doses similar to LIPUS in underwater mode, on tibiae of mice in the late lactation period.

At the morphometric level, the LIPUS samples showed a significant increase in the thickness and area of the ZP. Similar results were found by Wiltink *et al*. (1994) who, when evaluating the effects of LIPUS on endochondral ossification observed an increase in ZP, with no change in ZH, in metatarsal cultures of mice of 16 and 17 days of age lifetime. Likewise, Takeuchi *et al*. and Zhang *et al*. (2003) observed an increase in chondrocyte proliferation following LIPUS application in samples obtained from phalangeal metatarsal articulation of 6-month-old pigs and sternum of embryos of 16-day chicken respectively.

The morphometric changes in the ZP could be explained by the activation of the mechanoreceptor Integrina / Pax-Fax, located in the chondrocytes, which through mechanical stimulation generates activation of the pathway PI3k / Akt, inhibiting the phosphorylation of ß-Catenin, which on accumulation migrate to the nucleus promoting cell proliferation and MEC secretion genes (Takeuchi *et al*.). This phenomenon is strongly related to the intensity of application of the US, being low intensities, as in LIPUS

the most efficient in its activation (Zhang *et al*.). Another possible mechanism involved in the increase of chondrocyte proliferation is that LIPUS through its mechanical waves can generate extracellular fluid flows, redistributing the nutrients inside the PC allowing a better use of these for cell proliferation (Zauhar *et al*., 2006).

In the case of the ZH, no significant differences were observed in the area and thickness of this zone, which could be explained because the pathways stimulated by LIPUS mainly induce the proliferation of chondrocytes and in the case of ZH the capacity Proliferative function of the chondrocytes is diminished by the differentiation process that these cells have undergone (van der Eerden *et al*.).

At the histological level in the samples submitted to underwater LIPUS a significant increase in the number of CC and of cells per column in the ZH was observed. When evaluating the effects of LIPUS on the temporomandibular joint of rats, Oyanarte *et al*. evidenced a significant increase in height and variations in the columnar organization of rodents stimulated with LIPUS for 26 days for 10 minutes daily. A similar result was obtained by El-Bialy *et al*. to observe an increase in the columnar organization of ZH in CP of temporomandibular joints of male rabbits, after the application of LIPUS for 4 weeks for 20 minutes a day.

These results are in contrast to those described by Abramovich *et al*. (1970) and Lyon *et al*.; Lyon showed a significant decrease in the organization of both ZH and ZP after application of high doses (2.2 W/cm^2) in tibia and femur of 6-week rabbits, with a 6-week application of 20 minutes daily. On the other hand, Abramovich when using samples of tibia and femur of female Wistar rats of 30 days observed a disorganization product of the application of US at higher intensities (7 W / cm^2) for 10 minutes. However, the high intensity used by Abramovich and Lyon is related to an increase in the temperature of the deep tissues, which could explain the observed effects (Abramovich *et al*.; Lyon *et al*.; Poliachik *et al*.). In the present study no similar results were observed possibly because this thermal effect has been diminished by the intensities of LIPUS (30 mW/cm^2) and the underwater application modality.

The results obtained in the present study could be explained by an increase in the cellular proliferation triggered by mechanoreceptors located mainly in the chondrocytes of the ZR and ZP (Nilsson *et al*., 2004, Takuchi *et al*., 2008, Padilla *et al*.). The increase in proliferation generates a greater number of cells per unit area, causing an accumulation of cells that will differentiate and enter together in the ZH, thereby increasing the number of cells per column and the increase of complete columns (Nilsson *et al*., 2004).

Another possible mechanism is directly related to the mechanical waves generated by LIPUS, it has been described that these waves can organize the cellular distribution through extracellular fluid flows (Zauhar *et al*.), or through the direct interaction with the Proteins of the cell membrane, stimulating the organization between contiguous cells and between these cells and the MEC (Padilla *et al*.).

In relation to the contraindication of applying ultrasound on growth plates, it has been described that it is one of the most named in the literature (Batavia). And it relies mainly on studies that have shown that high doses of therapeutic ultrasound can produce PC closure (De Forest *et al*., 1953), where one of the mechanisms possibly implicated is the thermal effects of the US. In the case of the present study, the thermal effects were minimized by the underwater application methodology and the calibration of the equipment in relation to the distance of the fields. In this sense the main mechanisms that could account for the observed results would be mechanical effects on the tissues. In analyzing the results observed in the present study we realize that they do not necessarily tend to a PC closure as described in the contraindication, it is for this reason that we can argue that possibly the US has effects on the PC, but that these are differentiated according to the dosage, modality of application or even, to the predominance of thermal effects or mechanical effects on the fabric.

For future studies it is important to evaluate the effects of LIPUS on the production of local factors of the PC that could be controlling the growth of plaque.

Based on the results obtained in the present study it is possible to conclude that LIPUS in underwater modifies the histomorphometry of the proximal epiphyseal PC of the tibia of rodents in the late postnatal stage, promoting proliferation and columnar organization in the proliferative zone and hypertrophic, respectively. In relation to the contraindication of applying US on PC, the observed results support the theory that the US modifies the development of CP, but probably its effects are intensity dependent, where intensities similar to LIPUS in underwater mode would not produce a premature closure of The PC. More studies are needed in the area to elucidate the real effects of the US at different intensities and their possible utilities in bone tissue.

RESUMEN: El ultrasonido es una de las herramientas más utilizadas en fisioterapia, su modalidad LIPUS permite tratar lesiones músculo-esqueléticas, pero al igual que el ultrasonido convencional está contraindicada su aplicación en zonas próximas a cartílago de crecimiento por un posible cierre de éste. En la literatura no existe evidencia contundente de esta contraindicación, por lo cual el objetivo del presente estudio fue evaluar los efectos de dosis equivalentes a LIPUS subacuático sobre la histomorfometría de la placa de crecimiento de la epífisis proximal de tibia en un modelo animal. Metodología. Se aplicó LIPUS en modalidad subacuática en el miembro pélvico derecho a 8 ratones en p 15 por 4 días, 2 veces al día. Al cumplir 8 sesiones se sacrificaron y se evaluó la histología de la placa de crecimiento del miembro estimulado. Al evaluar la histomorfometría de la placa se observó un aumento significativo en el espesor y el área de la zona proliferiva estimulada con LIPUS (p=0.048). En la zona hipertrófica no se observaron diferencias significativas en el espesor ni en las áreas, pero si existieron cambios significativos en la organización histológica de la zona, observando un aumento significativo de las columnas completas en la placa estimulada (p=0,04). El LIPUS en modalidad subacuática modifica la histomorfometría de la PC epifisiaria proximal de la tibia de roedores en el etapa postnatal tardía, promoviendo la proliferación y la organización columnar en la zona proliferativa e hipertrófica, respectivamente.

PALABRAS CLAVE: Ultrasonido pulsátil de baja intensidad; Ratón CF-1; Lactancia tardía; Placa de crecimiento.

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