*Osteoarthritis and Cartilage* (2010) **18,** 424–432

doi:10.1016/j.joca.2009.09.008

# Osteoarthritis and Cartilage

## Mitochondrial DNA damage is involved in apoptosis caused by pro-inflammatory cytokines in human OA chondrocytes

J. Kim†, M. Xu†, R. Xo†, A. Mates†, G. L. Wilson‡, A. W. Pearsall IV† and V. Grishko†‡*
† *Department of Orthopaedic Surgery, University of South Alabama, College of Medicine, USA*
‡ *Department of Cell Biology and Neuroscience, University of South Alabama, College of Medicine, Mobile, AL 36693, USA*

### Summary

*Objective*: Pro-inflammatory cytokines play a pivotal role in cartilage destruction during the progression of osteoarthritis (OA). Additionally, these cytokines are capable to generate reactive oxygen and nitrogen species within chondrocytes. Mitochondrion is a prime target of oxidative damage and an important player in aging and degenerative processes. The purpose of the present study was to investigate whether these cytokines will alter the mitochondrial DNA (mtDNA) integrity and mitochondrial function in both normal and osteoarthritic human chondrocytes.

*Design*: Primary normal and osteoarthritic human chondrocyte cultures were exposed to various concentrations of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) for different time. Following exposure, chondrocytes were evaluated for mitochondrial DNA damage, ATP production, changes in mitochondrial transcription, and apoptosis. Adenoviral vectors were used to deliver DNA repair enzyme hOGG1 to mitochondria.

*Results*: Pro-inflammatory cytokines IL-1β and TNF-α disturb mitochondrial function in human chondrocytes by inducing mitochondrial DNA damage, decreasing energy production and mitochondrial transcription, which correlated with the induction of apoptosis. Increased NO production was the key factor responsible for accumulation of mtDNA damage after cytokine exposure. Mitochondrial superoxide production was also enhanced following pro-inflammatory cytokine exposure. OA chondrocyte mitochondria were more susceptible to damage induced by pro-inflammatory cytokines then mitochondria from normal chondrocytes. Protection of human chondrocytes from mtDNA damage by the mitochondria-targeted DNA repair enzyme hOGG1 rescued mtDNA integrity, preserved ATP levels, reestablished mitochondrial transcription, and significantly diminished apoptosis following IL-1β and TNF-α exposure.

*Conclusion*: Mitochondrion is an important target in pro-inflammatory cytokine toxicity, maintaining of mitochondrial DNA integrity is necessary to prevent chondrocytes from apoptosis induced by IL-1β and TNF-α.


*Key words*: Cytokines, Mitochondria, Mitochondrial DNA, Apoptosis, Reactive oxygen species, Nitric oxide, Osteoarthritis, Chondrocytes.

### Introduction

It is well established that cytokines play an important role in the regulation of chondrocyte function. It is firmly believed that pro-inflammatory cytokines, most notably interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), play a pivotal role in cartilage destruction and that elevated levels of these cytokines occur in the synovial fluid and cartilage of patients with osteoarthritis (OA)[1,2].

Oxidative stress, disrupted mitochondrial respiration, and mitochondrial damage have been found to promote cell death, functional failure, and degeneration. Recently, the studies of mitochondrial dysfunction in OA have received additional scrutiny. It has been reported that respiratory chain activity and mitochondrial membrane potential are reduced in cultured human chondrocytes from patients with OA, when compared to normal donors[3]. Multiple studies have implicated a decrease in mitochondrial bioenergetic reserve as a pathogenic factor in degenerative cartilage diseases[4,5]. A recent study of comparative proteomics on chondrocytes from normal donors and OA patients showed significant changes in mitochondrial proteins participating in energy production, maintenance of mitochondrial membrane integrity, and free radical detoxification[6]. This study also revealed a significant decrease in mitochondrial superoxide dismutase (SOD) levels and, as a possible output for this antioxidant deficiency, an increase of intracellular ROS generation in OA chondrocytes. This data supports our recent study, which shows a diminished mtDNA integrity and repair capacity in OA chondrocytes compared to normal donors[7].

Because pro-inflammatory cytokine production is up regulated in osteoarthritic cartilage and this likely contributes to the enhanced NO and ROS production observed in OA cartilage, it is important to evaluate the effects of these cytokines on chondrocyte mitochondrial function. The mitochondrion is the predominant site for intracellular ROS production and a prime target for oxidative damage. ROS production

*Address correspondence and reprint requests to: Valentina Grishko, University of South Alabama, 307 University Blvd, MSB 1200, Mobile, AL 36688, USA. Tel: 1-251-460-7100; Fax: 1-251-460-6771; E-mail: vgrishko@jaguar1.usouthal.edu
Received 8 April 2009; revision accepted 27 September 2009.

induces damage to lipids, proteins, and nucleic acids in mitochondria, and also, leads to mitochondrial permeability transition (MPT). All these events are implicated in the mitochondrial pathway of apoptosis. To date, controversy still remains as to whether mitochondria are involved in the chondrocyte death induced by pro-inflammatory cytokines. There are data that mitochondrial activity is modulated by TNF-$\alpha$ and IL-1$\beta$ in normal human chondrocytes[8]. Additionally, it has been shown that IL-1$\beta$ disturbs the antioxidant enzyme system in bovine chondrocytes, which can explain why oxidative stress generation and mitochondrial damage is observed in OA[9]. Moreover, studies have been performed to show that TNF-$\alpha$ and IL-1$\beta$ have differential effects on cell death in human articular chondrocytes[10].

There is a growing body of evidence, indicating that mtDNA damage, caused by ROS or RNS, is involved in cellular dysfunction and death and could play a causal role in disorders linked to the excessive generation of reactive oxygen species. While there is published work which shows that IL-1$\beta$ leads to oxidative nuclear DNA damage associated with OA[11], no data has been published about the effects of pro-inflammatory cytokines on mtDNA integrity. Previously, we found that mtDNA integrity and mtDNA repair are diminished in OA chondrocytes compared to normal donors[7]. Therefore, we hypothesized that the destructive effects of pro-inflammatory cytokines on chondrocytes include the induction of mtDNA damage and the concomitant onset of mitochondrial dysfunction. The purpose of the present study was to investigate whether these cytokines will alter mitochondrial DNA integrity and function, and cause apoptosis in both normal and OA human chondrocytes. Also, we investigated whether overexpression of the DNA repair enzyme hOGG1 in human chondrocyte mitochondria will protect chondrocytes from mtDNA damage and have an effect on chondrocyte death induced by both IL-1$\beta$ and TNF-$\alpha$.

## Materials and methods

### CARTILAGE SPECIMENS AND CHONDROCYTE PRIMARY CULTURES

The cartilage was obtained from OA patients undergoing total knee replacement and from normal donors available through the University of South Alabama Anatomical Gifts Program. Primary chondrocyte cultures from OA patients were generated as previously described[7,12]. Each experiment was carried out with cultures generated from a single specimen, including all necessary controls. Normally, about 15–20 confluent 100 mm dishes containing about $5–7 \times 10^6$ cells were obtained from culture preparations. Cells grew to confluence in 7–10 days and then were used for experiments.

### CYTOKINE EXPOSURE

For initial dose- and time response experiments normal and OA human chondrocytes were exposed to 10, 20, or 50 ng/ml of IL-1$\beta$ or 50 or 100 ng/ml of TNF-$\alpha$ (both human recombinant from Roche Inc.) for 48 h in DMEM/F12 media supplemented with 4% FBS. Several experiments using a low dose of IL-1$\beta$ (10 ng/ml) were performed for extended time periods of 72, 96, and 120 h. In some experiments, primary chondrocyte cultures were pretreated with 0.5 mM of the iNOS inhibitor aminoguanidine (AG) for 6 h before and during treatment with cytokines. Following exposure, cells were collected and used for further analysis.

### ASSESSMENT OF MITOCHONDRIAL DNA DAMAGE

Following cytokine treatment, primary chondrocyte cultures were lysed overnight in buffer containing proteinase K. DNA was isolated by standard phenol/chloroform extraction and subjected to Southern blot analysis in alkaline conditions as described before in detail[13]. DNA damage was evaluated as the amount of DNA breaks per 16.6 kb fragment. Break frequency was determined using the Poison expression ($s = -\ln P_0$, where $s$ is the number of breaks per fragment, and $P_o$ is the fraction of fragments free of breaks).

### ATP BIOLUMINESCENCE ASSAY

Following the selected cytokine treatment time, primary chondrocyte cultures were lysed and the ATP levels in cells were evaluated using an ATP bioluminescence assay kit (Roche). This technique is well established and uses the ATP dependency of the light omitting luciferase-catalyzed oxidation of luciferin for the measurement of extremely low concentrations of ATP.

### NITRIC OXIDE RELEASE ASSAY

Total nitrite concentration in the tissue culture media following cytokine treatment was measured in duplicate using a microplate assay employing the Griess reagent (0.5% sulfanylamide and 0.05% naphthalene diamine dihydrochloride in 2.5% orthophosphoric acid). Nitrite values were determined using sodium nitrite as the standard. Background nitrite values in the media without cells were subtracted from values in the media with cells.

### NORTHERN BLOT ANALYSIS

To analyze changes in mitochondrial transcription, Northern blot analysis was employed. Following cytokine treatment for 48 h, total RNA was isolated using Trizol reagent (Gibco BRL). Ten $\mu$g of each RNA sample was loaded onto a gel and standard Northern blot analysis was performed. Membranes were hybridized with cytochrome c oxidase subunit III (COX III) or ATPase subunit 6 (ATP 6) – specific PCR-generated radioactive DNA probes, which are both mitochondrially encoded[14]. DNA probe for $\beta$-actin was used to ensure equal loading of RNA samples.

### MITOCHONDRIAL ROS PRODUCTION

Following 48 h of treatment with cytokines, OA chondrocytes cells were analyzed for mitochondrial ROS production. MitoSOX Red, a mitochondrial superoxide indicator for live cell imaging (Invitrogen Inc.), was employed to analyze mitochondrial superoxide generation within chondrocyte mitochondria following pro-inflammatory cytokine exposure. This agent is live-cell permeant and is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX Red reagent is readily oxidized by superoxide but not by other ROS or RNS, and oxidation of the probe is prevented by SOD. MitoTracker Green was used to label mitochondria in live cells (Invitrogen Inc.). Cells were incubated first for 30 min in normal growth media with MitoTracker Green at final concentration of 100 nM. After loading cells with MitoTracker Green, cells were replenished with DMEM/F12 – serum free media containing MitoSOX Red at final concentration of 2 $\mu$M, incubated for 10 min, replenished with normal media, and viewed in a fluorescent microscope. Additionally, cells were analyzed in a fluorescent plate reader, and the increase in ROS production was calculated as percentage increase compared to control.

### APOPTOSIS EVALUATION

#### *Flow cytometry*

The Aposcreen Annexin V apoptosis kit (Southern Biotech Inc), containing fluorescein-labeled annexin V (V-FITS) in concert with propidium iodide (PI) was used in this study. Following cytokine exposure, chondrocytes were trypsinized and collected by centrifugation. To ensure that all cells were harvested, cell culture media from each dish was combined with the resulting cell suspension from the same dish following trypsinization. Cells were washed twice in cold PBS and labeled with V-FITS and PI for 30 min according to the manufacturer's suggestions. Samples were analyzed by a FacsDIVA flow cytometry machine (Beckson Dickinson, Franklin Lakes, NJ) to identify apoptotic (V-FITS – labeled), necrotic (PI-labeled), and viable cells.

#### *DAPI staining*

To further identify the appearance of apoptosis, DAPI (4′-6-diamidino-2-phenylindole) staining was performed to observe condensed and fragmented nuclei. Following cytokine exposure, cells were washed with PBS and fixed with 4% of formaldehyde for 30 min at 37°C. Formaldehyde was removed from the cells by three subsequent PBS washes. Cells were incubated with DAPI for 10 min at a final concentration of 500 nM/ml and viewed in a fluorescent microscope.

#### *Western blot analysis*

Cells were lysed in cell lysing buffer (Cell Signaling Inc.) and processed according to the manufacturer's suggestions. The cell suspensions were briefly sonicated on ice, centrifuged once more at 5000 g to pellet any

remaining debris, and the supernatant protein was used for Western blot assays. The protein concentration was determined using the Bio-Rad protein dye micro-assay according to the manufacturer's recommendation (Bio-Rad). Caspase-9 antibodies (Cell Signaling Inc.) were used to evaluate the mitochondrial pathway of apoptosis. Antibodies against actin (Sigma Inc.) were used to ensure equal loading of samples.

### *hOGG1 adenoviral transduction*

Confluent primary chondrocyte cultures were transduced with adenoviral vectors, containing the gene for the human DNA repair enzyme 8-oxoguanine DNA glycosylase/AP lyase (hOGG1). Also, these vectors contained the mitochondria-targeting sequence (MTS) from mitochondrial SOD to ensure that the enzyme will be delivered to mitochondria and the gene for green fluorescent protein (GFP) to monitor the efficiency of transduction. Vectors without hOGG1 insert, but containing MTS and GFP, were used as negative controls (designated as empty vectors). Adenoviruses containing hOGG1 under MTS were kindly provided by Dr. M Kelly (Indiana State University). Initial experiments were done to find the most efficient and less harmful multiplicity of infection (MOI). We found that an MOI = 6 (six virus particles per cell) gave us the most GFP-positive cells 48 h after virus was added (from 72 to 95% in different experiments). Adenoviral vectors were added to cells in normal growth media and 48 h later those cells were exposed to cytokines for another 48 h before further analysis was performed.

### *Statistical analysis*

Statistical analyses were performed using either Student's *t* test, or one- or two-way analysis of variance (GraphPad Prism) where appropriate. A difference of $P < 0.05$ was considered significant. The Bonferroni *posthoc* test was used to determine the source of difference.

## Results

The results of this work will be presented as two parts. The first part gives the results of comparative studies on the effects of IL-1β and TNF-α on mitochondria from normal and OA chondrocytes. The second part explodes the effect of overexpression of a DNA repair enzyme, hOGG1, on mtDNA damage, mitochondrial function and apoptosis in OA chondrocytes following exposure to IL-1β or TNF-α.

In the first set of experiments, we incubated confluent primary human chondrocyte cultures, obtained from OA patients and normal donors, with increasing concentrations of the pro-inflammatory cytokines IL-1β and TNF-α to investigate whether mtDNA damage and mitochondrial dysfunction could be induced and whether this would correlate with cell death. As can be seen in Fig. 1, 10 ng/ml of IL-1β did not cause cell death in either normal or OA chondrocytes, while some mtDNA damage was observed. Following exposure to higher doses of IL-1β, a significant amount of mtDNA damage accumulated in association with an increasing number of apoptotic cells in the OA chondrocytes. Exposure of chondrocytes obtained from normal donors to high doses of IL-1β resulted in a lesser degree of mtDNA damage and a smaller increase in the number of apoptotic cells. Similar results were obtained when confluent chondrocyte cultures were exposed to increasing doses of TNF-α: the accumulation of mtDNA damage with a concomitant increase in the number of apoptotic cells. Therefore, these data show that OA chondrocytes sustained more extensive mtDNA damage and had a larger increase in the number of apoptotic cells when exposed to cytokines. Additionally, because 10 ng/ml of IL-1β is the most frequently used concentration in previous studies and it did not cause apoptosis in either OA or normal human chondrocytes, we decided to expand the treatment time up to 120 h. We hypothesized that through the secondary production of ROS caused by initial mtDNA damage, we would observe enhanced mtDNA damage and apoptosis later, even after low doses of cytokine. As can be seen in Fig. 2, time-extended treatment of OA chondrocytes with 10 ng/ml of IL-1β caused more mtDNA damage and increased apoptosis. Although the differences between each time point were not found to be statistically significant, the percentage of apoptotic cells was increased with increased time of exposure.

To evaluate the cause of the mtDNA damage, the next set of experiments was performed on OA chondrocytes using the iNOS inhibitor AG. Previously we demonstrated that NO is capable of inducing mtDNA damage and mitochondrial dysfunction in other cell types[15,16]. The results of our experiments showed that when AG was present during cytokine treatment, mtDNA damage was abolished [Fig. 3(A, B)]. To prove that AG was blocking NO production by iNOS, we measured nitrite levels in the culture media collected from chondrocytes exposed to pro-inflammatory cytokines alone or in combination with AG. The results of these studies showed that nitrite levels were significantly reduced when AG was added to the cells with cytokines [Fig. 3(C)]. Moreover, addition of AG to the media during cytokine exposure significantly reduced the apoptosis induced by both IL-1β and TNF-α [Fig. 3(D)].

Because energy production is an important mitochondrial function, we analyzed the changes in ATP levels following cytokine treatment. As can be seen in Fig. 4, both IL-1β and TNF-α decreased total ATP in a dose-dependent manner, and OA chondrocytes exhibited a more significant decrease in ATP levels following cytokine exposure compared to normal cells.

Based on our previous work on other cell types[17,18], we hypothesized that mtDNA damage may be the major factor in the mitotoxicity caused by pro-inflammatory cytokines and protection of chondrocytes from mtDNA damage may prevent this mitochondrial dysfunction and apoptosis, which was observed following exposure to IL-1β and TNF-α. To test this hypothesis, we transduced our primary OA chondrocyte cultures with adenoviral vectors containing a DNA repair enzyme hOGG1, an MTS, and GFP. Vectors containing an MTS and GFP only were used as negative controls.

The first set of experiments was performed to evaluate the effect of hOGG1 overexpression on mtDNA damage induced by both cytokines. As can be seen in Fig. 5, hOGG1 targeted to OA chondrocyte mitochondria was able to protect human chondrocytes from the accumulation of mtDNA damage following exposure to pro-inflammatory cytokines IL-1β and TNF-α.

Additionally, ATP levels were preserved and chondrocyte apoptosis was significantly decreased in hOGG1-transduced chondrocytes [Fig. 5(E)]. As another functional outcome of these experiments, we evaluated the status of mitochondrial transcription in hOGG1 and empty vector-hOGG1-transduced OA chondrocytes following exposure to IL-1β for 48 h. We found that exposure to IL-1β significantly decreased mRNA levels for the two mitochondrially encoded genes analyzed, subunit III of cytochrome c oxidase and subunit VI of ATPase, in the cells transduced with adenoviral vectors without hOGG1 [Fig. 5(D)]. Similar results were obtained for the TNF-α, which also significantly decreased mitochondrial transcription (data are not shown). However, targeting of hOGG1 to chondrocyte mitochondria preserved mitochondrial transcription and mRNA levels.

In the next set of experiments we investigated whether overexpression of hOGG1 would have an effect on ROS generation induced by IL-1β and TNF-α. We hypothesized that mtDNA damage would affect electron transport chain function and more free radicals would be generated as

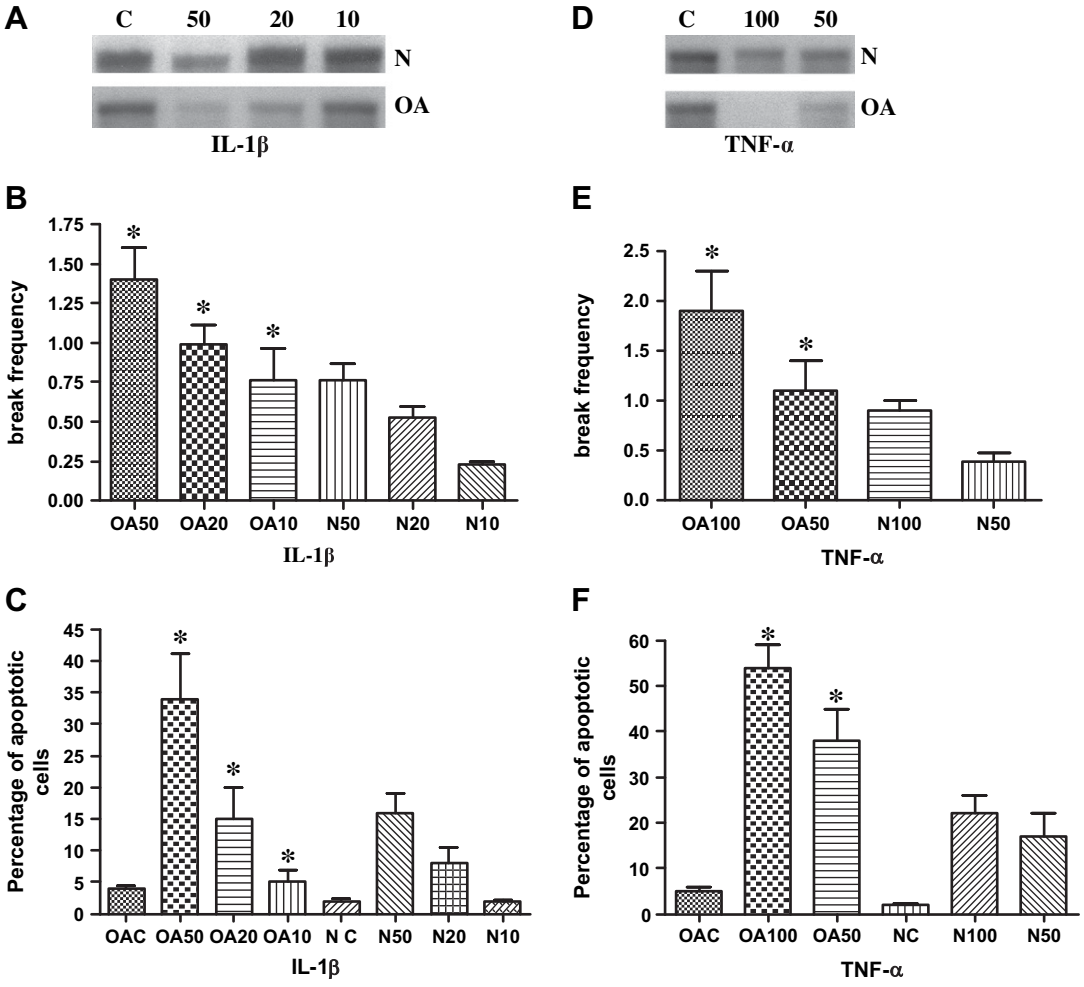


Fig. 1. Southern blot analysis of mtDNA damage induced following exposure to 50, 20, and 10 ng/ml of Il-1β (**A**) or 100 and 50 ng/ml of TNF-α (**D**) for 48 h in normal (N) and OA chondrocytes; the amount of DNA breaks per mitochondrial genome accumulated following exposure to various doses of IL-1β (**B**) or TNF-α (**E**) in normal and OA chondrocytes; quantitation of apoptosis in normal and OA chondrocytes induced by the same concentrations of Il-1β (**C**) or TNF-α (**F**) after 48 h according to flow cytometry analysis. OA C and N C represent non-treated controls. The results were obtained from a minimum of seven independent experiments, and the values represent the mean break frequency ±S.E.M. (**B**, **E**), and the mean percentage of apoptotic cells ±S.E.M. (**C**, **F**). *indicates a significant difference ($P < 0.05$) between normal and OA chondrocytes.

a consequence. OA chondrocytes, transduced with both empty and hOGG1-containing vectors, were exposed to both IL-1β and TNF-α for 48 h and analyzed for mitochondrial ROS production. As can be seen in Fig. 6, cytokine treatment with cytokines of OA empty vector-transduced chondrocytes resulted in enhanced mitochondrial superoxide generation compared to untreated controls, while hOGG1-transduced OA chondrocytes exhibited just a slight increase in ROS.

The final set of experiments was performed to investigate the effect of hOGG1 overexpression on apoptosis induced by IL-1β and TNF-α. Importantly, targeting of hOGG1 to OA chondrocyte mitochondria greatly diminished the apoptosis induced following exposure to 50 ng/ml of IL-1β and 100 ng/ml of TNF-α for 48 h, as can be seen by DAPI staining and flow cytometry analysis [Fig. 7(A, B)]. Moreover, the analysis of procaspase 9 cleavage, performed by Western blot analysis, demonstrated that hOGG1-transduced OA chondrocytes cleaved much less of this caspase to the active form following a 48 h exposure to either cytokines [Fig. 7(C)].

## Discussion

The principal findings of the present work are that: (1) the pro-inflammatory cytokines IL-1β and TNF-α disturb normal mitochondrial function in both normal and OA human chondrocytes by inducing mtDNA damage, decreasing energy production and decreasing mitochondrial transcription; (2) increased NO production is a key factor responsible for accumulation of mtDNA damage after cytokine exposure; (3) OA chondrocyte mitochondria are more susceptible to damage induced by pro-inflammatory cytokines than mitochondria from normal chondrocytes; (4) mtDNA damage plays a pivotal role in the mitochondrial dysfunction and apoptosis induced by pro-inflammatory cytokines. Protection of human chondrocytes from mtDNA damage by the mitochondria-targeted DNA repair enzyme hOGG1 rescues mtDNA integrity, decreases mitochondrial ROS production, preserves ATP levels, reestablishes mitochondrial transcription, and greatly diminishes apoptosis following IL-1β and TNF-α exposure. To the best of our knowledge, this is the first report to explore the involvement of mtDNA damage

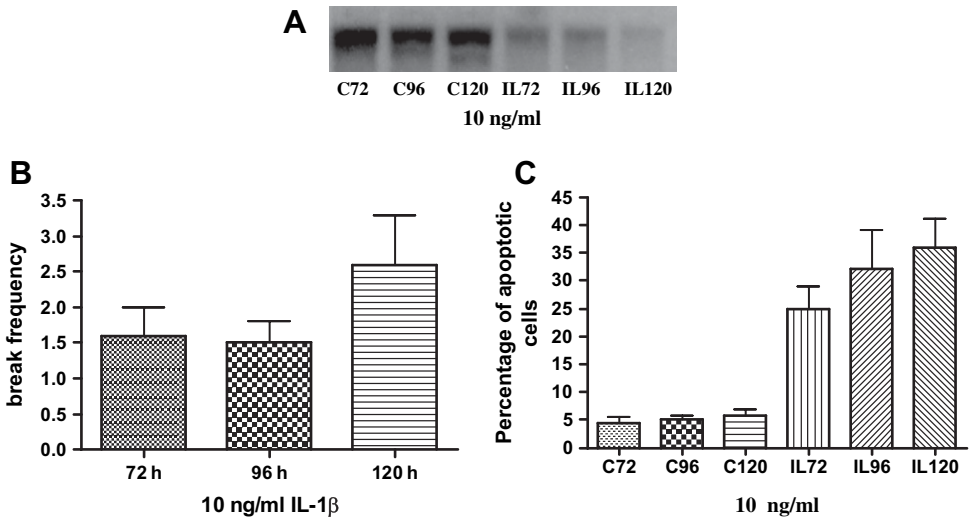


Fig. 2. **A** – Southern blot analysis of mtDNA integrity in non-treated (C72, C96, C120) OA chondrocytes and OA chondrocytes exposed to 10 ng/ml of Il-1β for 72, 96, and 120 h (IL72, IL96, IL120); **B** – quantitation of mtDNA damage as the amount of DNA breaks per mitochondrial genome; **C** – quantification of apoptosis according to flow cytometry analysis induced in non-treated and 10 ng/ml of IL-1β-treated OA chondrocytes. The results were obtained from a minimum of six independent experiments, the values represent the mean break frequency ±S.E.M. (**B**), and the mean percentage of apoptotic cells ±S.E.M. (**C**).

in the dysfunction and death of cartilage cells following the exposure to pro-inflammatory cytokines.

Mitochondria are important for regulating cell survival, and the signs of cell death usually are preceded by mitochondrial alterations. Recent studies in chondrocytes have shown that NO production, which is up regulated in OA chondrocytes and enhanced as the result of IL-1β and TNF-α exposure, suppresses respiration and ATP

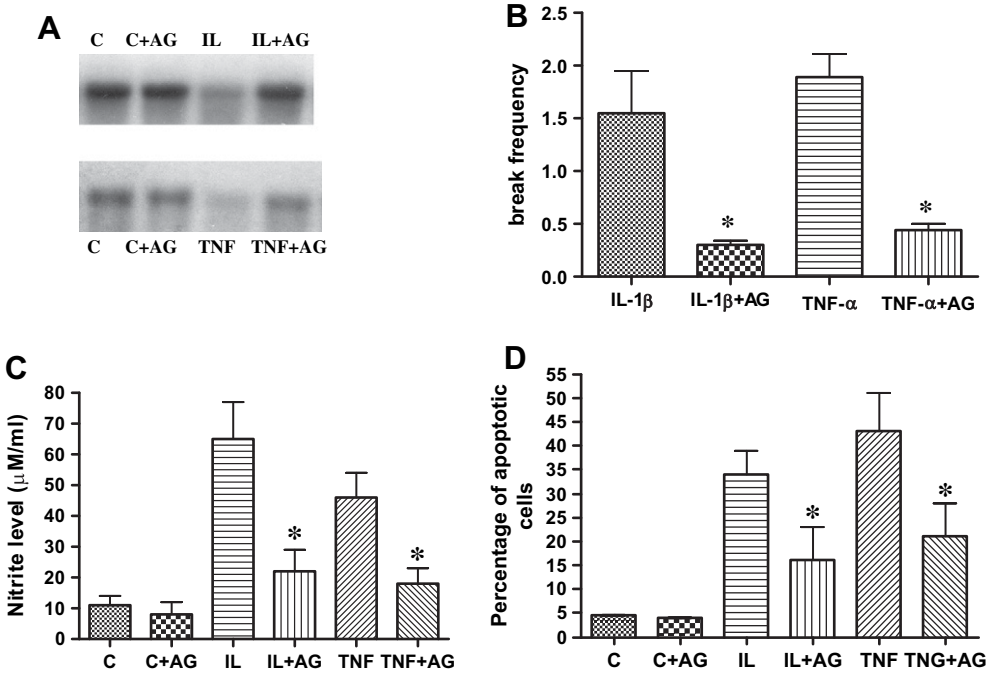


Fig. 3. **A** – Amelioration of the mtDNA damage induced by pro-inflammatory cytokines in OA chondrocytes with iNOS inhibitor AG. Cells were treated with 50 ng/ml of Il-1β and 100 ng/ml of TNF-α for 48 h alone or in combination with 0.5 mM of AG; **B** – quantitation of mtDNA damage as the amount of DNA breaks per mitochondrial genome; **C** – the effect of AG on nitrate levels following cytokine exposure; **D** – the effect of AG on the induction of apoptosis following pro-inflammatory cytokine exposure. The results were obtained from a minimum of five independent experiments, and the values represent the mean break frequency ±S.E.M. (**B**), the mean nitrate levels ±S.E.M. (**C**), and the mean percentage of apoptotic cells ±S.E.M. (**D**). * indicates a significant difference in mtDNA break frequency ($P < 0.05$) between AG + cytokine treated and cytokine alone treated chondrocytes, in the nitrate levels between chondrocytes treated with cytokines alone or with cytokines and AG ($P < 0.05$), and also in the percentage of apoptotic cells between chondrocytes treated with cytokines alone or with cytokines and AG ($P < 0.05$).

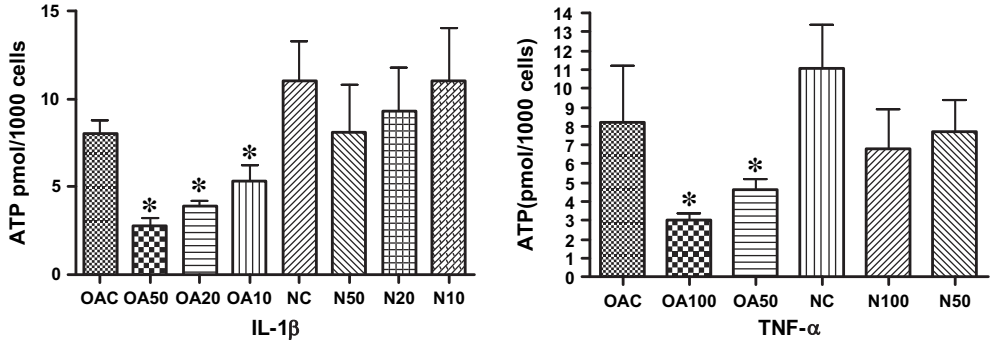


Fig. 4. Effect of pro-inflammatory cytokines on ATP levels in normal (N) and OA chondrocytes following 48 h of exposure. The results were obtained from a minimum of five independent experiments, and the values represent the mean ATP concentration ±S.E.M. * indicates a significant difference in ATP levels ($P < 0.05$) between cytokine-treated and non-treated normal or OA chondrocytes.

generation[19,20]. Recently, several studies have addressed the effects of NO on chondrocyte mitochondria. It has been shown that NO activates mitochondria-dependent events which induce apoptosis in human chondrocytes[21,22]. Moreover, NO is capable of affecting the antioxidant status of chondrocytes. Recently, it has been reported that IL-1β and IL-6 disturb the antioxidant enzyme system in bovine chondrocytes, which may explain the generation of oxidative stress and cartilage damage during OA[9]. In the present work, we demonstrate that, along with NO, mitochondrial ROS production also is enhanced in OA chondrocytes by both IL-1β and TNF-α. Protection of mtDNA from damage by overexpression of the DNA repair enzyme hOGG1 significantly reduces this production, pointing to mtDNA damage as an important initiating factor responsible for the increase in mitochondrial superoxide production following cytokine treatment. This notion is supported by our recent work[7] where we show that OA chondrocytes have diminished mtDNA integrity and mtDNA repair capacity as a result of OA progression.

There is a growing body of evidence which indicates that mitochondrial function is altered in the OA chondrocytes. Several studies recently have been published addressing the possible connection between cytokines, mitochondrial dysfunction, and apoptosis in OA. Studies from the Blanco lab have dealt with this problem[8,10,23]. While these authors demonstrate that mitochondrial dysfunction is induced following cytokine exposure, no apoptosis by has been accumulated. However, it is difficult to compare these results with ours because the experimental conditions used by this group and our approach are very different. The experiments from the Blanco lab were performed using serum

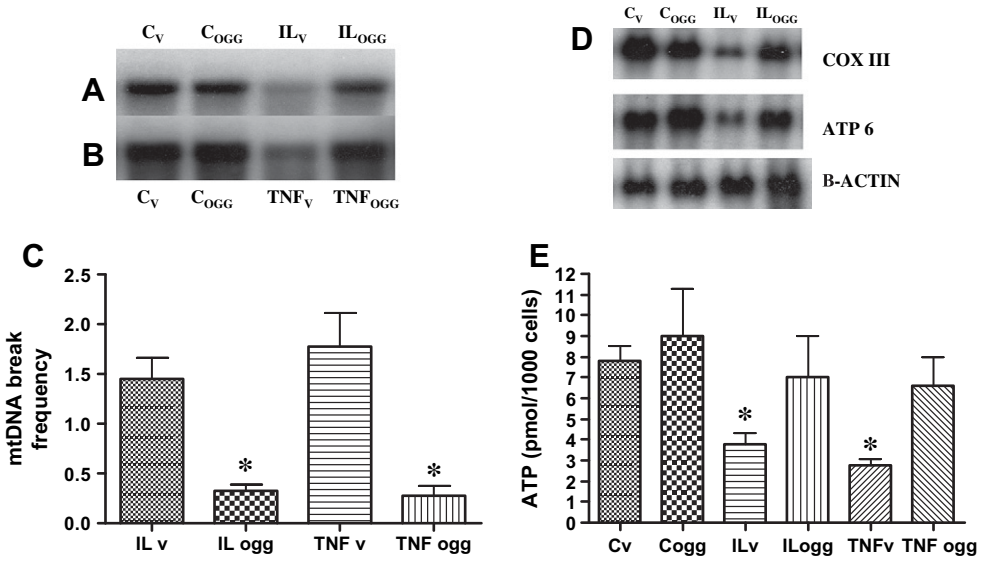


Fig. 5. Effect of hOGG1 transduction on OA chondrocyte mitochondrial function and apoptosis following pro-inflammatory cytokine exposure. Southern blot analysis of mtDNA damage following exposure for 48 h to 50 ng/ml of Il-1β (**A**) or 100 ng/ml of TNF-α (**B**) in OA chondrocytes transfected with adenoviral vector without hOGG1 insert ($IL_V$) and with hOGG1 containing vector ($IL_{ogg}$); **C** – quantitation of mtDNA damage as the amount of DNA breaks per mitochondrial genome; **D** – Northern blot analysis of mitochondrial transcripts following IL-1β treatment of chondrocytes transduced with empty or hOGG1 containing vectors; **E** – ATP levels in a same groups of transduced chondrocytes following both cytokines treatment. The results were obtained from a minimum of six independent experiments, and the values represent the mean break frequency ±S.E.M. (**C**), and the mean ATP ± S.E.M. (**E**). * indicates a significant difference ($P < 0.05$) between vector without hOGG1 and vector with hOGG1-transduced cells.

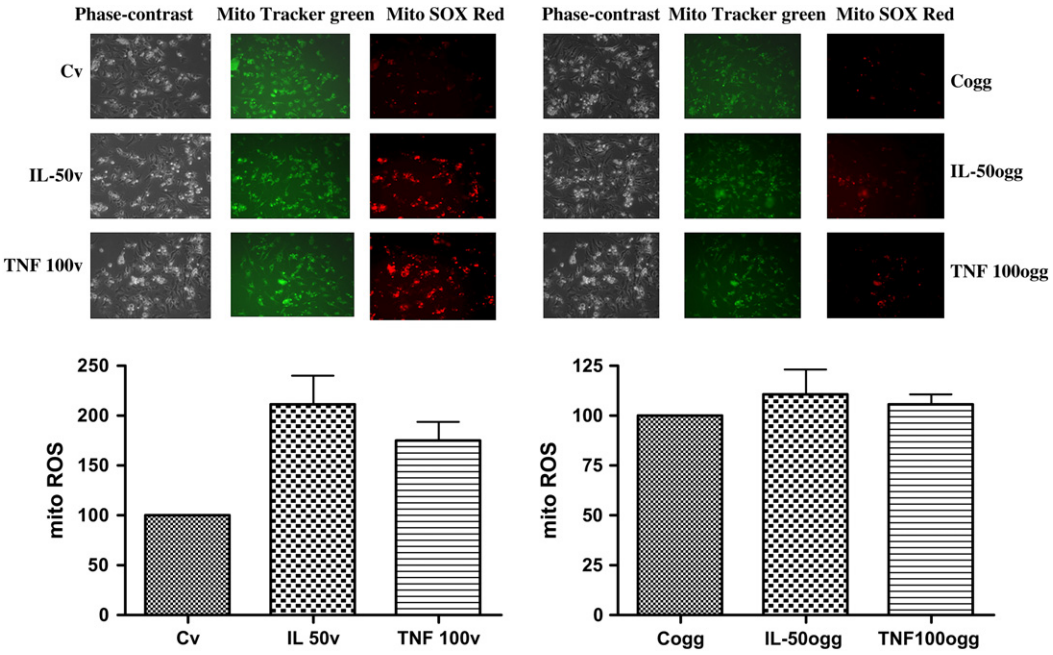


Fig. 6. Mitochondrial superoxide production in OA chondrocytes transduced with empty vectors (V) and hOGG1 (ogg) containing vectors following exposure to 50 ng/ml of IL-1β or 100 ng/ml of TNF-α for 48 h. Cells were exposed to cytokines, loaded with MitoTracker green and MitoSOX Red, and viewed in fluorescent microscope. Additionally, cells were analyzed in fluorescent plate reader, and increase in ROS production was calculated as percentage increase compare to control.

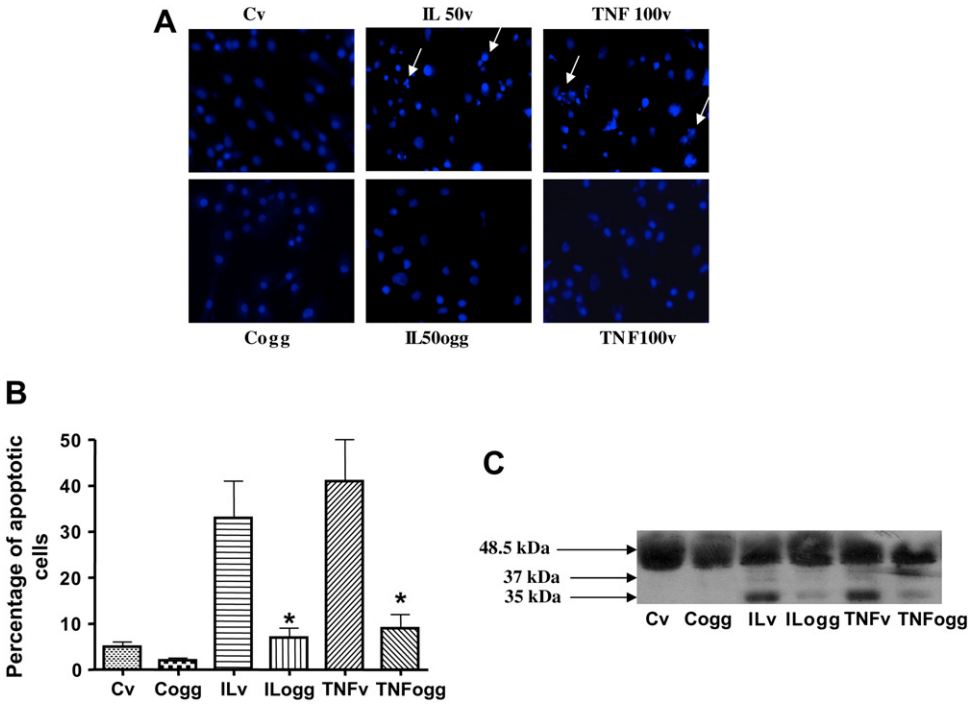


Fig. 7. Transduction of OA chondrocytes with hOGG1 containing vectors diminishes apoptosis induced following exposure to IL-1β and TNF-α. A – DAPI staining of OA chondrocytes transduced with empty and hOGG1 containing vectors and exposed to both cytokines for 48 h; B – quantitation of the percentage of apoptotic cells according to flow cytometry analysis; C – Western blot analysis of caspase 9 cleavage in the same groups of cells. The results were obtained from a minimum of six independent experiments, and the values represent the mean percentage of apoptotic cells ±S.E.M. *indicates a significant difference in the number of apoptotic cells ($P < 0.05$) between empty vector and hOGG1 containing vector cytokine-treated OA chondrocytes.

starvation (0.5% FCS in cell media) for 24–48 h followed by cytokine exposure for another 48 h. We simply exposed our chondrocytes to cytokines in regular media containing 4% FBS (Hyclone Inc) for 48 h. Additionally, they used a combination of cytokines with Actinomycin D (ActD) (the inhibitor of transcription) to induce apoptosis. ActD, itself, was found to cause a significant reduction in OA chondrocyte viability. There was an additive effect using a combination of TNF-$\alpha$ and ActD, resulting in more profound viability loss, while the combination of IL-1$\beta$ (5 ng/ml) with ActD was found to enhance cell viability compare to ActD alone. Moreover, most of this work was performed on normal chondrocytes. In our study, we report that OA and normal chondrocytes exhibit different sensitivity to pro-inflammatory cytokines. Our work shows that there is a connection between mitochondrial dysfunction and apoptosis following pro-inflammatory cytokine exposure. Experimental conditions may contribute to the differences observed, as well as possible variations in the specimens used.

Our work, along with others, on various cell types suggests that mtDNA damage is ultimately linked to cell viability and apoptosis[24–26]. Protection of human chondrocyte mitochondria with a DNA repair enzyme, hOGG1, not only preserves mtDNA integrity following IL-1$\beta$ treatment, but also reduces the number of apoptotic cells. While there is a study, which shows the accumulation of oxidative nuclear DNA damage in porcine chondrocytes following IL-1$\beta$ exposure, no, prior to ours, studies have been published analyzing mtDNA integrity following cytokine treatment of cartilage cells[11]. Extensive work has been performed on cell types other than cartilage to seek a possible link between mtDNA damage, oxidative stress in mitochondria, and cell death after pro-inflammatory cytokine treatment. Oxidative stress mediates TNF-$\alpha$ – induced mtDNA damage and dysfunction in cardiac myocytes[27]. Studies on pancreatic $\beta$-cells show that their mtDNA is more sensitive to damage induced by IL-1 than nuclear DNA, and cytokine-induced $\beta$-cell apoptosis is NO-dependent and mitochondria-mediated[28]. Additionally, pro-inflammatory cytokines are capable of inducing nitric oxide-mediated mtDNA damage and apoptosis in oligodendrocytes[29]. We believe, our study is the first to demonstrate the accumulation of mtDNA damage as a result of pro-inflammatory cytokine treatment of human chondrocytes and to show the role which this damage plays in mitochondrial dysfunction and apoptosis. An important aspect of our findings is that OA chondrocyte mitochondria are more sensitive to the DNA damaging effects of the pro-inflammatory cytokines L-1$\beta$ and TNF-$\alpha$ than mitochondria from normal chondrocytes. Previously, it has been shown by other investigators that the production of NO by isolated OA chondrocytes was significantly higher than in normal chondrocytes both under basal conditions and after stimulation with L-1$\beta$ or TNF-$\alpha$[30]. Our work, and that of others, suggests that mitochondria in OA chondrocytes are already damaged and data from the present study contribute to this knowledge.

Although we did not directly prove, in the present work, that NO is responsible for the apoptosis induced following pro-inflammatory cytokine exposure, our data indicates this may be the case because mtDNA damage and apoptosis induced by IL-1$\beta$ and TNF-$\alpha$ were both abolished by the iNOS inhibitor AG. Our data also suggest the involvement of oxidative stress in the damaging effects of cytokines on chondrocytes function. Because AG did not completely block apoptosis, we believe that ROS also are involved in this process, NO probably only initiates the destructive events, including mtDNA damage, which are later enhanced by ROS.

It is well established that ROS and RNS can directly react with DNA leading to the formation of apurinic (AP) sites resulting in single-strand breaks. Single-strand breaks will abort replication and transcription of mtDNA, which in turn affects mitochondrial synthesis of electron transport chain proteins. Previously we observed that targeting of the DNA repair proteins 8-oxoguanine DNA glycosylase/AP lyase (hOGG1), endonuclease III or endonuclease VIII into mitochondria augmented mtDNA repair of oxidative damage in this organelle, and enhanced cellular survival by blocking the formation of abasic sites[16–18].

In conclusion, our work strongly suggests that the mitochondrion is an important target for chondrocyte damage induced by the pro-inflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ and the protection from this damage ameliorates mitochondrial dysfunction and diminishes cell death induced by these agents.

## Conflict of interest

The authors disclose that while performing the study there were not any commercial affiliation or conflict of interests for neither author with any commercial or financial institutions.

## Acknowledgements

The authors disclose that while performing the study there were not any commercial affiliation or conflict of interests for either author.

### References

1. Guilak F, Fermor B, Keefe FJ, Kraus VB, Olson SA, Pisetsky DS. The role of biomechanics and inflammation in cartilage injury and repair, Clin Orthop Relat Res 2004;423:17–26.
2. Goldring SR, Goldring MB. The role of cytokines in cartilage matrix degeneration in osteoarthritis. Clin Orthop Relat Res 2004;23:S27–36.
3. Maneiro E, Martin MA, deAndres MC, Lopes-Armada MJ, Fernandes-Sueiro JL, del Hoyo P, *et al*. Mitochondrial respiratory activity is altered in osteoarthritic human articular cartilage. Arthritis Rheum 2003;48:700–8.
4. Terkeltaub R, Johnson K, Murphy A, Ghosh S. Invited review: the mitochondrion in osteoarthritis [review]. Mitochondrion 2002;1:301–19.
5. Johnson K, Jung AS, Andreyev A, Murphy A, Dykens J, Terkeltaub R. Mitochondrial oxidative phosphorylation is a downstream regulator of nitric oxide effects on chondrocyte matrix synthesis and mineralization. Arthritis Rheum 2000;43:1560–70.
6. Ruiz-Romero C, Calamia V, Mateos J, Carriera V, Martinez-Gomariz M, Fernandez M, *et al*. Mitochondrial dysregulation of osteoarthritic human articular chondrocytes analyzed by proteomics: a decrease in mitochondrial superoxide dismutase points to a redox imbalance. Mol Cell Proteomics 2009;8:171–89.
7. Grishko VI, Ho R, Wilson GL, Pearsall AW. Diminished mitochondrial DNA integrity and repair capacity in OA chondrocytes. Osteoarthritis Cartilage 2009;17(1):107–13.
8. Lopez-Armada MJ, Carames B, Martin MA, Cillero-Pastor B, Lires-Dean M, Fuentes-Boquete I, *et al*. Mitochondrial activity is modulated by TNF $\alpha$ and IL-1$\beta$ in normal human chondrocyte cells. Osteoarthritis Cartilage 2006;14:1011–22.
9. Mathy-Hartert M, Hogge L, Sanchez C, Deby-Dupont G, Grielaard JM, Henrotin Y. Interleukin-1beta and interleukin-6 disturb the antioxidant enzyme system in bovine chondrocytes: a possible explanation for oxidative stress generation. Osteoarthritis Cartilage 2008;16:756–63.
10. Carames B, Lopez-Armada MJ, Cillero-Pastor B, Lires-Dean M, Vaamonde C, Galdo F, *et al*. Differential effects of tumor necrosis factor-alpha and interleukin-a beta on cell death in human articular chondrocytes. Osteoarthritis Cartilage 2008;16(6):715–22.
11. Davie SM, Guilak F, Weinberg JB, Fermor B. Reactive nitrogen and oxygen species in interleukin-1-mediated DNA damage associated with osteoarthritis. Osteoarthritis Cartilage 2008;16(5):624–30.

12. Grishko V, Xu M, Mates A, Watson S, Kim JT, Wilson GL, *et al*. Effects of hyaluronic acid on mitochondrial function and mitochondria-driven apoptosis following oxidative stress in human chondrocytes. J Biol Chem 2009;284(14):9132–9.
13. Grishko VI, Rachek LI, Spitz D, Wilson GL, LeDoux SP. Contribution of mitochondrial DNA repair to cell resistance from oxidative stress. J Biol Chem 2005;280(10):8901–5.
14. Sanchez-Alcazar JA, Schneider E, Hernandez-Munoz I, Ruiz-Cabello J, Siles-Rivas E, de la Torre P, *et al*. Reactive oxygen species mediate the down-regulation of mitochondrial transcripts and proteins by tumor necrosis factor alpha in L929 cells. Biochem J 2003;370:609–19.
15. Grishko VI, Druzhyna N, LeDoux SP, Wilson GL. Nitric oxide- induced damage to mtDNA and its subsequent repair. Nucleic Acids Res 1999;27:4510–5.
16. Rachek LI, Grishko VI, LeDoux SP, Wilson GL. Role of nitric oxide-induced mtDNA damage in mitochondrial dysfunction and apoptosis. Free Rad Biol Med 2006;40(5):754–62.
17. Rachek LI, Grishko VI, Musiyenko SI, Kelley MR, LeDoux SP, Wilson GL. Conditional targeting of the DNA repair enzyme hOGG1 into mitochondria. J Biol Chem 2002;277:44932–7.
18. Dobson AW, Grishko V, LeDoux SP, Kelley MR, Wilson GL, Gillespie MN. Enhanced mtDNA repair capacity protects pulmonary artery endothelial cells from oxidant-mediated death. Am J Physiol Lung Cell Mol Physiol 2002;283:L205–10.
19. Abramson SB, Attur M, Amin AR, Clancy R. Nitric oxide and inflammatory mediators in the perpetuation of osteoarthritis. Curr Rheumatol Rep 2001;3:535–41.
20. Brookes PS, Levonen AL, Shiva S, Sarti P, Darley-Usmar VM. Mitochondria: regulators of signal transduction by reactive oxygen and nitrogen species. Free Radic Biol Med 2002;33(6):755–64.
21. Aigner T, Kim HA. Apoptosis and cellular vitality. Issues in osteoarthritic cartilage degeneration. Arthritis Rheum 2002;46:1986–96.
22. Kuhn K, Shikhman AR, Lotz M. Role of nitric oxide, reactive oxygen species, and p38 MAP kinase in the regulation of human chondrocyte apoptosis. J Cell Physiol 2003;197(3):379–87.
23. Kim HA, Blanco FJ. Cell death and apoptosis in osteoarthritic cartilage. Curr Drug Targets 2007;8(2):333–45.
24. Santos JH, Hunakova L, Chen Y, Borthner C, Van Houten B. Cell sorting experiments link persistent mitochondrial DNA damage with loss of mitochondrial membrane potential and apoptotic cell death. J Biol Chem 2003;278:1728–34.
25. Ballinger SW, Patterson C, Yan CN, Doan R, Burow DL, Young CG, *et al*. Hydrogen peroxide-and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. Circ Res 2000;86:960–6.
26. Grishko VI, Rachek LI, Musiyenko SI, LeDoux SP, Wilson GL. Involvement of mtDNA damage in free fatty acids-induced apoptosis. Free Radic Biol Med 2005;38(6):755–62.
27. Suematsu N, Tsutsui H, Wen J, Kang D, Ikeuchi M, Ide T, *et al*. Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. Circulation 2003;107(10):1418–23.
28. Wilson GL, Patton NJ, LeDoux SP. Mitochondrial DNA in beta cells is a sensitive target for damage by nitric oxide. Diabetes 1997;46(8):1291–5.
29. Druzhyna N, Musiyenko SM, Wilson GL, LeDoux SP. Cytokines induce nitric oxide-mediated mtDNA damage and apoptosis in oligodendrocytes. Protective role of targeting 8-oxoguanine glycosylase to mitochondria. J Biol Chem 2005;280(22):21673–9.
30. Clancy RM, Amin AR, Abramson SB. The role of nitric oxide in inflammation and immunity. Arthritis Rheum 1998;41:1141–51.