



Article Ultrasound Protects Human Chondrocytes from Biochemical and Ultrastructural Changes Induced by Oxidative Stress

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Abstract: The aim of the study was to assess the effects of therapeutic ultrasound (US) on oxidative stress (OS)-induced changes in cultured human chondrocytes (HCH). For this, monolayer HCH were randomized in three groups: a control group (CG), a group exposed to OS (OS group), and a group exposed to US and OS (US-OS group). US exposure of the chondrocytes was performed prior to OS induction by hydrogen peroxide. Transmission electron microscopy (TEM) was used to assess the chondrocytes ultrastructure. OS and inflammatory markers were recorded. Malondialdehyde (MDA) and tumor necrosis factor (TNF)-α were significantly higher (p < 0.05) in the OS group. Finally, in the US-OS group MDA and TNF-α were lower than in CG, but without statistical significance. TEM showed normal chondrocytes in CG. In the OS group TEM showed necrotic chondrocytes and chondrocytes with a high degree of vacuolation and cell organelles damages. In the US-OS group the chondrocytes from biochemical (lipid peroxidation, inflammatory markers synthesis) and ultrastructural changes induced by OS and could stimulate autophagosomes development.

Keywords: ultrasound; chondrocytes; assessment; oxidative stress; TNF- α ; autophagosomes

1. Introduction

Osteoarthritis (OA) is the most common cause of chronic disability in older people [1]. An increase in oxidative stress (OS) and a decrease in autophagy in aging cartilage are the main factors involved in OA pathogenesis in the elderly [2].



Citation: Ungur, R.A.; Muresan, A.; Olteanu, D.E.; Florea, A.; Ciortea, V.M.; Irsay, L.; Borda, I.M.; Codea, R.A.; Ober, C.A.; Bâlici, Ș.; et al. Ultrasound Protects Human Chondrocytes from Biochemical and Ultrastructural Changes Induced by Oxidative Stress. *Appl. Sci.* 2022, *12*, 2334. https://doi.org/10.3390/ app12052334

Academic Editor: Elena Amaricai

Received: 19 December 2021 Accepted: 18 February 2022 Published: 23 February 2022

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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Although the entire joint structure may be affected in OA, the main structural changes occur in the cartilage. These changes include extracellular matrix (ECM) damage and chondrocyte death by apoptosis, necrosis, or chondroptosis. Proinflammatory cytokine excess and OS underlie these structural changes, and autophagy deficiency allows chondrocytes and ECM's defects to accumulate in aging cartilage.

In OA cartilage, chondrocytes synthesize reactive oxygen species (ROS) and reactive nitrogen species (RNS) in a large amount and have impaired antioxidant defense, both leading to OS. As a result, OA patients' plasma and synovial liquid contain a considerable amount of oxidative products: malondialdehyde (MDA) and carbonylated protein [3–5]. ROS and RNS promote ECM degradation [6], impair chondrocytes ultrastructure [7], and may induce chondrocytes death [8,9].

In older people, the persistent ROS production, the subsequent mitochondrial dysfunction, and the dysregulation of the glutathione antioxidant system increase OS and get chondrocytes more susceptible to death [9,10] and cartilage more susceptible to OA development.

Moreover, chondrocytes ROS upregulate the expression of proinflammatory genes and stimulate the synthesis of the main proinflammatory cytokines [11,12]. Furthermore, proinflammatory cytokines stimulate ROS synthesis [13,14] and generate a vicious circle of "OS-inflammation" that perpetuates cartilage's damage [15].

The main cytokines involved in OA pathogenesis are interleukin (IL)-1 β and tumor necrosis factor (TNF)- α .

TNF- α is a common member of OA and senescence-associated secretory phenotype (SASP) that is present in senescent chondrocytes. SASP is characterized by proinflammatory cytokines, ROS, RNS, and matrix metalloproteinases (MMPs) synthesis, all involved in OA joint damage [16,17].

TNF- α inhibits proteoglycan synthesis, suppresses link protein and type II collagen expression in chondrocytes [18,19], and induces the expression of MMPs, the main proteolytic enzymes involved in ECM degradation: MMP1 (interstitial collagenase), MMP3 (stromelysin 1), and MMP13 (collagenase 3), and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-5 [20,21].

TNF- α can also induce nitric oxide (NO), prostaglandin E₂ (PGE₂), and IL-6 synthesis and activate c-Jun N-terminal kinases (JNKs), which are responsible for upregulating proapoptotic genes and inactivating antiapoptotic proteins Bcl-2/Bcl-xL in OA chondrocytes [22–25].

The proapoptotic and damaging ECM action of TNF- α can be counteracted by antimicroRNA-30b, by enhancing autophagy [26].

Autophagy, known as type II programmed cell death, is an important mechanism that protects cartilage homeostasis. Moreover, an antiapoptotic role was also attributed to autophagy [27]. There are three types of autophagy: chaperone-mediated autophagy (CMA), macroautophagy, and microautophagy. Usually the term autophagy indicates the macroautophagy.

Previous studies demonstrated that age-related OA is associated with compromised autophagy and an increase in chondrocytes apoptosis [2].

Although OS can activate CMA that contributes to the efficient removal of oxidized proteins [28], this phenomenon is less important quantitatively than macro- and microautophagy, because only particularly soluble cytosolic proteins and not organelles can be degraded by CMA [29]. In living mammalian cells the main role belongs to macroautophagy, which is able to break down the damaged cell organelles and to provide the integral parts of these for new cell organelles synthesis.

In the elderly, the effects of OS on the insulin receptor-signaling pathway have a critical role in macroautophagy's decrease [29]. In NIH3T3 cells (murine fibroblast line) the OS, induced by hydrogen peroxide (H_2O_2), inhibits the Adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling pathway responsible for autophagocytosis [30]. In chondrocytes AMPK activation leads to activation of the tuberous sclerosis complex, inhibits mTOR activity, and stimulates autophagy that recycles cells nutrients and provides ATP synthesis

substrate [31]. In some cells, the senescence phenotype induced by OS may be accompanied by an increase in autophagic structures, but the lysosomal dysfunction impairs autophagic flux and makes it inefficient [32]. On the other hand, excessive OS and excessive autophagy can induce chondrocytes death in articular cartilage [33,34].

 H_2O_2 induces OS in chondrocytes and mediates their senescence via the p53-p21-pRb pathway, induction of caveolin 1 synthesis, and activation of p38 mitogen-activated protein kinase (MAPK) [35]. Because H_2O_2 has a stable structure, it was used in many studies to induce OS in cartilage or in chondrocytes' cultures [36].

Therapeutic Ultrasound (US) is an excellent option to avoid adverse events induced by non steroidian anti-inflamatory drugs in OA patients. US is used as a treatment option in OA for more than 50 years for reducing pain and improving functional articular parameters [37]. It has been shown that US stimulates chondrocyte metabolic activity [38], increases chondrocyte proliferation [39] and viability [40], and reduces proinflammatory cytokine synthesis in cartilage [41]. On the other hand, there are few studies on the US effect on OS, with inconsistent results. To date there is no study showing the effects of US on the OS level, chondrocytes ultrastructure, and autophagocytosis process.

The aim of this study was to assess the effects of US exposure on OS-induced changes in human chondrocytes (HCH). For that, we exposed HCH cultures to the sequenced action of US and H_2O_2 and assessed the ultrastructural changes of chondrocytes, and the synthesis of MDA—a marker of oxidative damage, and TNF- α , a very important cytokine for OA development, whose synthesis is stimulated by H_2O_2 [42].

2. Materials and Methods

2.1. Human Chondrocyte Culture

Primary human chondrocytes (HCH) were purchased from PromoCell (Heidelberg, Germany) and were cultured as monolayers in Petri dishes 38 mm in diameter (Nunclon Delta Surface, Nunc, Roskilde, Denmark), in chondrocytes growth medium (PromoCell, Heidelberg, Germany) at 37 °C, in a humidified atmosphere, 5% CO₂, to 80% confluence. Chondrocytes were divided into three groups (Figure 1):

- 1. control group—chondrocytes with no treatment;
- group exposed to OS (OS group)—chondrocytes exposed to H₂O₂;
- group exposed to US and OS (US—OS group)—chondrocytes exposed to US and H₂O₂.



Figure 1. Experimental set-up chart. (H₂O₂—hydrogen peroxide exposure, C—control group, OS—oxidative stress group, US-OS—ultrasounds—oxidative stress group, US device—ultrasound generator device).

These experiments were performed in triplicate, and the results are provided as mean values of three independent experiments.

2.2. US Exposure

Exposure to US (Figure 2) was performed using a Misonic 12M device (Misonix, Cluj-Napoca, Romania). Petri dishes were placed on the transducer of US device by means of the aqueous gel layer, Ultraschall gel (Dahlhausen & Co. GmbH, Köln, Germany), that prevented the loss of ultrasonic waves in the air environment. Chondrocytes from the US-OS group were exposed to the US at the frequency of 850 KHz and the intensity of 100 mW/cm² for 5 min, on 3 consecutive days before H_2O_2 treatment. Chondrocytes of the normal control group and those of the OS group were also placed on the transducer for 5 min, on 3 consecutive days, but with the power button of the US device in the OFF position.



Figure 2. Ultrasound exposure. (D₁—Petri dish diameter, D₂—transducer diameter, h—chondrocyte growth medium height, US device—ultrasound generator device).

The experimental model used by us involves generation of standing waves at the air—liquid interface, which can increase the absolute peak rarefaction pressure. As the amplitude reflection coefficient at the interface between chondrocyte growth medium and air is -0.9994 (close to -1), almost 100% of the energy is reflected here, according to Hensel. A standing wave with nodes and antinodes develops temporarily, resulting in a 200% amplitude increase in the antinodes position.

Due to the larger size of the Petri dish compared to the transducer, the additional reflections of the waves propagated on the outer walls of the Petri dish were eliminated, and an additional increase in pressure and intensity in the center of the Petri dish was avoided [43].

For exposure to US, the chondrocyte cultures were moved from the culture environment at 37 °C to the room temperature of 25 °C for a time interval of 5 min. During this period the temperature increase caused by the ultrasonic waves was canceled by the lower temperature of the room environment, so that at the end of the US exposure the temperature measured with the thermometer in the culture medium was 34.3 °C.

2.3. OS Exposure

OS was induced by treating HCH with H_2O_2 . H_2O_2 in a concentration of 100 µmoL/L was added to cell culture medium in the OS group, and in the OS-US group, 12 h after the last US exposure. This concentration resulted in a prooxidant environment [36], similar to that existing in OA, but allowed HCH to remain viable. After 30 min H_2O_2 was removed by washing with phosphate buffered saline (PBS).

2.4. Protein Assay

After H_2O_2 exposure, chondrocytes were incubated for 24 h in chondrocytes growth medium, and afterwards they were detached using a trypsin/EDTA solution. Then, after 3 washings with PBS and centrifugations, chondrocytes were treated with a lysis buffer containing Igepal-nonidet 1% (Sigma), 1% protease inhibitor complex (Sigma) in PBS, for

1 h, on ice. Cell extracts were centrifuged at $14,000 \times g$, for 30 min, at 4 °C. Supernatant was removed, and a fixed volume (50 µL) was used to determine the protein content by the Bradford method [44] (Biorad, CA, USA).

2.5. Quantification of MDA

MDA is a stable product of the reaction between high-energy radicals and lipids in the cell membrane [45]. MDA content was assessed using a spectrofluorimetrical method (Esterbauer, 1990) [46]: lysate obtained from 5×10^6 cells was used for MDA dosage. The method employed thiobarbituric acid (TBA) that stains MDA (2.5 mL H₂SO₄ 50 mM and 3 mL TBA 2 M in Na₂SO₄ 2M, boiled for 30 min in a water bath). After the staining reaction, the colored product was extracted with n-butanol; then, the organic phase was separated from the inorganic one by vigorous shaking and centrifugation. The absorbance of the colored dye was read spectrofluorimetrically. MDA values were expressed as nanomol per mg protein (nmol/mg prot). Three biological replicates were performed with three technical replicates per experiment.

2.6. Enzyme-Linked Immunosorbent Assay

Chondrocytes were cultured and stimulated as described above, and supernatant was collected after chondrocyte lysate was obtained. The release of TNF- α was assessed by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The optical density of each well was measured within 30 min, using a microplate reader set to 450 nm. TNF- α values were expressed as nanomol per mg protein (nmol/mg prot). Three biological replicates were performed with three technical replicates per experiment.

2.7. Transmission Electron Microscopy Assay

Chondrocytes aggregates were processed for transmission electron microscopy (TEM) according to the usual protocols (Hayat, 2000; Watt, 2003) [47,48]. The chondrocyte aggregates were prefixed with 2.7% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 M PBS for 2 h, washed four times with 0.1 M PBS, postfixed with 1% osmium tetroxide (Fluka, Buchs, Switzerland) in 0.15 M PBS for 1.5 h, and washed with 0.15 M PBS. Next, they were dehydrated in an acetone series (30–100%) and embedded in Epon 812 (Fluka). Ultrathin sections obtained using glass knives on a Bromma 8800 ULTRATOME III (LKB, Bromma, Sweden) were contrasted with alcoholic uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Fluka). The sections were examined on a JEOL JEM 1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan), and images were captured using a Mega VIEW III camera (Olympus, Soft Imaging System, Munster, Germany).

2.8. Statistical Analysis

The software used for statistical analysis was MedCalc 12. Each individual measurement was performed in triplicate. Results were expressed as the mean of the three measurements \pm standard deviation (SD). Statistical analysis was performed by the unpaired 2-tailed *t*-test. *p* values less than 0.05 were considered significant.

3. Results

3.1. US in the Chosen Regimen Effectively Combated the Oxidant Effects of H_2O_2 Treatment

In the OS group the MDA value was $11.9 \pm 1.2 \text{ nmoL/mg prot}$, which was significantly higher (p < 0.05) than in the control group ($7.2 \pm 0.6 \text{ nmoL/mg prot}$). In the US-OS group, where chondrocytes had been exposed to the US at the frequency of 850 KHz and the intensity of 100 mW/cm² for 5 min, on 3 consecutive days before H₂O₂ treatment, MDA ($5.0 \pm 0.8 \text{ nmoL/mg prot}$) failed to increase. We found that MDA was significantly lower (p < 0.05) in the US-OS group than in the OS group. In the US-OS group MDA levels were even lower than in the control group, but without statistical significance (p > 0.05) (Table 1, Figure 3).

OS Markers	OS Group (Mean \pm SD)	US-OS Group (Mean \pm SD)	CG (Mean \pm SD)
MDA (nmoL/mg prot)	11.9 ± 1.2	5.0 ± 0.8	7.2 ± 0.6
TNF (nmoL/mg prot)	13.7 ± 1.1	6.5 ± 0.8	5.7 ± 0.7

Table 1. Oxidative stress markers in the three groups.

OS—oxidative stress, MDA—malonildialdehyde, TNF- α —tumour necrosis factor – α , OS group—oxidative stress group, US-OS group—ultrasounds- oxidative stress group, CG—control group, SD—standard deviation.



Figure 3. MDA level in control group, OS group, and US-OS group. MDA level increased in OS group compared to control group (p < 0.05). In the US-OS group the MDA level decreased compared to the OS group (p < 0.05). (MDA—malonildialdehyde, OS group—oxidative stress group, US-OS group—ultrasounds- oxidative stress group, CG—control group).

3.2. The Increase in TNF- α Level, Induced by H₂O₂ Treatment, Was Prevented by US Exposure

As shown in Figure 4, the highest concentration of TNF- α (13.7 ± 1.1 nmoL/mg prot) was found in chondrocytes treated with H₂O₂. The level of TNF- α production in the OS group was approximately two-fold higher than in the control group (5.7 ± 0.7 nmoL/mg prot) and significantly higher (p < 0.05) than in the US-OS group. Exposure to US significantly decreased the TNF- α concentration (p < 0.05) in the US-OS group (6.5 ± 0.8 nmoL/mg prot) to levels similar to those in the control group (p > 0.05) (Table 1, Figure 4).



Figure 4. TNF- α level in control group, OS group, and US-OS group. TNF- α level increased in OS group compared to control group (p < 0.05). In US-OS group TNF- α level decreased compared to OS group (p < 0.05). (TNF- α —tumour necrosis factor– α , OS group—oxidative stress group, US-OS group—ultrasounds- oxidative stress group, CG—control group).

3.3. US Exposure Protected Chondrocytes from Ultrastructural Changes Induced by OS

TEM images from each HCH group were examined to assess the changes in chondrocyte ultrastructure.

In the control group chondrocytes had a normal aspect of the nucleus and cytoplasm. Nuclei presented a polymorphic aspect, with a prevalence of euchromatin and with a peripheral ring of heterochromatin. A small amount of endoplasmic reticulum; many



glycogen granules, sometimes crowded in large areas; and secretory vesicles were observed in cytoplasm (Figure 5).

Figure 5. Panels (**A**–**D**) illustrate ultrastructure of normal cells in control group: chondrocytes had a normal aspect: nuclei (N) presented a polymorphic aspect and euchromatic, with a peripheral ring of heterochromatin, endoplasmic reticulum (rer) in small amount, many glycogen granules (g), normal mitochondria (m).

In the OS group, we identified two different types of chondrocyte response to OS induced by H_2O_2 (Figure 6). As a first type of response, in cells with otherwise a quite normal aspect, we identified many electron-transparent vacuoles, as well as a large amount of expanded rough endoplasmic reticulum (RER). Mitochondria displayed a more condensed matrix. Glycogen was present in all cells, but its amount varied in different cells. Some chondrocytes had an excessive amount of fibrillar material, which occupied large areas of the cytoplasm. The fibrillar material was more compact and electron-dense. Lipid droplets appeared in some cells. Nuclei were unchanged as compared to control group. As a second type of chondrocyte response to OS, the same chondrocytes displayed a high degree of vacuolation and even signs characteristic for cell death by necrosis. In this group we identified numerous destroyed cells. In the remaining cells, there were many vacuoles and disorganized organelles in the cytoplasm. Nuclei were ballonized with lysated chromatin.

In the US-OS group the analysed chondrocytes exposed to US prior to H_2O_2 exposure were able to conserve their ultrastructure and viability under OS conditions. In this group we did not identify necrotic chondrocytes. In the US-OS group chondrocytes ultrastructure was only slightly changed compared to that in the control group. In this group nuclei had a normal appearance, shape, and chromatin aspect. Mitochondria had a normal matrix, without condensation and a normal aspect of cristae. In the US-OS group the cytoplasm contained a large number of vacuoles, but less compared to the OS group. Rough endoplasmic reticulum was dilated in many cells from the US-OS group, but fibrillar material was generally less than in the OS group, with perinuclear and intracytoplasmic disposition. In some chondrocytes of the US-OS group lysosomes were more numerous than in the OS and control groups, and we also identified autophagosomes (Figure 7).



Figure 6. Oxidative stress (OS) group: (1) chondrocytes with cvasinormal aspect (**A**–**F**): many electron-transparent vacuoles (v), lysosomes (ly), large amount of expanded rough endoplasmic reticulum (rer), more condensed matrix of mitochondria (m), excessive amount of fibrillar material more compact and electron-dense (f); (2) chondrocytes with signs characteristic for necrosis (**G**–**I**): many vacuoles (v) and disorganized organelles in cytoplasm, ballonized nuclei (N) with lysated chromatin.



Figure 7. US-OS group: chondrocytes with normal morphology (**A**–**F**): nuclei (N) with normal appearance, shape, and chromatin aspect, mitochondria (m) with normal matrix, without condensation and normal aspect of cristae, but rough endoplasmic reticulum (rer) dilated in many cells, fibrillar material (f) generally less and lysosomes (ly) more than in the OS group, autophagosome (a).

We assume that the findings described here can apply only to the cells that were examined at the TEM. As quantitative evaluations of necrosis, apoptosis, autophagy, and chondroptosis markers were not carried out, we may not completely exclude one or more of these mechanisms in the alterations encountered.

9 of 17

4. Discussion

Increasing evidence in both experimental and clinical studies suggests that OS is one of the main causal factors in the OA pathogenesis [3]. An increased production of ROS and a deficient antioxidant protection coexist in OA. In chondrocytes the primarily formed radical is superoxide ($O_2^{\bullet-}$), which is converted to H_2O_2 by superoxide dismutase (SOD). Usually, catalase (CAT) and glutathione peroxidase (GPx) neutralise H_2O_2 , but in OA these enzymes are deficient and allow H_2O_2 accumulation. In late-stage OA, SOD synthesis declines, and a $O_2^{\bullet-}$ neutralization deficit appears.

 H_2O_2 and $O_2^{\bullet-}$ impairs mitochondrial membrane permeability, leads to cytochrome c release into the cytoplasm [49], and may cause chondrocytes apoptosis [50,51]. H_2O_2 also induces the expression of some proinflammatory cytokines, such as IL-1 β and TNF- α , that exert stimulating effects on the expression of MMPs and contribute to the degradation of ECM [52].

Due to the analgesic and anti-inflammatory effects, US therapy has many applications, both in human and veterinary pathology, even when classical non-steroidian therapy is contraindicated [53–59]. A large number of clinical and experimental studies that support US use in OA treatment have been published over the last years. Nevertheless US influences on OS in OA are unclear. Some studies performed in cancer therapy have demonstrated that low-intensity US (LIUS) may induce apoptosis in human hepatocarcinoma cells in vitro, by mitochondrial dysfunction and OS [60]. This effect was also reported in human leukemic cells [61]. On the other hand, some studies reported US antioxidant effects. According to Bertuglia, LIUS therapy diminished OS during reperfusion in ischemia-reperfusion experimental animal models [62]. In our previous studies we demonstrated that US therapy improved plasma antioxidant capacity in OA patients [63], and recently antioxidant and anti-inflammatory effects of US have been reported in experimental model of muscle injury in rats [64]. Moreover, Lai and coworkers communicated that LIUS, at a lower intensity (<3 W/cm²), induced" more of antioxidant effects in non-cancer cells compared to cancer cells" [65].

Since US effects depend on the dose and the irradiated tissues, it is not surprising that the results of studies regarding the influences of US on OS are contradictory.

In our study we applied US at the frequency and the intensity used in OA therapy to assess their effects on chondrocytes exposed to OS.

Because the chondrocytes cultures are more vulnerable to stress than the in vivo chondrocytes, we chose to use US at low therapeutical intensity.

In our study H_2O_2 was used for inducing OS in chondrocyte cultures because H_2O_2 is an important factor in OA pathogenesis [66]. It was also used to reproduce oxidative environment in chondrocytes cultures.

In a previous study, H_2O_2 demonstrated its capacity to increase the level of ROS and mRNA levels for pro-inflammatory and prooxidative genes (cyclooxygenase-2 and inducible nitric oxide synthase) and to induce a significant increase in caspase 3-like activity and chondrocyte death [67].

In our study H_2O_2 added to chondrocyte cultures increased MDA and TNF- α synthesis and induced the death of some chondrocytes by necrosis and ultrastructural damage for most viable chondrocytes. After H_2O_2 exposure only a small number of chondrocytes remained quite normal in appearance. Pre-exposure to US-protected chondrocytes from MDA and TNF- α increased as did ultrastructural damages.

4.1. Oxidative Stress-Induced Ultrastructural Chondrocyte Damages

Four types of death are described for chondrocytes: apoptosis (type I), autophagic chondrocytes death (type II), necrosis (type III), and chondroptosis, the last involving both apoptotic and autophagic components [68].

In our study we found that H_2O_2 exposure led to variable morphological changes in primary human chondrocytes, and the ultrastructural damages were correlated with OS increase. The main ultrastructural changes in chondrocytes exposed to OS were found in mitochondria and RER. Mitochondria displayed a more condensed matrix, and RER was expanded and in large quantities in most viable chondrocytes. In almost all cells we identified a large amount of electron-dense fibrillar material. All these ultrastructural changes found in chondrocytes exposed to H_2O_2 were recognised as markers for cellular OS. Condensed matrix of mitochondria was reported as consequence of mitochondrial membrane potential (mMP) damage induced by OS [69]. Previous studies communicated that RER exposure to OS induced accumulation of unfolded proteins, RER expanding, and endoplasmic reticulum stress (ERS) occurrence [70]. Accordingly, in our study the large amount of expanded RER in most viable chondrocytes was the proof of ERS induced by OS. At the same time, the abundant fibrillar material in chondrocyte exposed to OS was a marker for chondrocyte injury. Fibrillar material was also found in ear chondrocytes exposed to papain, an enzyme with proteolytic effect on cartilage [71], and in rabbit OA chondrocytes [72].

4.2. Ultrastructural Damages in Chondrocytes Exposed to OS Were Correlated with MDA and TNF- α Increase

In our study MDA and TNF- α levels increased in chondrocytes exposed to H₂O₂ and were associated with chondrocytes necrosis, mitochondria condensed matrix, and a large amount of expanded RER. Previous studies also demonstrated elevated MDA and TNF- α levels in rat chondrocyte cultures exposed to H₂O₂ [73,74].

MDA is a stable product of the reaction between high-energy radicals and lipids [45] and the most used indicator of OS in clinical and experimental studies. In previous studies it was demonstrated that MDA was involved in collagen oxidation [75], reacted with histidine and lysine to form stable adducts, and might induce DNA cross-linking in cartilage [76]. The chondrocytes necrosis, ERS, and mitochondrial damages were found to also be correlated with MDA increase in previously published studies. In Kashin Beck disease MDA enhancing was correlated with chondrocytes necrosis in the deep-zone of cartilage [77]. In rat monosodium iodoacetate OA, a MDA increase was associated with elevated levels of TNF- α and biochemical markers of ERS [78]. In human OA chondrocytes higher MDA values coexist with lower respiratory capacity and higher proton leak in mitochondria correlated with structural mitochondria damage [79].

In our experimental model, cell necrosis, damaged mitochondria matrix, and expanded RER occurrence were associated with a significant TNF- α increase in in chondrocytes exposed to H₂O₂. In accordance with our results, in a previous study the H₂O₂ capacity to induce TNF- α synthesis was proven [80]. TNF- α was also found to stimulate NF- κ B mediated inflammatory response and ERS in human chondrocytes. [81] In another study Han communicated that in TNF- α -treated chondrocytes mitochondria cristae and ATP decreased as consequence of the mitochondria injury [82].

Previous studies demonstrated that TNF- α can induce both apoptosis and necrosis, depending on cell types and the experimental model [83,84]. Although most studies have associated TNF- α synthesis with apoptosis, our study showed that most chondrocytes died from necrosis. This leads us to believe that TNF- α was not the major decision factor for chondrocyte death and that most likely chondrocytes necrosis was caused by the cascade of ROS, synthesized following mitochondrial damage caused by chondrocyte exposure to H₂O₂.

In a recently published study cell necroptosis, also called "programmed necrosis" [85], was found to be correlated with ROS synthesis in a model of cartilage damage induced by mechanical stress [86].

4.3. US Exposure of Human Chondrocytes Cultures Prevented Ultrastructural Changes Induced by OS

Previous data indicated that US had cytoprotective effects by modulating cell proliferation and by anti-inflammatory effects in non-cancer cells. Most of the genes downregulated by LIUS in non-cancer cells are responsible for cell death and also for inflammatory signaling pathways correlated with ROS and RNS synthesis [65]. It is not surprising that in our study US reduced MDA and TNF- α synthesis, concomitantly with increasing chondrocytes' viability and improving chondrocytes' ultrastructure.

The MDA level assessed in the US-OS group was significantly lower than in the OS group. This result is most likely due to the increased capacity of chondrocytes to neutralize H_2O_2 , thus blocking generation of new ROS and the synthesis of oxidative compounds. Our findings are similar to the results published by Rosa, who found that US treatment decreased lipid peroxidation and TNF- α synthesis, concomitantly increasing antioxidant enzymes, CAT and SOD, in muscle injuries [64]. CAT is an important antioxidant enzyme that prevents OS by neutralizing H_2O_2 and inhibiting OH⁻ and O⁻ synthesis. SOD is the first defense line against the $O_2^{\bullet-}$, synthesized by damaged mitochondria under H_2O_2 action. The antioxidant effects of US were also communicated by Wang, who showed that US promoted expression of antioxidant genes GPX3 and GPX7 and decreased expression of prooxidant Nitric Oxide Synthase (NOS) genes [65]. US also influenced synthesis of various antioxidant compounds in plants, like terpenes and antioxidant enzymes [87,88]. The antioxidant effects of US were shown by Chen, who demonstrated that US pretreatment could help plants to eliminate the ROS generated after cadmium and lead exposure, by enhancing activities of CAT, SOD, and glutathione reductase and by reducing MDA and O^{-} synthesis [89].

In our US-OS group the TNF- α level decreased significantly after US exposure, compared to the OS group. Our results are in accordance with a previous studies in which US exposure reduced the TNF- α plasmatic level in rabbit OA [41] and tissular TNF- α synthesis after muscle contusion [64]. In a study published by Tilwani, it was demonstrated that in chondrocytes' cultures biomechanical signals attenuated TNF- α -induced synthesis of NO, PGE₂, and MMPs [22], molecules involved in chondrocytes and cartilage injury. Similarly, in our experimental model mechanical energy provided by US attenuated OS, TNF- α synthesis, so alleviating ultrastructural chondrocyte damages.

In our study the enhanced antioxidant capacity and the decreased TNF- α level allowed chondrocytes to remain viable and to conserve their ultrastructure in US-OS group, where only a few changes in organelles were observed. In another study Wang demonstrated that US could inhibit expression of several inflammatory markers involved in non-cancer cell death [65].

4.4. US Therapy Induced Autophagosomes Occurence

The most important ultrastructural change in the US-OS group was the large number of lysosomes and the occurrence of autophagosomes. A variety of essential cellular pathways are associated with lysosomal function, including the clearance of substrates, autophagy, exocytosis, and cell membrane repair. Cells with a larger number of lysosomes have an enhanced degradative capacity against lysosomal substrates such as glycosaminoglycans and autophagy substrates such as protein aggregates [90]. The autophagosomes occurrence is a characteristic feature for macroautophagy, a stress-induced form of autophagy that allows removal and continuous replacement of nonfunctional proteins and organelles. After fusion of lysosomes with autophagosomes to form autophagolysosomes, and after degradation of their content, a secondary lysosome may result [29]. This phenomenon explains how the number of lysosomes may remain higher under the autophagy process.

In our study autophagosomes' presence in the US-OS group confirms that the autophagy process is stimulated by US. Autophagy is a cellular mechanism that supports adaptation and survival of cells in OS and other stress conditions [91,92]. It has been shown that human age-related OA and surgically induced OA in mice were associated with a reduction in key regulators of autophagy and were accompanied by an increased rate of apoptosis [2]. In rat chondrocytes cultures, blocking autophagy also accelerated apoptosis [93].

The senescence—defective autophagy—OA axis was illustrated by Caramés, who demonstrated that early changes occurring in aging cartilage were due to an autophagy

defect. Thus, he hypothesized that "agents preventing aging-related loss of autophagy might promote chondrocyte survival and cartilage biosynthetic capacity" [94]. Autophagy defects cause an accumulation of dysfunctional mitochondria in chondrocytes [32]. This indicates that mitochondria function and autophagy are interdependent. In our study US preserved mitochondria ultrastructure most probably by autophagosomes occurrence. Recently, pre-treatment with vitamin D was found to be able to protect endothelial cells from mitochondrial lesions induced by H_2O_2 and to prevent endothelial cell death by reducing the apoptosis-related gene expression and activation of pro-autophagic molecule Beclin 1 [95].

In previous studies it was found that stimulation of autophagy attenuated mitochondrial injuries and ERS in chondrocytes exposed to OS [96] and ERS in mice hepatocytes exposed to a high-fructose diet [97]. In our US-OS group mitochondria had a normal aspect, and RER was swollen in most chondrocytes, but in a small amount. It could mean: firstly—US inhibited ROS production and ROS induced mitochondrial damages, secondly—autophagocytosis rapidly removed damaged cell organelles, or thirdly—both previous mechanisms were involved.

In accordance with previous research, two molecular pathways could explain the US-mediated autophagy increase: MAPK pathways and phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway.

In his study Kim demonstrated that US can improve viability of chondrocytes exposed to H_2O_2 by modulation of p38 MAPK autophagy inhibitory pathways [34,98]. In another study Wu showed that US induced autophagy in chemotherapy-resistant prostate cancer cells via the PI3K/AKT/mTOR signaling pathway [99].

When mTOR is inhibited, the activity of PI3K/Akt pathway increases, and the autophagy is activated to protect chondrocytes [100]. Recently Sun summarised in a narrative review the effects of the PI3K/AKT/mTOR signaling pathway in OA showing that PI3K/AKT could prevent chondrocyte death by autophagy stimulation [101].

To date only few studies demonstrated US capacity to induce autophagocytosis, and, to our knowledge, none of them proved this effect in chondrocytes. US-induced autophagy was found in different cancer cells, such as nasopharyngeal carcinoma cells [102], prostate cancer cells [99,103], murine sarcoma 180 cells [104], but also in THP-1 macrophages and derived foam cells [105], as well as in mice neurons [106]. This effect was, nevertheless, not found in all cellular types. Thus, the inhibitory effect of LIPUS on autophagocytosis was published for mesenchymal stem cells [107].

Assessment of US effects on OS-exposed chondrocyte cultures suggests that US increased capacity of chondrocytes to preserve their ultrastructure by an enhanced autophagic process, simultaneously with increased antioxidant capacity.

4.5. Study Limits

The metabolic and ultrastructural effects of US on chondrocytes were assessed only in vitro and were not correlated with an experimental animal model in vivo.

Autophagocytosis was assessed only by TEM, with no specific biological markers determined. The mechanism by which US induced autophagocytosis has not been specified. HCHs have been used, and they could have a different response to US exposure than chondrocytes in OA cartilage. The study makes no statement either on the persistence of chondrocyte downregulation after US or on the stage of OA most receptive to US. It is possible that US does not have the same efficacy in all stages of osteoarthritis, and this aspect should be clarified by future research.

Another important study limit was that no cell viability quantitative assay was performed.

A main limitation of the study was also the model used for US exposure, with the test well placed on a planar ultrasound transducer. The reflecting liquid–air interface and the thin coupling layer providing contact between the test well and the transducer surface, where standing waves occur, represent the main weak points of this model, as identified by Hensel [43].

5. Conclusions

US protected chondrocytes from biochemical and ultrastructural changes induced by OS, by decreasing MDA and TNF- α synthesis and increasing autophagic process.

Author Contributions: Conceptualization, R.A.U., A.M., D.E.O., V.M.C. and I.M.B.; methodology, R.A.U., D.E.O., A.F., L.I., E.D. and Ş.M.S.; software, Ş.B.; validation, R.A.U., R.A.C., Ş.B., E.D. and Ş.M.S.; formal analysis, D.E.O.; investigation, R.A.U., D.E.O. and A.F.; resources, R.A.U., C.A.O., S.C. and Ş.M.S.; data curation, R.A.U., V.M.C. and I.M.B.; writing—original draft preparation, R.A.U., D.E.O., V.M.C., I.M.B., and Ş.B.; writing—review and editing, R.A.U., A.F., V.M.C., I.M.B., S.C. and I.A.O.; visualization, R.A.U., V.M.C., I.M.B., R.A.C., C.A.O., G.S.M. and I.A.O.; supervision, R.A.U., A.M., L.I., Ş.B. and Ş.M.S.; project administration, R.A.U., I.M.B. and Ş.M.S.; funding acquisition, R.A.U., R.A.C., Ş.B., G.S.M. and Ş.M.S., D.E.O., V.M.C. and Ş.B. equally contributed as the first author to this work. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Available upon request.

Acknowledgments: This work was supported by a grant of the Romanian Ministry of Education and Research, CNCSIS-UEFISCSU, project number PNII-IDEI code 2623/2008. The publication was supported by funds from the National Research Development Projects to finance excellence (PFE)-14/2022-2024 granted by the Romanian Ministry of Research and Innovation.

Conflicts of Interest: The authors declare no conflict of interest.

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