Comparing different physical factors on serum TNF-α levels, chondrocyte apoptosis, caspase-3 and caspase-8 expression in osteoarthritis of the knee in rabbits

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A B S T R A C T
Objective: To study the therapeutic effects that different physical factors may have on rabbits with osteoarthritis of the knee.

Methods: A total of 64 rabbits were randomised and organised into eight groups, eight of which were each assigned a different physical factor, in which the rabbits received one type of physical therapy: millimetre waves for 20 min, pulsed electromagnetic fields, millimetre waves for 40 min, ultrasound, low-level laser therapy or ultrashort wave diathermy. The two remaining groups, the normal group and the model group, served as controls. The efficacy of the different treatments were determined by observing the configuration and structure of the cartilaginous tissue by haematoxylin and Eosin staining, measuring the serum tumour necrosis factor-α levels by enzyme immunoassay, evaluating the expression levels of caspases-3 and -8 by immunohistochemistry, and calculating the ratio of chondrocytes apoptosis by TdT-mediated dUTP nick end labelling. The values obtained for each assessment of the eight groups were analysed by a One-way ANOVA.

Results: By applying upmentioned physical treatments, the organisational configuration and structure of cartilage cells from the knees of rabbits with osteoarthritis increased. These treatments also decreased serum tumour necrosis factor-α levels, reduced the expression of caspase-3 and caspase-8 and reduced chondrocyte apoptosis, resulting in an overall delay in osteoarthritis development.

Conclusion: The application of pulsed electromagnetic fields, millimetre waves for 40 min, ultrasound, or low-level laser therapy had significant effects in improving osteoarthritis; in particular, treatment with pulsed electromagnetic fields or ultrasound yielded the greatest therapeutic effect.

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1. Introduction

Osteoarthritis (OA) is the most common joint disease and is a leading cause of chronic disability [1]. This disease is compounded by age, genetic factors, and mechanical forces, and is characterised by distinct changes in both superficial and interior cells within the cartilage [2]. The pathogenesis of OA remains unclear, but recent studies have revealed that apoptosis is associated with the onset and development of OA [3]. Apoptosis, a form of programmed cell death, is critical not only during development and tissue homeostasis, but also in the pathogenesis of a variety of diseases [4]. Blanco et al. have found that the proportion of apoptotic cells in OA is greater when compared to that in normal or healthy cartilage [5]. The greater proportion of apoptotic cells in OA implies that apoptosis plays an important role in the development of OA. In molecular studies, the expression of several caspases, such as caspase-3 and caspase-8, is increased in human osteoarthritic cartilage and in animal models [6,7]. Additionally, tumour necrosis factor-α (TNF-α) is a pleiotropic cytokine that plays a central role in inflammation and apoptosis [8].

Millimetre waves (MW), pulsed electromagnetic fields (PEMFs), ultrasounds (US), low level laser therapy (LLLT) and short wave diathermy (SWD) are non-invasive techniques that are considered safe and effective forms of physiotherapy for the management of OA [9–14].

We hypothesised that an effective treatment can significantly improve the progression of OA and can be monitored through the analysis of several key disease indicators, such as histological...
evaluation of the articular cartilage, serum TNF-α levels, expression of caspase-3 and caspase-8 and apoptosis of chondrocytes. In this study, we quantitively evaluated the effects of MW for 20 min, PEMFs, MW for 40 min, US, LLLT and SWD therapies on serum TNF-α levels, chondrocyte apoptosis, and caspase-3 and caspase-8 expression in rabbits with osteoarthritis of the knee. Our results may shed light on the efficacy of various physical therapies available to patients in the management OA of the knee.

2. Materials and methods

2.1. Animals

A total of 64 New Zealand White (NZW) rabbits, 32 male and 32 female, 3 months of age and weighing 2–2.5 kg, were used for this study.

All animals were given a standard laboratory diet with drinking water and housed in individual cages with a 12-hour light-dark cycle at 20–26 °C. The research complied with national legislation and with Ministry of Health of the People’s Republic of China Guide for the Care and Use of Laboratory Animals, and had local ethical committee approval. All the rabbits were sacrificed by air embolisation 8 weeks after the OA model induction operation. NZW rabbits were supplied by the Sichuan University, Huaxi animal testing center, batch number was: SCXK (Sichuan) 2004-14.

2.2. OA of the knee joint model

The rabbits were in given intraperitoneal injections of 5% chloral hydrate (3 ml/kg). The surgical operations performed to create the knee OA model in rabbits were conducted as described in the reported literature [15]. Briefly, the right knee was shaved, then sterilised and draped in sterile fashion before a medial arthrotomy was performed. OA was induced by anterior cruciate ligament transaction (ACLT). All the rabbits were sacrificed 8 weeks after the operation. After the surgeries, the rabbits were injected with Gentamicin (15 mg/kg, 40 mg/ml) once a day for 5 days and allowed to move freely in a cage. OA of the knee joint was successfully induced in rabbits that were caged post-operatively for 6 weeks.

2.3. Intervention

After 6 weeks of OA modelling, rabbits in each group (A-F) were treated with one physical factor for 10 days:

- **Group A**: MW for 20 min (MV 20): 10mw/cm2, 35 GHz;
- **Group B**: PEMFs for 30 min: 8mw/cm2, 75 Hz. Two sets of red and white alternating electrodes were placed on the skin of the upper and lower edge of the patella;
- **Group C**: MW for 40 min (MV 40): conditions were the same as that of group A;
- **Group D**: US for 10 min: 300mw/cm2, 1 MHz;
- **Group E**: LLLT for 10 min: wavelength of 810 nm, the output power of the laser ranged from 0 mW to 1000 mW and could be adjusted by a controller. The radius of laser spot was approximately 5 mm with a distance of 1 cm between the surface of the knee and the lens in all the treatments;
- **Group F**: SWD for 20 min: 5 mw/cm2, 27.12 MHz;
- **Group G**: the normal group (NORMAL). These rabbits did not undergo surgery to induce OA or receive treatment;
- **Group H**: the model group (MODEL). These rabbits underwent surgery for induction of knee joint OA, but did not receive any treatment.

2.4. Histological analysis

Cartilaginous tissue was prepared by staining 5 μm sections with Hematoxylin and Eosin (H&E). The sections were mounted with neutral balsam and morphology of the tissues was observed by light microscopy. Sections were scored using a modified Mankin scoring system [16]. The distal femur of the cartilage tissue from the right knee of each rabbit had been previously evaluated.

2.5. Detection TNF-α serum levels

After 10 days of treatment, serum from all experimental animals was collected. The concentration of TNF-α in the serum was detected by an enzyme immunoassay (ELISA) using a TNF-α kit, purchased from Kemei Biological Technology Co., LTD (Peking, China; batch number QRCT-301332322133EIA\(,\)UTL). The assays were performed according to the manufacturer’s instructions.

2.6. Immunohistochemistry assessment of caspase-3 and caspase-8

To investigate the level of caspase-3 and caspase-8 protein expression, immunohistochemistry assessment was conducted on the cartilaginous tissue from the rabbit knees. Tissues were prepared in 5 μm sections. The commercial kits for immunohistochemistry assessment of caspase-3 and caspase-8 were purchased from Boster Biological Technology Co., LTD (Wuhan, Hubei, China); the primary monoclonal antibody was used at a dilution of 1:150. An immunohistochemical SP assay was performed following the manufacturer’s instructions. The digital images were captured by an optical microscope with a 400x magnification. Cells positive for caspase-3 or caspase-8 expression appeared brown. The expression levels of the caspases were calculated using the image processing software, Image-Pro Plus 6.0 (Media Cybernetics Co. USA), and the mean densities (MD) of caspase-3 or caspase-8 expression were determined.

2.7. TUNEL assay to observe Apoptosis of Chondrocytes

The degree of Chondrocyte apoptosis was measured by a fluorometric TUNEL system (G3250, Promega Co., USA). Cartilaginous tissue was prepared in 5 μm sections and stained with TUNEL following the manufacturer’s instructions. Images of the tissues sections were immediately captured under the fluorescence microscope at wavelengths of 520 ± 20 nm and > 620 nm. The apoptosis ratio was obtained using the image processing software, Image-Pro Plus 6.0.

2.8. Statistics

Statistical analysis was completed using the statistical software program, SPSS 17.0 (SPSS Co. USA). One-way ANOVA was used to assess the statistic differences among values obtained from each of the eight treatment groups. Measurement values from the assessment of the eight groups were expressed as the mean ± SD (standard deviation). Differences were considered to be statistically significant at P < 0.05.

3. Results

3.1. The appearance of cartilage sections by HE staining

In Fig. SI (see the supplementary material associated with this article online), we found that normal rabbit cartilage (Group G) appeared as a thick layer of cartilage neatly arranged in a grid-like pattern. In the model group (Group H), the
cartilage layer was thinner and contained fewer cartilage cells; these cells appeared in a less ordered structure. After treatment, the number of cartilage cells increased and the cells re-established an ordered pattern. The modified Mankin scores of the MW for 20 min, PEMFs, MW for 40 min, US, LLLT, SWD, NORMAL and MODEL groups (Group A, B, C, D, E, F, G and H) were respectively shown in Table 1. The scores of the groups that underwent the 6 physical intervention factors (A, B, C, D, E and F) were significantly lower than those of MODEL group (Group H) (P<0.01).

3.2. Post-intervention serum TNF-α level in all groups

TNF-α serum levels in the samples evaluated were compared with baseline levels. The serum TNF-α (pg/ml) levels in the model group and the 6 physical factor groups were increased compared with that in the normal group (Group G) (Fig. 1). The level of TNF-α in the PEMFs group (Group B) and the US group (Group D) were significantly lower than that in the model group (Group H) (P<0.05) and were similar to that of the normal group (Group G) (Table 2).

3.3. Positive expression of caspase-3 and caspase-8 in articular cartilage

Immunohistochemistry analysis revealed the positive expression of caspase-3 (Fig. 2) and caspase-8 (Fig. 3) in the articular cartilage in each of the eight groups. Differences in the average optical density of caspase-3 (Table 3) and caspase-8 (Table 4) were observed among the eight groups. Caspase-3 and caspase-8 expression levels in the 6 intervention groups were increased compared to that of the normal group, but were decreased when compared to that of the model group. The expression of caspase-3 in the groups treated with PEMFs, MW40 and US were significantly lower than that of the MODEL group (P<0.05) (listed in the Table 3). However, the expression of caspase-8 in the groups intervened by MW for 40 min, US and LLLT were significantly lower than that of the MODEL group (listed in the Table 4).

3.4. Apoptosis of chondrocytes

It can be seen in Fig. 2 that apoptosis of chondrocytes occurred in each of the eight groups. Compared to the NORMAL group, the 6 intervention groups had an insignificantly higher ratio of chondrocytes apoptosis (P<0.05). However, in comparison with the MODEL group, all of intervention groups, groups A-F, had a significantly lower ratio of chondrocytes apoptosis (P<0.01), with respect to the alphabetical ordering of the group (listed in the Table 5).

3.5. Scoring of each intervention factor

For quantitative analysis of the effect each of the 6 treatments had on OA, the individual intervention factors were graded and the total score for each intervention factor is shown in Table 6. The lower values indicate more significant differences as compared to the model group, suggesting that the particular treatment was more effective than others. This study demonstrated that exposure to PEMFs (scores of 26), MV (MW) for 40 min (scores of 18), US (scores of 26) and LLLT (scores of 16) were all effective at improving OA in the knee joints of the rabbits, whereas the effects of MW for 20 min (scores of 9) and SWD (scores of 10) treatments were not evident (Tables 1–6). By establishing a grading system to evaluate the 6 treatments analyzed in this study, we can conclude that these treatments can be classified as ineffective, effective, or inconsistent at improving OA.

4. Discussion

In this study, we evaluated the effects of six intervention factors on OA in the knee by using four indices: histological evaluation of the articular cartilage by H&E staining, analysis of serum TNF-α levels by ELISA, assessment of caspase-3 and caspase-8 expression by immunohistochemistry and apoptosis of chondrocytes by TUNEL. According to the graded scores based on the four indices (shown in Table 6), PEMFs and US were confirmed as the two most optimal physical intervention factors, whereas MW for 20 min and LLLT were confirmed as feasible but non-optimal physical intervention factors. The resulting effects of MW for 20 min and SWD were inconclusive for treating OA in the knee.

4.1. Optimal treatments: PEMFs and US

PEMFs had been confirmed to be a safe and effective physiotherapy for OA, leading to increased cartilage weight and providing a greater surface as a stent for supporting bone formation [17]. Ryaby et al. had suggested that magnetic fields could promote the short-term secretion of insulin-like growth factor II and could result in long-term effects of electromagnetic stimuli due to the development of a dependent mechanism involving growth factors as a dependent mechanism [18]. TNF-α concentration was significantly lower in PEMFs-treated animals compared to the control group. It is speculated that treatment with PEMFs could largely prevent articular cartilage degeneration by providing a protective effect on the whole articular cartilage OA [19]. Of the proinflammatory cytokines, TNF-α is the most prominent in the early stages of inflammation in OA and can stimulate the production of these cytokines, thus causing further OA.
Table 2
Comparison of TNF-α (pg/ml) serum levels in all kinds of physical factors groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>MW20</th>
<th>PEMFs</th>
<th>MW40</th>
<th>US</th>
<th>LLLT</th>
<th>SWD</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD</td>
<td>117.5 ± 23.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>146.9 ± 42.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>124.5 ± 52.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>134.4 ± 35.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>129.8 ± 38.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>152.3 ± 32.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>154.8 ± 48.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>182 ± 63.8</td>
</tr>
</tbody>
</table>

*P < 0.05*.

<sup>a</sup> Compared to the Normal group, P = 0.304, P = 0.774, P = 0.507, P = 0.605, P = 0.176, P = 0.193, respectively

<sup>b</sup> Compared to the Model group, P = 0.210, P = 0.021, P = 0.062, P = 0.030, P = 0.233, P = 0.327, respectively

of other cytokines, such as IL-8, IL-6, and leukaemia inhibitory factor, leading to accelerated damage of articular tissue [20]. Moreover, Lopez-Armada MJ et al. indicated that TNF-α can affect chondrocytes apoptosis and is related to the caspase family in articular tissue [21]. Further evidence has confirmed that apoptosis may play an important role in the development of OA [22]. In contrast with normal cartilage in animal models and humans, cartilage stricken with OA has a higher ratio of chondrocytes

Fig. 2. Caspase-3 postive expression in the each group, ×400 magnification. Arrows indicate positive cells. A: millimeter waves for 20 min; B: pulse electromagnetic fields for 30 min; C: millimeter waves 40min; D: ultrasound for 10 min; E: Low level laser therapy for 10 min; F: Ultrasound Wave Diathermy for 20 min; G: the normal control group without any treatment; H: the model group only produce OA of knee joint model without any interferent (n = 8 in each group).
apoptosis and may result in a contribution to cartilage matrix loss [23]. The caspase family of proteins had been confirmed to play key roles in apoptosis signalling, especially caspase-3, which was considered to be the executor of apoptosis [24]. Molecular studies have reported that expression of caspase-3 and caspase-8 were observed in human osteoarthritic cartilage and in animal model [25]. Caspase-8 is activated by membrane death receptors or by cytochrome C, which is released by the mitochondria;

Fig. 3. Caspase-8 positive expression in each group. ×400 magnification. Arrows indicate positive cells. A: millimeter waves for 20 min; B: pulse electromagnetic fields for 30 min; C: millimeter waves 40 min; D: ultrasound for 10 min; E: Low level laser therapy for 10 min; F: Ultrasound Wave Diathermy for 20 min; G: the normal control group; H: the OA model group (n: 8 in each group).

Table 3
Comparison of mean density of all kinds of physical factors on expression of caspase-3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>MW20</th>
<th>PEMFs</th>
<th>MW40</th>
<th>US</th>
<th>LLLT</th>
<th>SWD</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD</td>
<td>158.5 ± 6.1</td>
<td>167.0 ± 5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>160.3 ± 9.4&lt;sup&gt;b&lt;/sup&gt;***</td>
<td>163.8 ± 8.7&lt;sup&gt;b&lt;/sup&gt;***</td>
<td>162.6 ± 9.9&lt;sup&gt;b&lt;/sup&gt;***</td>
<td>165.0 ± 8.8&lt;sup&gt;b&lt;/sup&gt;***</td>
<td>163.7 ± 8.6&lt;sup&gt;b&lt;/sup&gt;***</td>
<td>170.7 ± 7.3</td>
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</table>

<sup>a</sup> Compared to the Normal group, P= 0.027, P= 0.688, P= 0.199, P= 0.298, P= 0.083, P= 0.226, respectively.

<sup>b</sup> Compared to the Model group, P= 0.232, P= 0.008, P= 0.045, P= 0.014, P= 0.058, P= 0.058, respectively.
Comparison of positive ratio of all kinds of physical factors on expression of chondrocyte apoptotic index.

Table 5
Comparison of mean density of all kinds of physical factors on expression of caspase-8.

Table 6
Comparison of positive ratio of all kinds of physical factors on expression of chondrocyte apoptotic index.

Table 7
Grading of 6 physical factors in the expression of caspase-3, caspase-8 levels and the ratio of Chondrocytes Apoptosis mean (comparing with that of model group).

in turn, caspase-8 activates the downstream effector, caspase-3 [26].

US transforms electrical energy into an acoustic waveform that is subsequently converted into heat through tissues of varying resistance to treat musculoskeletal disorders, such as tendinitis, tenosynovitis, bursitis and OA [27]. Huang et al. found that US can affect the release of inflammatory mediators in addition to enhancing microcirculation, the rates of protein synthesis and the repair of articular cartilage [28]. Although effective ultrasound treatment can reduce pain and improve the functional ability of people with OA of the knee, the quantitative results were inconclusive when applied to the treatment of symptomatic OA of the knee [29]. According to the grading score system described in the present study, US obtained the highest score and was regarded as an optimal treatment. Furthermore, the highest score reflected the ability of US treatment to effectively improve all of the 4 indices investigated in this study, which was consistent with a previous report [27–29]. In sum, our hypothesis was supported by the observation that both PEMFs and US gained high scores, which is can be regarded as a critical index of optimal treatment for OA.

4.2. Secondary optimal treatments: MW 40 min, LLLT

More than 50 diseases and conditions have been successfully treated with MW [30]. In previous studies, the most commonly reported indications for MW were a variety of diseases associated with pain relief [31]. MW treatment can inhibit apoptosis of chondrocytes through the p38 MAPK pathway [32]. The results of the grading scores of MW for 40 min indicated that this treatment was regarded as a feasible but non-optimal treatment for OA. Our results were consistent with previously published research [33–35]. In sum, hypothesis was validated because both MW for 40 min and LLLT were feasible but non-optimal treatment for OA.

4.3. Inconclusive treatments: MW for 20 min and SWD

The previous study regarding the length of exposure did not include data on the outcome of MW treatment after shorter or longer exposures [36]. In the present study, the effect of MW for 40 min on OA received a higher grading score than MW for 20 min, suggesting that a longer exposure of MW was important in the treatment for OA of the knee. Marks et al. reported that that SWD was commonly used in clinical treatment of knee OA, but the effect of SWD on improving knee OA was inconclusive in this study [37]. A recent review suggested that further study on the benefits of undergoing knee OA treatments that yield inconclusive would be addressed in the future [38].

In this study, using the levels of histological morphology, immunohistochemistry and apoptosis of chondrocytes, and expression of caspase-3 and caspase-8 we confirmed that four physical intervention factors, PEMFs, MW for 40 min, US and LLLT, improve the health of knee joint cartilage rabbit with OA. In particular, treatment with PEMFs and US resulted in the greatest improvements across the indices we examined. Our study suggested that these two physical intervention factors may be used for clinical management of OA of the knee.

Disclosure

We declare that we have no conflict of interest statement.
Authors' contributions

Hua Guo conceived the study, performed the statistical and data analysis and drafted the manuscript. Qinglu Luo, Jinglong Zhang and Haidan Lin participated in experimental design of the study and data acquisition. Chengqi He conceived the study and is the primary author of the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary material

Supplementary material (Figs. S1–S6) associated with this article can be found at http://www.sciencedirect.com, at Appendix A. Supplementary material

References