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Chondroprotective effects of pulsed shortwave therapy in rabbits with experimental osteoarthritis

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Abstract

Introduction: Osteoarthritis (OA) represents a public health challenge since the pathogenic treatment, able to induce cartilage regeneration, still remains unknown. Ageing of the population and increasing OA prevalence have led to a lot of research, aiming to identify treatments acting on chondrocytes that play a determinant role in cartilage degeneration/regeneration balance. Pulsed shortwave therapy (with the classical application form – Diapulse) is a physiotherapy method with anabolic effects demonstrated on nervous, conjunctive and vascular tissues, but its effects on OA cartilage are not known. *Aim*: Our aim was to demonstrate the effects of Diapulse on the cartilage in experimental induced OA. *Materials and Methods*: Experimental OA was induced in 10 mature female rabbits by anterior cruciate ligament transection (ACLT). Ten weeks after ACLT, rabbits were randomized in a treatment group and a control group. Treatment group was exposed to Diapulse at a frequency of 27.12 MHz, pulse length of 65 μs, pulse frequency of 300 pulses/s (300 Hz) for 10 minutes/day. Control group was exposed to sham therapy. After treatment, rabbits were sacrificed and the cartilage was evaluated by histopathological examinations with Hematoxylin–Eosin (HE) staining and transmission electron microscopy (TEM). *Results*: OA characteristic changes were found in both groups. In the treatment group, we found that Diapulse influenced the degenerative process in the OA cartilage by improving the chondrocyte viability and the capacity to maintain cellular matrix integrity and structure. *Conclusions*: Diapulse can be considered a disease modifying therapeutic procedure and could be a reliable option for treatment of OA patients.

Keywords: cartilage, Diapulse, osteoarthritis.

Introduction

Osteoarthritis (OA) has become increasingly prevalent worldwide because of the combination of aging population and growing levels of obesity [1]. Despite of a multitude of possible therapeutic interventions for the symptomatic control of OA, it is important to note that none of the current therapies can actually cure OA. Current drugs have failed to demonstrate any morphological changes in the cartilage structure and many of them have numerous side effects [2]. Therefore, actual research is oriented in the direction of physiotherapeutic agents.

Numerous studies have emphasized the effects of physiotherapeutic agents on chondrocyte functions, while others have focused on global effects reflected in the cartilage morphology. Physiotherapeutic agents act as modulators of physiological and pathophysiological processes in chondrocytes, since they can change the viability and structure of chondrocytes from osteoarthritic lesions.

To date, ultrasound therapy [3, 4], low laser therapy [5, 6] and pulsed electromagnetic fields at low and high frequency [7, 8] have demonstrated their effects on cartilage and chondrocytes changes induced by OA.

Pulsed shortwave diathermy (PSWD) (also known as pulsed electromagnetic field therapy (PEMF), pulsed shortwave therapy (PSWT), pulsed radio frequency electromagnetic field therapy (PRFE) or radio frequency non-thermal diathermy) is an electrotherapy treatment that uses a specific radio-frequency band of the electromagnetic spectrum at 27.12 MHz [9] for generating electromagnetic fields.

The pulsed regime allows heat dissipation in tissues. High frequency electromagnetic fields at pulsed mode expose tissue to high energy levels with biomodular effects and without the risk of thermal injury. The heat dissipation in tissues appears to be more efficient in highly vascularized tissues. Because the cartilage is avascular, it is important that the electromagnetic energy exposure is done to perfect pulsed forms which eliminate the endothermic effects.

Diapulse represents a model of the device used for the PSWD. It generates high frequency pulsed electromagnetic waves without endothermic phenomena with proven regenerative effects in skin tissue [10] and peripheral nerves [11]. In clinical practice, Diapulse is used in OA treatment to relieve pain and to improve physical function [12]. However, the effects of Diapulse on the osteoarthritic cartilage structure are unknown.

The aim of this study was to demonstrate the effects of Diapulse on experimental induced OA. For this purpose, rabbits with experimental induced OA were exposed to Diapulse. The study was conducted using an experimental model in which OA develops as a result of anterior cruciate ligament transection (ACLT) in rabbits with closed growth plates. The efficiency of Diapulse in attenuating OA process was assessed after 10 days of treatment by histological and transmission electron microscopy (TEM) examination.

Materials and Methods

Experimental animals

Ten mature female rabbits were utilized in the study. The animals were divided in two groups: control and treatment. The study was approved by the Ethical Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca (Romania).

Anterior cruciate ligament transection (ACLT) surgery for induction of OA

Experimental OA in rabbits was induced by ACLT surgery in both control and treatment groups, according to the protocol described by Park *et al*. [13]. The rabbits were anaesthetized with an intramuscular injection of Ketamine [50 mg/kg body weight (bw)] and Xylazine (10 mg/kg bw) [14]. After shaving and disinfecting the surgical site, ACLT was performed using a para-medial approach with the skin incision in the right knee medial para-patellar area. To achieve optimal visualization of the anterior cruciate ligament, the patellar bone was displaced laterally and the knee was placed in full flexion. The anterior stability was confirmed by an anterior drawer test [15]. The synovium and the incised skin were sutured, and sterile dressing was applied. Following the surgical procedure, 5% Enrofloxacin (5 mg/kg bw) was injected intramuscularly, in single dose, to each rabbit. All animals were allowed normal cage activity.

Experimental protocol for treatment

After ACLT, the rabbits were divided into two groups of five rabbits each: control and treatment group, respectively. Generally, articular cartilage exhibits degenerative changes approximately 3–8 weeks after ACLT in experimental rabbits [16]. Because the lesions are most important at 10 weeks after ACLT and therapeutic effects are noticeable at this moment, we chose to begin Diapulse therapy 10 weeks after ACLT, similarly to other studies [17, 18].

Ten weeks after ACLT, the osteoarthritic joint changes were confirmed by radiological examination. All rabbits from treatment group were exposed to Diapulse therapy: mode of operation pulsed, 27.12 MHz frequency, pulse length 65 μs, pulse frequency 300 pulses/s (300 Hz), maximum pulsed power output 975 W, for 10 minutes/day. All rabbits from control group were exposed to sham Diapulse therapy, with the power button of Diapulse device in the OFF position.

Mean power [W] and total energy were calculated using following formulas:

$$\text{Mean power [W]} = \text{peak power [W]} \times \text{pulse duration [s]} \times \text{pulse frequency [Hz]}$$

$$\text{Total energy [kJ]} = \text{Mean power [W]} \times \text{application time [s]}$$

Mean power was 19.01 W, a value less than 20 W that minimized the thermal effects [12]. Total energy was 11.4 kJ.

After the last day of treatment, all rabbits were sacrificed by cervical dislocation. Hematoxylin–Eosin (HE) staining was applied to tissue samples from each of the groups. TEM images of the articular cartilage were also examined to confirm the histopathological findings in the two groups.

Histopathological examinations

After the rabbits had been sacrificed, their right knee joints were dissected. The medial femoral condyles of the rabbits were fixed in 10% phosphate-buffered formalin (pH 7.4) for seven days and decalcified in a 1:1 mixture of 8% formic acid and hydrochloric acid for two weeks. The decalcified specimens were trimmed longitudinally and embedded in paraffin wax using the laboratory routine protocol. Four μm thin sections were cut from the paraffin wax blocks and stained with HE for light microscopic examination [19].

TEM analysis

Articular cartilage samples for TEM analysis were taken from the medial tibial condyle. Specimens were processed for TEM according to usual protocols [20, 21]. Briefly, they were fixed for two hours in 2.7% (*w/w*) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and washed four times in the same buffer (one hour each). Samples were post-fixed in 2% osmium tetroxide for two hours, dehydrated in an acetone series, and embedded in Epon 812 epoxy resin. Ultrathin sections with yellow interference color (60–80 nm) were cut with glass knives on a Bromma 8800 ULTRATOME III (LKB, Stockholm, Sweden). The sections, collected on 300 mesh copper grids (Agar Scientific Ltd., Stansted, UK), were double contrasted with uranyl acetate and lead citrate solutions, and examined with a JEOL JEM 1010 TEM (JEOL Ltd, Tokyo, Japan) at 80 kV. Images were captured with a Mega VIEW III camera (Olympus, Soft Imaging System, Münster, Germany).

Results

Progression of OA in untreated limbs

Histology

As expected, degenerative modifications of cartilage were present in the untreated group (Figure 1, A–C). In this group, the articular cartilage showed typical OA changes in different stages of development. In the affected areas, we found reduction in articular cartilage thickness, superficial erosion coexisting with deep, multiple osteochondral cracks. In other areas, we found detached cartilage fragments (Figure 1A), calcification of cartilage matrix, or focal loss of chondrocytes (Figure 1B). In areas with normal thickness of articular cartilage, we observed changes in cartilage structures such as collagen proliferation, and increased matrix mineralization (Figure 1C).

TEM examination

TEM examination of cartilage from the OA control group showed chondrocytes containing nuclei with irregular outline or even polymorphic appearance and predominantly euchromatic (Figure 2, A and B). Cytoplasm was dense and heterogeneous. The mitochondria preserved the normal shape (round-oval), but the matrix was rarefied (Figure 2, C and E). Many profiles of rough endoplasmic reticulum were found in most of the cells (Figure 2C), whereas the Golgi apparatus was in normal amount and preserved normal structure (Figure 2E). In the cytoplasm, deposits of lipids (l) were also identified, forming relatively large, grouped droplets (2–9/cell) (Figure 2, A, B, D and E).

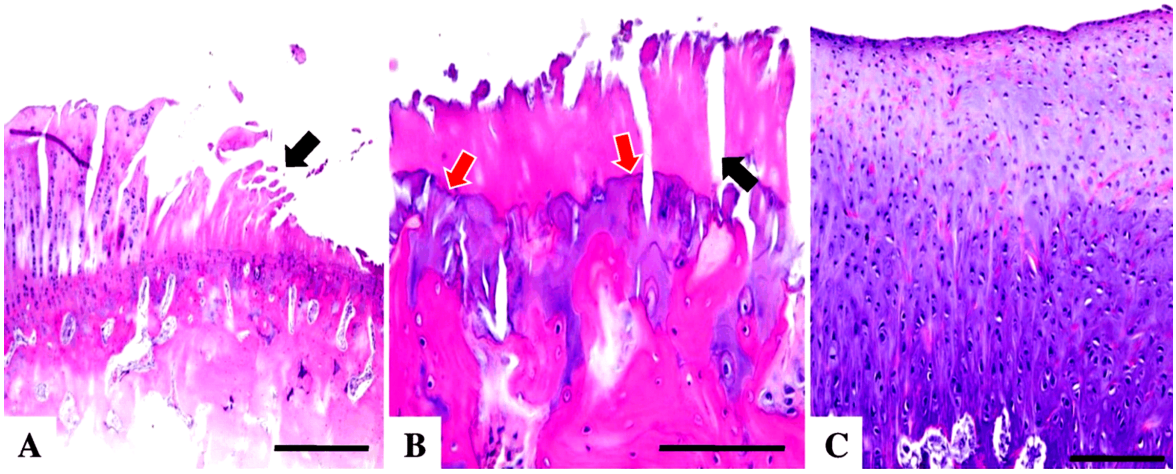

Figure 1 – *Cross-section of OA femur cartilage in the control group: (A) Osteochondral cracks (arrow) with deep cleavage of cartilage; (B) Chondral bone deep fissures (arrow), calcification of matrix and chondroplasts (red arrow); (C) Fibrous collagen proliferation (eosinophilia), chondrocytes "through" in the mineralized matrix. HE staining: ×100 (A and C); ×200 (B). Scale bar: 400 μm (A and C); 200 μm (B).*

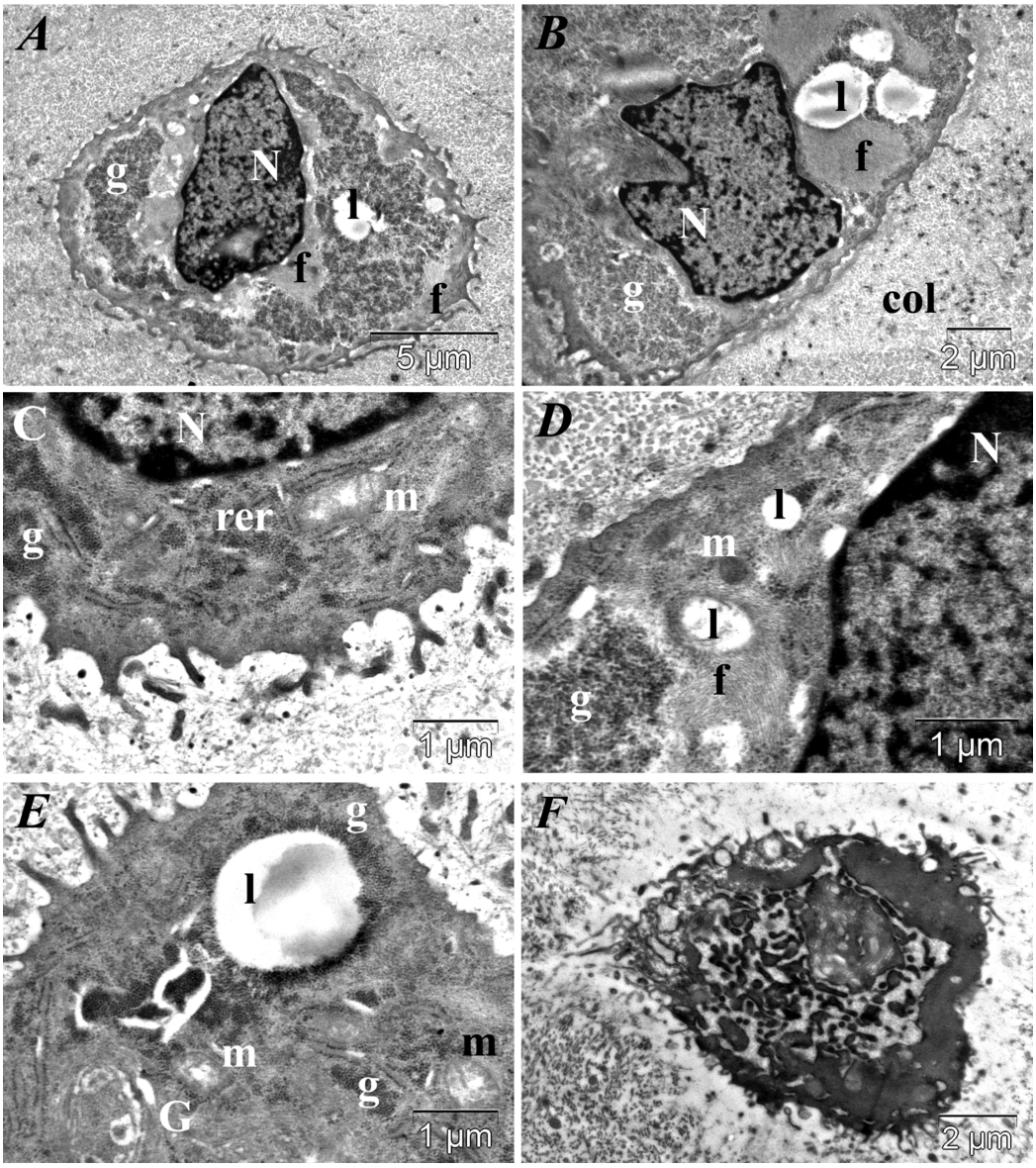

Figure 2 – *OA control group (TEM): Chondrocytes with euchromatic and polymorphic nuclei (N) (A and B), mitochondria (m) with rarefied matrix (C and E), large rough endoplasmic reticulum (rer) (C), Golgi apparatus (G) (E), large droplets of lipids (l) (A, B, D and E), numerous glycogen granules (g) (A–E) and fibrillar material (f) (A, B and D). Collagen fibers (col) in low density (B) and apoptotic cells in advanced stage (F).*

Glycogen granules were compactly grouped, but occasionally appeared dispersed within the cytoplasm (Figure 2, A–E). They occupied between 1/4 and 1/2 of the cytoplasm volume (Figure 2, A–E). In chondrocyte cytoplasm, we found fibrillary material, which was disposed between the nucleus and the regions rich in glycogen (Figure 2, A, B and D). Extracellular matrix consisted of collagen fibers with heterogeneous density (Figure 2, A–F). In the same places, we observed apoptotic cells with disorganized structure and without any contact with the surrounding extracellular matrix (Figure 2F).

Effect of Diapulse treatment

Histology

Representative histological findings are shown in Figure 3. Diapulse treatment regenerated the articular cartilage consecutively to changes induced by ACLT through preserving chondrocytes viability and extracellular matrix (ECM) structure. In treatment group, chondrocytes conserved normal arrangement in three zones. Moreover, histological examination demonstrated that Diapulse increased chondrocytes density. Also, the treatment preserved intact the cartilage surface, few areas showed superficial mild loss of chondroid matrix staining.

TEM examination

TEM examination of cartilage from the treatment group showed high cell density. The chondrocytes were grouped in clusters of 2–3 cells (Figure 4, A and B) and the extracellular matrix separating these cells was more electron-lucent than in the rest of the tissue, where the collagen fibers had a normal structure and density. In chondrocytes, we observed large, polymorphous and euchromatic nuclei (Figure 4, A–E). In the cytoplasm, mitochondria with oval shape and dense matrix were observed (Figure 4E), rough endoplasmic reticulum was abundant, but without dilated profiles (Figure 4, A–E). In some cells, we observed the Golgi apparatus, also having a normal appearance (not shown). Lipid droplets were found in large number, about 2–5/cell (Figure 4, B–D and F), up to 13 lipid droplets in some cells. The glycogen granules were also present on large areas (Figure 4, C and D), sometimes taking up to 1/4 of the cell (Figure 4D), and in most of the cases were clustered around the droplets of lipids. Fibrillar material was observed only in some of the examined chondrocytes, occupying especially areas adjacent to the nucleus (Figure 4, D and E). Rare apoptotic cells were observed, but they were in early stages (Figure 4F).

Discussion

Although OA involves the "whole joint", cartilage degradation, a multi-faceted and complex process that characterizes all forms of OA, is the hallmark of the progression and irreversibility of the disease [22].

Under normal physiological conditions, chondrocytes (the only cells present in cartilage) maintain an equilibrium between anabolic and catabolic activities and express various proteolytic enzymes such as aggrecanases and matrix metalloproteinases (MMPs), which mediate a very low matrix turnover, responsible for cartilage remodeling [18]. Recent studies suggest that chondrocyte death is a key player in cartilage degeneration. Reduced cellularity of cartilage means that the chondrocytes are no longer able to maintain the vast ECM and therefore a net degradation and a loss of cartilage results in OA [23]. In consequence, an ideal treatment for OA must improve viability and metabolic activity of chondrocytes and restore the ECM structure.

The ACLT is a well-known model for OA induced by mechanical stress, which is used for demonstrating the effects of numerous therapeutically agents on OA. ACLT is associated with immediate and severe joint instability [24]. In our study, articular cartilage exposed to mechanical stress after ACLT developed OA specific structural changes.

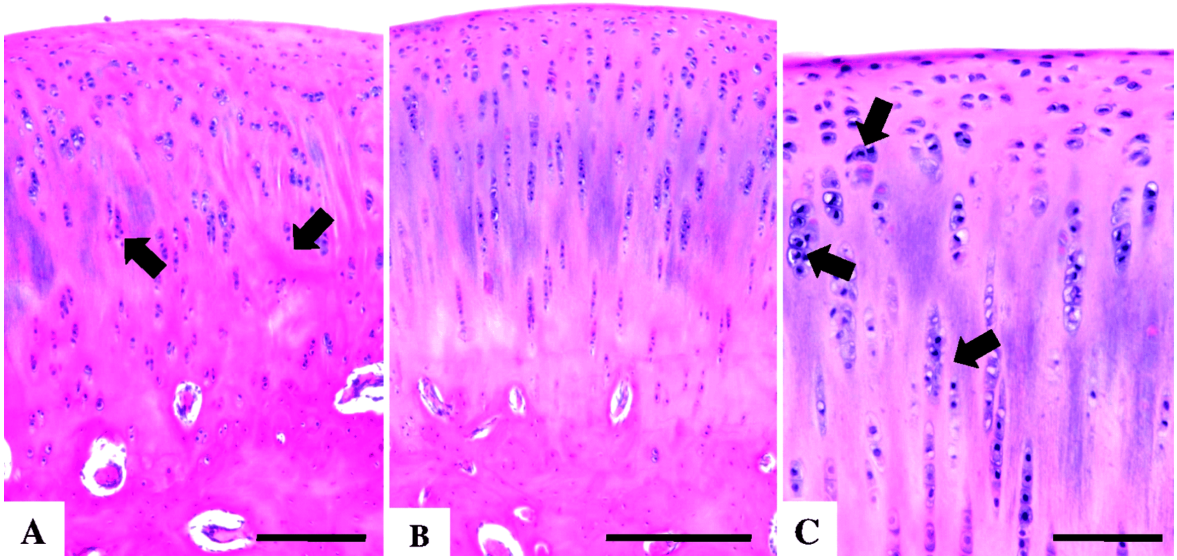

Figure 3 – ***Cross-section of femur cartilage in the Diapulse group, with soft surface, normal thickness and cellularity: (A) Focal eosinophilia (right arrow), poor delineation of cancellous bone–cartilage; (B) Poor delineation of cancellous bone–cartilage and chondrocytes normal distribution in fasciculated area; (C) Focal loss of chondrocytes in the fasciculated area (right arrow) and few chondrocytes with pyknotic nucleus (left arrows). HE staining: ×100 (A and B); ×200 (C). Scale bar: 400 µm (A and B); 200 µm (C).***

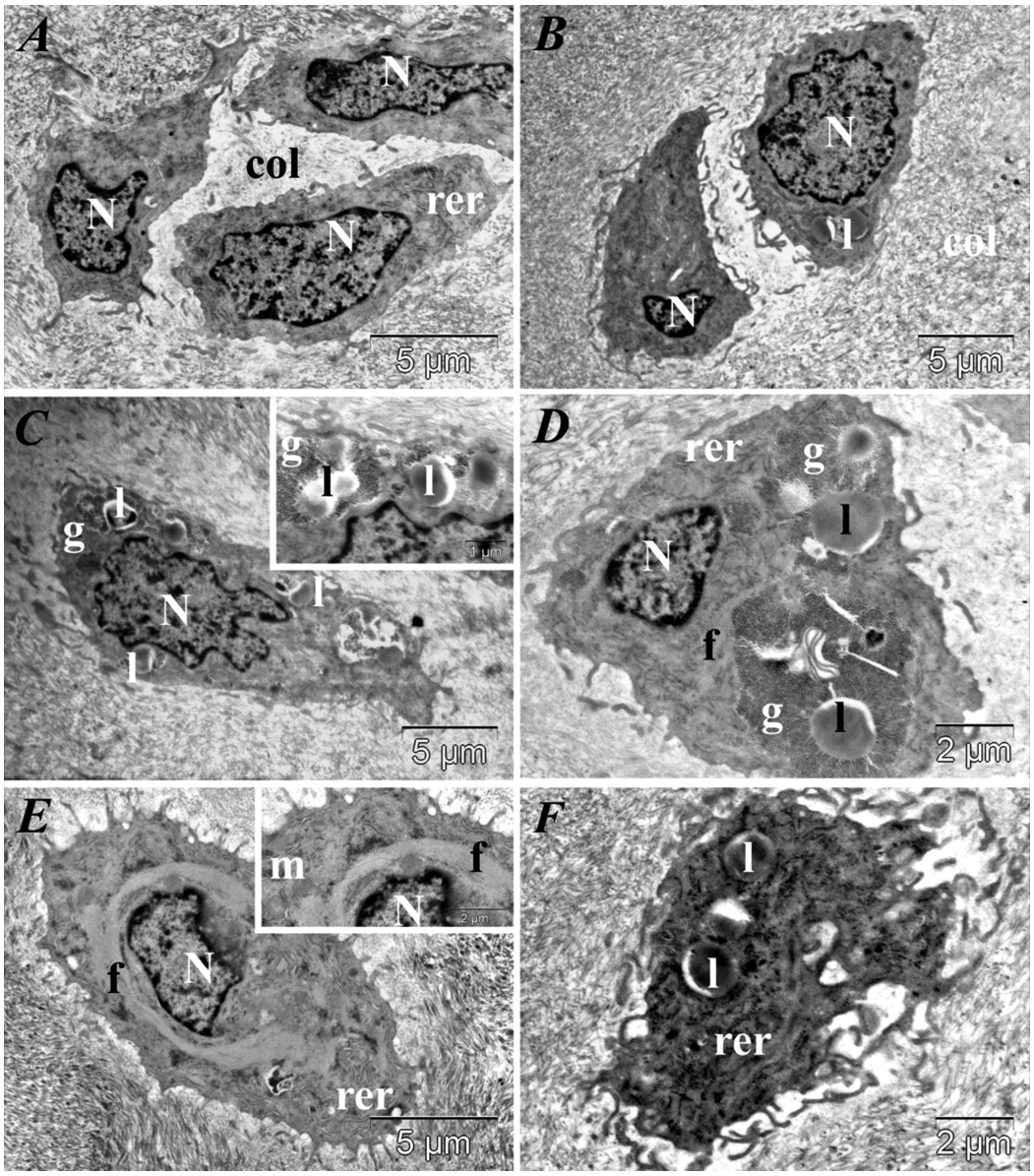

Figure 4 – ***Diapulse group (TEM): Grouped chondrocytes (A and B) with large, polymorphous and euchromatic nuclei (N) (A–E), rough endoplasmic reticulum (rer) (A–E) and mitochondria (E) with normal aspect, lipid droplets (l) (B–D and F) and glycogen granules (g) (C and D) in large number, and fibrillar material (f) in low amounts (D and E). Apoptotic cells in early stages (F). Collagen fibers (col) in low density (B).***

Mechanical compression of cartilage induces deformation of cells and the surrounding matrix, hydrostatic pressure, fluid flow, changes in osmotic pressure, streaming potentials [25–27] and, depending on the mechanical stimulus, chondrocytes may be killed directly (necrosis) or may die through apoptosis [28, 29]. In mechanically injured cartilage, the increase of MMP-3 induces ECM degradation and proteoglycan (PG) depletion [30]. The more intact (less degenerated) deeper OA cartilage would appear to mount a "reparative" response, characterized by increased synthesis of type II procollagen [31]. Microscopically, severe OA is characterized by extensive fissuring and fibrosis, clustering of chondrocytes and loss of cartilage. Zonal cartilage organization is disturbed. The superficial zone degradation produces rough surface, fissures, and cracks extending to the calcified zone [32].

According to these records, we found that in control group animals developed severe OA with destruction of the chondrocytes, deep cleavage of cartilage, deep swelling and separation of fragments of cartilage. Also, areas with intense calcification of ECM were observed. In control group, in areas where the cartilage was less degenerated, the disorganized collagen proliferation attested that regenerative process is poorly coordinated and ineffective [33]. Previous studies have shown that chondrocytes isolated from OA cartilage were prone to induce calcifications *in vitro*. Additionally, calcified areas were found in cartilage samples from OA patients [34]. Mineralization of articular cartilage is a common event in end-stage OA and is associated closely with chondrocyte hypertrophy and disease progression [35].

In treatment group, we found that Diapulse modified the evolution of degenerative process in OA cartilage by improving the chondrocyte viability and capacity to maintain ECM integrity and structure. The cartilage surface was smooth and only focally the fibrous structure was

changed. This change corresponds for OA stage 0, according to the *Osteoarthritis Cartilage Histopathology Assessment* system [36].

Phenotype variability in normal and OA chondrocytes has been demonstrated by many authors. The fact that cartilage disintegration is a focal and not synchronic process, could explain the great diversity of OA chondrocyte phenotypes described at the ultrastructural level [37].

In control group, we found chondrocytes with near normal appearance close to the chondrocytes in apoptosis. The chondrocytes presented specific alterations for degeneration, abundant rough endoplasmic reticulum and decreased mitochondrial cristae, similarly to those found by Liu *et al.* in endemic degenerative osteoarthritis [38]. The prominent rough endoplasmic reticulum suggests that chondrocytes headed to secretory phenotype, which was involved in chondroptosis within OA chondrocytes. Although chondroptosis has some features in common with apoptosis, it differs by prominent Golgi apparatus and rough endoplasmic reticulum [37]. The fibrillary material accumulated in large amounts in the cytoplasm represents another marker for advanced chondrocyte degeneration [39, 40].

Despite the fact that lacunae presence attests chondrocytes death in treatment group, this process was significantly less than in control group. We found chondrocytes with pyknotic nuclei corresponding to apoptotic early stage [41]. In treatment group, chondrocytes were also involved in replicative process and formed clusters observed in the middle zone of cartilage. This disposition, associated with the electron-transparent aspect of the extracellular matrix separating such cells (looking as newly-formed), and corroborated with the low number of apoptotic cells are indicatives for stimulation of cellular proliferation, explaining at the same time the higher cell density noted for the treatment group. Previous studies confirmed that chondrocytes clustering is present in the first three stages of OA and absent in stages 4, 5 and 6 [36]. In the treatment group, TEM revealed that rough endoplasmic reticulum was abundant in chondrocyte cytoplasm, with normal profile signifying absence of cellular stress [42]. The secretory activity in treatment group was increased, but to a lesser extent than in control group. The fibrillar material present in cytoplasm only in same cells was in small amount. In a previous study, intracytoplasmic fine filaments were also present in small amounts throughout the cytoplasm in normal chondrocytes [43].

There are several possible mechanisms by which Diapulse could modulate the progression of OA: thermal and non-thermal. In case of thermal mechanism, Diapulse increases tissue temperature with only 0.1^0C, but this increase has significant biological effects that include vasodilatation and metabolism acceleration. Non-thermal mechanisms include Ca^{2+}-calmodulin-signaling leading to nitric oxide production [44], up-regulation of gene families for tissue inhibitors of metalloproteinases (TIMPs) [45] and for fibroblast growth factor-2 (FGF-2) [46].

Previous studies demonstrated that Diapulse has many effects on wound healing, by reducing the inflammation phase, increasing cell proliferation associated with collagen I synthesis [47] and reducing of interleukin (IL)-1β in the wound exudates [48]. Diapulse can also stimulate the synthesis of fibronectin [49], an essential component for tissue regeneration [50].

In treated palatal shelves, Diapulse showed induction of cartilage within the mesenchymal compartment and loss of the overlying compartment independent of thermal changes and probably due to calcium flux within the tissue [51].

Several studies reported that mechanical stress induced ECM degradation by the increasing synthesis of MMPs [30, 52]. Chondrocytes have receptors for ECM components, many of them being responsive to mechanical stimulation. Activation of these receptors stimulates the production of MMPs and inflammatory cytokines [53]. The main cytokines at this level are IL-1β and tumor necrosis factor-alpha (TNF-α), which suppress ECM synthesis and promote cartilage catabolism [54].

One possible explanation for the Diapulse effects in osteoarthritis may be the reduced synthesis of IL-1β and the neutralization of degenerative effects of MMPs on ECM by increasing levels of TIMPs, the natural inhibitors of MMPs. These effects allow the cartilage to conserve ECM structure under mechanical stress.

Another explanation could be the conservation on chondrocytes number by stimulation of chondrocyte proliferation and by inhibition of chondrocyte death. In treatment group, we found that Diapulse reduced chondrocyte death and stimulated chondrocyte proliferation and clustering. Our results are in concordance with those published by D'Lima *et al.* The authors used caspase inhibitors to block chondrocyte death and reported cartilage lesions of lower severity in a rabbit model of post-traumatic OA [55].

To the best of our knowledge, this is the first report which demonstrates that Diapulse modifies the degenerative process in cartilage exposed to OA changes induced by mechanical stress.

Conclusions

Diapulse can protect the cartilage from OA progression by stimulation of chondrocyte proliferation and by improving chondrocyte viability and capacity to maintain ECM integrity and structure. In agreement with these data, our results suggest that Diapulse can be considered a disease modifying OA treatment and could be a reliable option for treatment of OA patients.

Conflict of interests

The authors declare that they have no conflict of interests.

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