Improvement of total antioxidant status, a possible bioeffect of the ultrasound therapy - a pilot study

Creşterea statusului antioxidant total, un posibil bioefect al ultrasonoterapiei - studiu pilot

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ABSTRACT

The importance of the oxidative stress in the pathogenesis of osteoarthritis (OA) is well recognized at present. Nowadays, the ultrasound (US) therapy is successfully used to treat OA, but the precise mechanism of action is not yet completely understood. The present study monitored the effects of the US therapy over the clinical and functional parameters and the antioxidant/oxidant status, in patients with knee OA. A group of 12 patients diagnosed with knee OA were treated with US therapy (0.5 watt/cm^2, for 5 minutes, during 10 sessions, 5 times/week). Before and after the US therapy the Womac score (WS), the Lequesne index (LI), and the total antioxidant status (TAS), the total oxidant status (TOS), superoxide dismutase (SOD), reduced/total glutathione and malonyldialdehyde (MDA) levels were measured. A statistically significant improvement ($P < 0.05$) was observed regarding both studied clinicaly parameters (WS, LI) and TAS. However, there were no significant differences between levels of TOS, SOD, reduced/total glutathione and MDA before and after US therapy. The increase of TAS could represent one of the possible mechanism through which the US therapy exerts its favorable effects in OA. Further studies will be required to investigate the biochemical nature of TAS increase.

Keywords: *ultrasound therapy, arthrosis, antioxidant status.*

REZUMAT

Importanţa stresului oxidativ în boala artrozică este pe deplin recunoscută în prezent. Ultrasonoterapia este utilizată cu succes în tratamentul bolii artrozice fără a se cunoaşte cu precizie mecanismul ei de acţiune.

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Studiul de faţă a evaluat efectul ultrasonoterapiei asupra parametrilor clinico-funcţionali şi asupra statusului antioxidant/oxidant la pacienţii cu gonartroză. Un număr de 12 pacienţi diagnosticaţi cu gonartroză au fost supuşi ultrasonoterapiei 0,5 watt/cm^2, timp de 5 minute, 10 şedinţe (5 expuneri/săptămână). Pacienţii au fost evaluaţi înainte de tratament şi la încheierea acestuia prin determinarea scorului Womac (WS), indicelui Lequesne (LI) precum şi prin măsurarea statusului antioxidant total (TAS), statusului oxidant total (TOS), superoxid dismutazei (SOD), raportului glutation redus/glutation total si malondialdehidei (MDA). A fost observată o îmbunătăţire semnificativă statistic ($p < 0,05$) a parametrilor clinici (WS, LI) şi TAS. În schimb, nu s-au întregistrat modificari semnificative statistic ale TOS, SOD, glutation redus/glutation total şi MDA în urma ultrasonoterapiei. Creşterea TAS poate reprezenta unul din mecanismele prin care ultrasonoterapia îşi exercită efectele favorabile în tratamentul bolii artrozice. Sunt necesare noi determinări pentru a investiga bazele biochimice ale creşterii TAS.

Cuvinte-cheie: *ultrasonoterapie, artroză, status antioxidant.*

Introduction

Osteoarthritis (OA) is one of the most common chronic diseases and a major cause of joint disability in elderly population (1). It affects all joint tissues, but cartilage degradation is the most important phenomenon.

Chondrocytes play a key role in cartilage degradation by releasing metalloproteinases and reactive oxygen species (ROS). The main ROS produced by chondrocytes are the nitric oxide (NO) and the superoxide anion ($O_2^{\cdot -}$), that both generate secondary radicals. The $O_2^{\cdot -}$ and NO production are stimulated by many factors, such as interleukin (IL)-β and tumor necrosis factor (TNF)-α (1). Chondrocytes have a variety of antioxidant defense mechanisms. These include a well-coordinated enzymatic antioxidant system, essentially formed by superoxide dismutases (SODs), catalase (CAT) and glutathione peroxidase (GPX). Oxidative stress results when the amount of ROS exceeds the antioxidant capacity of the cells. Ultrasound may possess significant healing benefits useful for the OA management, by facilitating the protein synthesis and the angiogenesis, and by promoting orderly collagen deposition and inflammation resolution (2).

Recently, it has been admitted that ultrasounds are able to increase the type II collagen synthesis in joint cartilage, possibly via the activation of chondrocytes and induction of type II collagen mRNA expression (3) and to stimulate the *in vitro* cartilage tissue repair; it was observed that chondrocytes previously treated with IL-1β have a diminished level of messenger RNA corresponding to matrix metalloproteinase (MMP)-1, but show an increased level of tumor growth factors (TGF)-β1 and -β3, and glycosaminoglycans (4). There are also studies showing the increased efficacy of hyaluronic acid in association with ultrasounds (5). Few studies though show contradictory results concerning the relationship between the ultrasounds and the oxidative stress. The studies performed in order to verify the ultrasounds effect on the oxidative stress (6), as well as the possibility of using the ultrasound-generated free radicals to influence the life span of certain cell populations, showed an increase in free radicals synthesis after ultrasounds exposure (7, 8). The studies on myocardial tissue injuries caused by the ischemia–reperfusion phenomenon, showed a decrease of the oxidative stress after ultrasounds exposure (9, 10). Up to now, no study has focused on ultrasound therapy and its effects on oxidative stress in OA.

Our research purpose was to evaluate the effects of the ultrasound (US) therapy over the clinical and functional parameters, and the antioxidant/oxidant status, in patients with knee OA.

Patients and methods

12 patients from the outpatient department of the Rehabilitation Hospital Cluj-Napoca were included in a pilot prospective cohort study (written informed consent was obtained from all subjects). The local ethics committee approved the study protocol.

Inclusion criteria were the following: age between 40 and 60, knee osteoarthritis on a frontal knee radiography (according to American College of Rheumatology criteria, stage 1 or 2 - modified Kellgren-Lawrence classification), normal erythrocyte sedimentation rate and C-reactive protein values, patient's ability to follow study instructions, no physiotherapy and no oxidative stress modifying treatments during the past 6 months.

Exclusion criteria were the following: smoking patients, chronic alcohol consumers, pregnant women, professional exposure to polluting factors or associated pathologies known to increase the oxidative stress and associated pathologies contraindicating ultrasound therapy.

Interventions. All subjects were treated with continuous ultrasonic waves (850±5% KHz frequency and 0.5 watt/cm^2) applied with a transducer which had an effective radiating area of 6.4 cm^2 (Misonic 12M, Misonix –Romania) on the OA knee for 5 minutes, in 10 sessions (5 times/week). US therapy was then applied using an aqueous gel as a coupling medium, by the same therapist stroking the applicator in circular movements. The transducer head was applied to the therapy region at right angles to ensure maximum absorption of the ultrasounds energy. The treatment area was 25 cm^2 and extended to both patello-femoral and tibio-femoral borders of the target knee on both the lateral and medial margins, avoiding the patella.

Concomitant use of corticosteroids, chondro-protective agents, analgesics and non-steroidal anti-inflammatory drugs, was not permitted throughout the study.

Main outcome measures. Before and after the US therapy the following clinical and biological parameters were measured: the Womac score (WS), the Lequesne index (LI), the total antioxidant status (TAS), the total oxidant status (TOS), superoxide dismutase (SOD), reduced/total glutathione and malonyldialdehyde (MDA).

Clinical scores

The WOMAC score (WS) was used to measure pain, stiffness and physical function. WS were recorded on a Likert scale of 0–4, where 0 = no pain/limitation; 1 = mild pain/limitation; 2 = moderate pain/limitation; 3 = severe pain/limitation; and 4 = very severe pain/limitation. Maximum scores for stiffness, pain and physical function were 8, 20 and 68 respectively with a total score of 96 (11). WOMAC pain scores (0–20), stiffness scores (0–8), and physical function scores (0–68) were calculated separately.

The Lequesne index (LI) included 11 questions regarding knee discomfort, endurance of ambulation, and difficulties in daily life. The disability may be graded as follows: 14 points = extremely severe; 11–13 points = very severe; 8–10 points = severe; 4–7 points = moderate; 1–3 points = mild (12).

Samples

Fasting venous blood was withdrawn, before and after the US therapy, into 3 different tubes (with heparin, K_3EDTA, and without anticoagulant), for plasma (centrifugation at 2,500 rpm for 10 minutes), washed erythrocytes (for SOD activity) and serum, which were stored at -80°C until analysis.

Biochemical parameters measurement

Superoxide dismutase (SOD) activity in erythrocytes

The whole blood (0.5 ml), collected into K_3EDTA tubes, was centrifuged for 10 minutes at 2,500 rpm and plasma was removed. The erythrocytes were washed three times with 0.9% NaCl solution, lysed by addition of up to 2.0 ml cold re-distilled water, followed by vigorous vortex-mixing and storage at +4°C for 15 minutes. The lysate was diluted with 0.01 mol/l phosphate buffer pH 7.0, so that the percent of inhibition falls between 30% and 60%.

SOD activity was measured with a RANSOD kit (cat. No. SD125; RANDOX Labs., UK). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-indophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity was then measured by the degree of inhibition of this reaction. The activity was mesured at 37^{238}_{92}C on

a Cobas Mira Plus (Roche), and the optical density was monitored at 505 nm. The unit of activity is defined as the amount of enzyme that inhibits the rate of the formazan dye formation by 50%. The activity of SOD was expressed in U/gHb.

Hemoglobin concentration (in whole blood – K_3EDTA)

Hemoglobin concentration was determined as cyanmethemoglobin by using Drabkin's method (13).

Reduced and total glutathione (in heparinized whole blood)

Reduced glutathione (GTr) and total (GTt) from whole blood were evaluated by using isocratic RP-HPLC with precolumn derivatisation and fluorimetric detection (excitation at 385 nm and emission at 515 nm). Sample preparation (the reduction step to convert oxidized glutathione into reduced glutathione, followed by protein precipitation and subsequent precolumn derivatisation) and chromatographic separation were performed according to the recommendations of use provided with the Chromsystems kit (Chromsystems Instruments and Chemical GmbH, Germany). The results were expressed in μmol glutathione/L.

Malonyldialdehyde (MDA) (in plasma – K_3EDTA)

Plasma MDA was estimated by using isocratic RP-HPLC system with fluorescence detector (excitation at 515 nm and emission at 553 nm) and Chromsystems kit (Chromsystems Instruments Chemicals GmbH, Germany). Sample preparation (protein precipitation followed by derivatisation) was made following the kit instructions. Results were expressed in μM MDA/L.

Total antioxidant status (TAS) in serum

TAS was measured according to the recommendations of use provided with the RelAssay Diagnostics kit (Turkey). The antioxidants in the sample reduce the dark blue-green colored ABTS radical to colorless reduced ABTS. The change of absorbance at 660 nm is related to the total antioxidant level in the sample. The assay is calibrated with a stable antioxidant standard solution, traditionally known as Trolox Equivalent (a vitamin E analogue). Results were expressed in μM Trolox/L.

Total oxidant status (TOS) in serum

TOS was evaluated by using RelAssay kit (RelAssay Diagnostics, Turkey). Oxidants (peroxides) present in the sample oxidize the ferrous ion–chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundantly present in the reaction medium. The ferric ion produces a colored complex with chromogen in an acidic medium. The color intensity, which can be measured spectrophotometrically (λ = 530 nm), is related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in μM H_2O_2/L.

Oxidative stress index (OSI)

OSI, an indicator of oxidative stress level, was calculated using the formula:

OSI = TOS (μM H_2O_2/L) x 100 / TAS (μM Trolox/L) (14)

Statistical analysis

For the statistical analysis, the SPSS software version 14 was used. The paired t-test was used to compare results obtained before and after the treatment. P values < 0.05 were considered statistically significant.

Results

The mean ± SD values of Womac score (WS) and the Lequesne index (LI), before and after the US therapy are reported in *Table 1*.

The WS values showed a statistically significant improvement ($P < 0.05$), both for pain and joint function and stiffness.

The LI showed a statistically significant decrease ($P < 0.05$) after the US therapy, which indicates an improvement of joint function and decrease of joint discomfort.

The mean ± SD values of TAS, TOS, OSI, SOD, GTr, GTr/GTt and MDA, before and after US therapy are presented in *Table 2*. There was a statistically significant increase of

Table 1. The mean ± SD values of Womac score (WS) and the Lequesne index (LI), before and after the US therapy

Parameters	Before US therapy	After US therapy	P values
WS	56.2 ± 17.14	39.08 ± 15.50	<0.05
WS-pain	11.67 ± 3.62	7.75 ± 3.54	<0.05
WS-stiffness	4.92 ± 1.73	3.17 ± 1.46	<0.05
WS-function	40.33 ± 12.33	28.17 ± 11.20	<0.05
LI	7.87 ± 2.42	5.41 ± 2.72	<0.05

Table 2. The mean ± SD values of TAS, TOS, OSI, SOD, GTr, GTr/GTt and MDA, before and after US therapy

Parameters	Before US therapy	After US therapy	P values
TAS (μM Trolox/L)	1589.42 ± 216.07	1824.50 ± 262.20	0.003
TOS (μM H_2O_2/L)	8.894 ± 5.891	10.032 ± 5.691	0.257
OSI	553.407 ± 314.377	554.231 ± 289.237	0.988
SOD (U/g Hb)	1031.541 ± 88.585	1026.375 ± 111.99	0.900
GTr (μM/L)	1025.517 ± 218.111	1011.955 ± 190.601	0.624
GTr/GTt	0.834 ± 0.052	0.856 ± 0.039	0.165
MDA (μM/L)	0.255 ± 0.040	0.250 ± 0.038	0.784

TAS value (1824.50 ± 262.20 vs. 1589.42 ± 216.07 μM Trolox/L; P = 0.003), but only a slight increase (without statistical signification) for TOS after the US therapy. The levels of other biochemical parameters were not changed.

Disscusion

Osteoarthritis (OA), the most common disabling condition in the Western world, is a disease affecting the articular system as a whole: the joints, including the cartilage, the subchondral bone, the synovial capsule and membrane and the periarticular (connective and muscular) tissues. The metabolic and structural changes in the articular cartilage are thought to play a leading role in the initiation and the progression of the disease process. Adult articular cartilage is an avascular and thus hypoxic tissue. The implications of this hypoxic environment are hardly understood at molecular level. Additionally, the role of changes in oxygen levels during the process of cartilage degeneration seems to be of great interest because oxygen can be processed into the so-called reactive oxygen species (ROS) that are involved, both, in intracellular signaling and, thus, cell physiology, but also in cellular destruction. When ROS production exceeds the antioxidant capacity of the cell, an "oxidative stress" occurs that contributes to structural and functional cartilage damage (15).

US therapy is one of the several physical therapies suggested for the management of pain and loss of function in OA. Despite its widespread use, the efficiency of therapeutic US in knee OA has only been subjected to limited studies and the results of these studies are somewhat conflicting.

The systematic review (up to February 2009) with meta-analysis without language limits of Loyola-Sánchez et al. concluded that US could be efficient for decreasing pain and improving physical function in patients with knee OA (16).

A recent study of Tascioglu et al. concluded that only the patients treated with pulsed US demonstrated significant improvements compared to the patients in the placebo group. No superiority was observed for continuous US over placebo (17).

Our pilot study, for the first time in Romania, investigates not only the therapeutic effect of US but also the antioxidant/oxidant status before and after US therapy.

The Womac score showed a statistically significant improvement ($P < 0.05$), both for pain and joint function and stiffness. These results are consistent with the results of a controlled randomized double-blind placebo study pulished by Ozgönenel et al., who also uses continuous US, at 1 MHz frequency or 1 watt/cm^2 power (18).

The Lequesne index showed a statistically significant decrease ($P < 0.05$) after the US therapy, which indicates an improvement of joint function and a decrease the articular discomfort.

The improvement of the Lequesne index and pain relief after continuous US were also reported by Huang (19) and Eyigör (20), but, because they used US treatment in association with isokinetic exercises, it is difficult to say whether the US or the isokinetic exercise were responsible for these effects.

As far as we know, this is the first study to prove the efficiency of the US therapy, as an exclusive treatment, in the improvement of the Lequesne index.

According to other studies (2, 21) these results prove the beneficial effects of the US therapy.

The TAS showed a statistically significant increase ($P = 0.003$) after the US therapy. This increase could be a compensatory response to increased oxidative stress. This hypothesis is sustained by the small increase of TOS ($P = 0.257$). It was observed a slightly increase of the GTr/GTt ratio ($P = 0.165$), without a significant alteration of the GTr value. On the other hand, the TAS improvement was not correlated with SOD, glutathione and MDA evolution. Against all odds, SOD activity and reduced glutathione showed a tendency to decrease, but without statistical significance.

The reason for these small differences could be the relatively small sample size, the influence of diet (22) and lifestyle (23) over the ROS levels, and the low levels of ROS generated after US.

Conclusion

This pilot study evaluates for the first time the oxidative/antioxidative balance showing an increase of total serum antioxidant capacity after US therapy.

In the light of our findings, it is possible to conclude that US therapy influences oxidative stress status. However, the nature of this link, and whether it is direct or indirect, remains to be explored. The limitation of our study is the relatively small sample size that could limit our ability to generalize the results to US therapy in general.

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