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Oxidative DNA damage in osteoarthritic porcine articular cartilage

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Abstract

Purpose—Osteoarthritis (OA) is associated with increased levels of reactive oxygen species. This study investigated if increased oxidative DNA damage accumulates in OA articular cartilage compared with non-OA articular cartilage from pigs with spontaneous OA. Additionally, the ability of nitric oxide (NO) or peroxynitrite (ONOO⁻) induced DNA damage in non-OA chondrocytes to undergo endogenous repair was investigated.

Methods—Porcine femoral condyles were graded for the stage of OA, macroscopically by the Collins Scale, and histologically by the modified Mankin Grade. Levels of DNA damage were determined in non-OA and OA cartilage, using the comet assay. For calibration, DNA damage was measured by exposing non-OA chondrocytes to 0–12 Gray of x-ray irradiation. Non-OA articular chondrocytes were treated with 0–500 μM of NO donors (NOC-18 or SIN-1), and DNA damage assessed after treatment and 5 days recovery.

Results—A significant increase ($p < 0.01$) in oxidative DNA damage occurred in OA chondrocytes in joints with Mankin Grades 3 or greater, compared to non-OA chondrocytes. The percentage of nuclei containing DNA damage increased significantly ($p < 0.001$) from early to late grades of OA. An increase of approximately 0.65–1.7 breaks/1000kB of DNA occurred in OA, compared to non-OA nuclei. NOC-18 or SIN-1 caused significant DNA damage ($p < 0.001$) in non-OA chondrocytes that did not undergo full endogenous repair after 5 days ($p < 0.05$).

Conclusion—Our data suggest significant levels of oxidative DNA damage occur in OA chondrocytes that accumulates with OA progression. Additionally, DNA damage induced by NO and ONOO⁻ in non-OA chondrocytes does not undergo full endogenous repair.

Keywords

Osteoarthritis; articular cartilage; Reactive oxygen species; DNA damage

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease that is the leading cause of disability in the United States (Lawrence et al., 2008). OA is characterized by the progressive degradation of articular cartilage over several decades resulting in inflammation, pain, and

decreased range of motion, particularly in large weight-bearing joints. The pathogenesis of OA likely involves the formation of reactive oxygen species (ROS) and the alteration of redox sensitive signaling pathways (Henrotin et al., 2003). Increased ROS, such as nitric oxide (NO) and peroxynitrite, occur in human OA cartilage (Abramson et al., 2001; Del Carlo and Loeser, 2002; Evans and Stefanovic-Racic, 1996; Yudoh et al., 2005).

NO is a relatively unstable molecule due to its unpaired electron. NO is formed by the conversion of L-arginine to citrulline, with oxygen serving as an electron acceptor. The removal of the terminal guanidino nitrogen is catalyzed by the enzyme nitric oxide synthase (NOS) (Kwon et al., 1990). NOS exists in three isoforms, NOS1 (nNOS), NOS2 (iNOS), and NOS3 (eNOS). The inducible NOS2 isoform (iNOS) is predominantly responsible for NO production in articular cartilage (Abramson et al., 2001). Some of the destructive effects of NO are linked to its ability to combine with superoxide anions (O_2^-). The unpaired radical electrons on NO and O_2^- can join to form the potentially more dangerous product peroxynitrite ($ONOO^-$) (Pacher et al., 2007).

Inflammation and mechanical stress are risk factors for OA (Agarwal et al., 2004; Guilak et al., 2004), and both are associated with the up-regulation of reactive species (Fermor et al., 2007). The up-regulation of reactive species can lead to several different effects on chondrocytes, including inhibition of adhesion-modulating signal transduction, modulation of cytokine expression, suppression of matrix collagen and proteoglycan synthesis, activation of matrix metalloproteinases, suppression of proliferation, promotion of chondrocyte apoptosis, and formation of nitrated collagen peptides (Deberg et al., 2005; Grabowski et al., 1997; Lotz et al., 1999; Pelletier et al., 1999; Studer et al., 2000). Additionally, porcine OA chondrocytes show increased nuclear DNA damage compared with non-OA chondrocytes when enzymatically isolated and grown in alginate culture for 3 days (Davies et al., 2008). Alginate is used for the growth of chondrocytes in cell culture to prevent the de-differentiation of the chondrocytes to fibroblasts. Increased nuclear DNA damage occurs in chondrocytes when they are passaged as monolayer cultures (Martin et al., 2004). However, DNA damage has not been investigated in chondrocytes that have not undergone cell culture.

Exposure of articular chondrocytes in alginate to NO or $ONOO^-$ can result in oxidative DNA damage (Davies et al., 2008). Oxidative DNA damage occurs naturally and does not cause adverse biological consequences unless the damage cannot be rectified by endogenous DNA repair mechanisms (Friedberg et al., 2006). Cells have developed several mechanisms to identify, remove, and repair DNA lesions. DNA repair enzymes, such as Endonuclease III (EndoIII), formamidopyrimidine (Fpg), or 8-oxoguanine-DNA glycosylase (Ogg1), can excise damaged bases (Friedberg et al., 2006). With increased oxidative stress, an adverse balance between DNA damage and repair can occur, resulting in cumulative un-repaired DNA damage. This accumulation could lead to altered gene transcription and the formation of altered proteins or the induction of apoptosis. Apoptotic chondrocytes are detected in 0-6% of OA articular chondrocytes (Aigner et al., 2001; Blanco et al., 1998; Kim et al., 2000; Kouri et al., 2000).

The goals of our study were to determine (i) if oxidative nuclear DNA damage increases in OA chondrocytes without cell culture compared with non-OA chondrocytes, (ii) the number of DNA breaks/chondrocyte associated with stage of OA, and (iii) if NO and $ONOO^-$ associated DNA damage in non-OA chondrocytes are able to undergo full endogenous repair *in vitro*. The results of these findings might indicate if accumulated nuclear DNA damage in articular cartilage is associated with the pathogenesis of OA.

MATERIALS AND METHODS

Tissue Collection

Pigs can develop spontaneous OA (Reiland, 1978; Turner et al., 1991). Synovial fluid was aspirated from porcine knee joints and centrifuged at 2500 rpm for 20 minutes to remove any cells. The supernatant was removed and stored at -20°C for the measurement of NO_x. Articular cartilage from the medial femoral condyles of skeletally mature pigs was graded macroscopically for the grade of OA using the Collins scale (Collins and McElligott, 1960). The Collins scale ranges from Grades 0-4, where Grade 0 is normal and smooth articular cartilage, Grade 1 has superficial flaking of the articular surface, Grade 2 has more extensive damage without denuded bone, and Grades 3 and 4 have no chondrocytes present. For each joint, full thickness articular cartilage with the most macroscopically severe OA was removed and a sample was saved for further histological analysis using the Modified Mankin Grading system for OA (van der Sluijs et al., 1992). Articular chondrocytes obtained from the same region that the histology sample had been obtained were enzymatically isolated using pronase and collagenase in Dulbecco's Modified Eagle's Media with 10% fetal bovine serum, 10 mM HEPES buffer, 0.01 mM essential amino acids, and 10 U/mL penicillin/streptomycin (Gibco, Gaithersburg, MD). The level and type of DNA damage were determined using the comet assay immediately after isolation to avoid any cell culture.

Single Cell Gel Electrophoresis

DNA damage was assessed using a single cell gel electrophoresis assay, the comet assay (Collins et al., 1997; Davies et al., 2008). Chondrocytes were embedded in agarose on microscope slides (Trevigen, Gaithersburg, MD). The nuclear and cellular membranes were lysed, and the DNA was treated with an alkaline solution (pH>13) to denature the DNA prior to electrophoresis. Nuclear DNA was stained with SYBR® green I. DNA damage was visualized using confocal microscopy as a "comet," with fragmented DNA (tail) being separated from undamaged nuclear DNA (head). DNA damage was quantified by calculating the "Olive Tail Moment" (OTM) (Konca et al., 2003). The presence of oxidative nuclear DNA damage was determined using the qualitative modified comet assay by incubating the nuclei with 1 µg/ml of DNA glycosylases (Trevigen) Endonuclease III (EndoIII), Formamidopyrimidine (Fpg), or 8-oxoguanine-DNA glycosylase (Ogg1) for 1 hr at 37°C, as part of the modified comet assay. Each assay was performed on a minimum of 3 pigs and analysis was performed on 50 comets per group.

Comet Analysis to Calculate the OTM

DNA damage was quantified by two methods. Analysis using the CASP™ software (Konca et al., 2003) measured the size of the comet head and tail to determine the OTM using the center of mass, area, and the sum of the pixel intensities. A caveat to the CASP™ software is the assumption that the comet head is larger in cumulative pixel intensity than the tail. Nuclei with extensive DNA damage may have more intense staining in the tail, resulting in an incorrect OTM using the CASP™ program. For these nuclei, a combination of the CASP program and ImageJ (NIH) was used. The CASP™ program could individually find the diameter of the head and tail. These values were used in the ImageJ program to create the head and tail regions of interest (ROI) and determined the comet characteristics of center of gravity, area, and cumulative intensity to measure the OTM. The same size ROI was used to ensure consistency in the measuring techniques across software programs.

Histology

Full thickness cartilage explants without calcified cartilage were fixed in 4% paraformaldehyde at 4°C, dehydrated in graduated levels of alcohol, and embedded in paraffin. 8 µm thick sections were cut and stained with Harris hematoxylin with glacial acetic acid (Poly Scientific, New York, NY), 0.02% fast green FCF in ethanol (Sigma-Aldrich, St. Louis, MO), and safranin-O (Sigma-Aldrich, St. Louis, MO). The samples were graded, according to the Modified Mankin System (van der Sluijs et al., 1992), by 5 blinded observers.

Cell Viability

In the recovery experiments, chondrocyte viability was determined using the Live/Dead assay (Molecular Probes, Carlsbad, CA) after treatment and recovery.

NOx Assay

NO production was assessed by determining the levels of total nitrate and nitrite, (termed “NOx”), in the media. Nitrate was first converted to nitrite using nitrate reductase and the total nitrite was measured using the Griess reagent (Fermor et al., 2001).

DNA Quantification

The total DNA in porcine chondrocytes was measured using the fluorescent picoGreen dsDNA quantification assay (Molecular Probes, Eugene, OR).

X-ray Dosing

Non-OA chondrocytes were enzymatically isolated using pronase and collagenase, embedded in agarose, placed on ice, and exposed to 0-12 Gray (Gy) of x-rays. The radiographic system used for this work included a 65 kW generator (EMD EPS 65RF) with a 0.3-1.0 mm focal spot W anode tube (SRO 0950 ROT 350, Philips Medical Systems, Hamburg, Germany). X-ray exposure was determined using an ionization chamber (Electrometer Model 9015, Probe 10×5-6, MDH Radcal, Monrovia, CA). Exposure (Roentgens) was converted to dose (Gray) using an f-factor of 0.9312 (Lin and Samei, 2006). The appropriate x-ray techniques (mAs at 70 kVp) were empirically tuned to produce 0-12 Gy of x-ray dose (Rossler et al., 2006). Immediately after x-ray exposure, levels of DNA damage were determined using the comet assay and the number of breaks per nucleus was calculated. X-ray dosing was repeated on chondrocytes isolated from 3 pigs.

Recovery Experiment

Articular chondrocytes were isolated from macroscopically non-OA full thickness articular cartilage, suspended in alginate (4×10^6 cells/ml), and cultured in media (DMEM, 10% FBS) at 37°C, 5% CO₂, and 95% air for 3 days. The chondrocytes embedded in alginate were then cultured for 24 hrs with either NOC-18 (0, 250, 500 µM), an NO donor, or SIN-1 (0, 250, 500 µM), an ONOO⁻ donor. Levels of DNA damage were measured using the comet assay after 24 hours treatment and 5 days after the media containing NO donors had been removed and replaced with fresh media. The amount of DNA damage using OTM was calculated for 50 nuclei from 3 different pigs.

Statistical Analysis

Statistical significance for the NOx assay was determined by the student's t-test assuming unequal variance. Statistical significance for the comet assays and modified comet assays was determined by analysis of variance (ANOVA) with Tukey post-hoc comparisons.

RESULTS

Levels of DNA damage associated with severity of OA

To determine if the DNA damage in OA accumulated over disease severity, the levels of DNA damage associated with different Mankin grades of OA were measured. A statistically significant increase in DNA damage, as determined by the OTM, was associated with OA chondrocytes in joints with modified Mankin Grades 3 OA or greater compared with non-OA chondrocytes (Figure 1).

Oxidative DNA damage in OA

In order to measure the type of DNA damage present in OA, DNA repair enzymes EndoIII, Fpg, or Ogg1 were added to OA nuclei during the comet assay to determine if a specific glycosylase could excise damaged bases. Excision of damaged bases by specific glycosylases formed abasic sites that were converted to strand breaks under alkaline conditions, increasing the OTM. When DNA repair enzymes were used in the modified comet assay, EndoIII, Fpg, or Ogg1 caused a significant increase in the OTM compared with OA chondrocytes without DNA glycosylases, demonstrating increased oxidative base DNA damage in OA (Figure 2). EndoIII excises oxidized pyrimidines, Fpg excises oxidized purines, and Ogg1 excises 8-oxoguanine.

Levels of NO associated with OA

A significant increase ($p < 0.05$) in NOx levels occurred in the synovial fluid of pigs with macroscopic OA (Collins Grades 1 or 2; $39.32 \pm 5.10 \mu\text{M}$, Mean $\pm$ SEM) compared with non-OA (Collins Grade 0; $14.17 \pm 3.78 \mu\text{M}$, Mean $\pm$ SEM).

DNA levels in porcine chondrocytes

The amount of DNA in porcine chondrocytes was $5.44 \pm 0.08 \text{ pg DNA/chondrocyte}$, which is equivalent to $5 \times 10^6 \text{ kB of DNA/chondrocyte}$.

Calibration of DNA damage with x-ray damage

The amount of DNA damage present in OA porcine chondrocytes was calibrated to levels of DNA damage induced by x-ray irradiation, as determined by the comet assay (Figure 3). From the calibration curve, the OTM can be predicted by the equation:

$$\text{OTM} = (1.4996\text{Gy}) + 2.738, \text{ where } r^2 = 0.985.$$

The number of DNA breaks calculated from 0 Gy exposure were assumed to be baseline nuclei undamaged by x-ray exposure.

The percentages of nuclei containing DNA damage greater than the mean value of the non-OA chondrocytes were calculated (Table 1). OA chondrocytes (Mankin Grades 3-5 and 6-7) showed a significant increase in the percentage of chondrocytes with increased DNA damage compared to non-OA chondrocytes (Mankin Grade 0). Using the x-ray calibration curve, the number of DNA breaks per chondrocyte, the percentage of nuclei with DNA damage greater than the mean value of the non-OA chondrocytes (Mankin Grade 0), and the increase in the number of DNA breaks per 1000 kB of DNA were calculated for different grades of OA (Table 1). These nuclei that contained DNA damage were compared to the nuclei of non-OA chondrocytes by calculating the approximate increase in the number of breaks (or damaged DNA bases) using the conversion factor $1 \text{ Gy} \approx 0.31 \text{ breaks}/10^9 \text{ Da of DNA}$ (Collins et al., 1997), which is equivalent to $0.0155 \text{ breaks}/1000\text{kB}$. Assuming a

chondrocyte contains 5×10^6 kB of DNA, 1 Gy causes 1016 DNA breaks/chondrocyte. The number of nuclei with increased DNA strand breaks per chondrocyte and the number of breaks/1000 kB of DNA increased significantly compared to Grade 0 chondrocytes. There was no statistical difference in the number of DNA strand breaks per chondrocytes between Mankin Grades 1-2 and Mankin Grades 3-5, but the number of breaks per chondrocyte showed a 3-fold increase for Grades 6-7 compared with the early grades of OA (Grades 1-2).

Endogenous DNA repair in NO and ONOO⁻ treated non-OA chondrocytes

To determine if DNA damage induced by NO donors in non-OA chondrocytes could undergo full recovery *in vitro*, DNA damage was induced by treatment with NO or ONOO⁻. The NO donor NOC-18 caused significant DNA damage in a concentration dependent manner. After 5 days of recovery, NOC-18 treated chondrocytes still contained significant DNA damage compared to controls (Figure 4). Similarly, treatment with ONOO⁻ donor SIN-1 resulted in significant DNA damage after 24 hrs and after 5 days of recovery (Figure 4). Cell viability was greater than 95% for all treatments at all time points (data not shown).

DISCUSSION

Our data suggest significant levels of oxidative DNA damage occur in OA compared with non-OA articular chondrocytes when not exposed to cell culture. These data further characterize the increased DNA damage that occurs in OA chondrocytes previously reported when cultured in alginate (Davies et al., 2008). Our data also suggest levels of DNA damage are greatest in the later stages of OA investigated, and that NO or ONOO⁻ induced DNA damage *in vitro* cannot undergo complete endogenous repair by 5 days. Therefore, the DNA damage in OA cartilage may accumulate due to increased ROS, such as NO and ONOO⁻ that are associated with OA.

The total amount of DNA damage in Mankin Grades 1-2 chondrocytes was not significantly increased compared to non-OA chondrocytes, but Grades 3-5 showed an approximate 50% increase and Grades 6-7 demonstrated a 3-fold increase compared to non-OA chondrocytes (Grade 0). In contrast, the percentage of OA chondrocytes with DNA damage did not show the same trend. 50% of the early Grade OA chondrocytes (Mankin Grade 1-2) contained DNA damage compared to non-OA chondrocytes. The percentage increased by 27% in Grades 3-5 over early OA, but there was little further increase in the percentage of chondrocytes with DNA damage in Grades 6-7 compared with Grades 3-5 chondrocytes. However, when the number of DNA strand breaks per nuclei were examined in only the damaged chondrocytes, the number of breaks were constant in the early stages of OA (Grades 1-5), but there was a 3-fold increase in the number of breaks in the damaged chondrocytes in grades 6-7 compared with the early stages of OA. These data might suggest that the endogenous DNA repair mechanisms can repair low levels of DNA damage, but when the chondrocytes contain very large amounts of DNA damage, endogenous repair mechanisms either become saturated or inactivated by nitration or nitrosylation of the repair proteins.

The percentage of chondrocytes containing DNA damage in all stages of OA in this study is in excess of varied reports of the percentage of OA chondrocytes undergoing apoptosis, which ranges from 0-6% (Aigner et al., 2001; Blanco et al., 1998; Kim et al., 2000; Kouri et al., 2000). The variation in the numbers of apoptotic chondrocytes detected in OA has been attributed to different techniques used to measure apoptosis (Aigner and Kim, 2002). However, since the percentage of apoptotic cells in previous studies are estimated to be orders of magnitude lower than those with DNA damage observed in this study, apoptosis alone is unlikely to explain the occurrence of DNA damage in OA. The DNA damage might

be associated with autophagy, which is suggested to occur in articular cartilage (Aigner et al., 2007).

Alternatively, the occurrence of DNA damage could have other biological consequences. The potential biological significance of articular chondrocytes inheriting damaged DNA is limited, since minimal cell division occurs in articular cartilage, apart from a small amount of cell cloning that occurs during the late grades of OA (van der Sluijs et al., 1992). The significance of the observed DNA damage may be partially via altered gene transcription, as observed in neurons in certain neurological diseases (Ishibashi et al., 2005). Free radical-induced DNA damage can modify transcription, causing transcription errors that result in erroneous protein formation or blocked transcription in other cell types (Doetsch, 2002; Ishibashi et al., 2005). In chondrocytes, the production of abnormal proteins could potentially contribute to the events leading to extracellular matrix breakdown.

Abnormal protein production could be a result of oxidative DNA damage in OA chondrocytes, as suggested by our data using the DNA repair enzymes in the modified comet assay. It is possible that the oxidative DNA damage occurs as a result of increased reactive oxygen species, such as NO and ONOO⁻, that occur in OA joints and are associated with chondrocytes, meniscus, and synovial fluid (Weinberg et al., 2007). In order to mimic the increased reactive species present in OA synovial fluid demonstrated by this and other studies (Karan et al., 2003), non-OA chondrocytes were treated with representative NO or ONOO⁻ donors. Our data showed that NO donor induced DNA damage was in excess of the ability of chondrocytes to undergo repair, under these culture conditions, in a 5-day period *in vitro*.

Apoptosis is an unlikely explanation for the significant DNA damage in chondrocytes treated with NO donors *in vitro*, since minimal cell death was observed under these conditions. Additionally, DNA damage is an early event in the apoptotic process, and the time course for the apoptotic process should be completed within the 5 days observed in our study. The lack of cell death is in agreement with previous studies showing that NOC-18 did not cause chondrocyte cell death (Del Carlo and Loeser, 2002). SIN-1 can cause significant cell death in human chondrocytes cultured in serum free media (Del Carlo and Loeser, 2002), but SIN-1 did not cause cell death in our system using serum. The ability to undergo endogenous repair was lower for peroxynitrite than NO induced DNA damage. These data support the findings of others, suggesting that peroxynitrite can be more damaging to a cell than NO (Pacher et al., 2007). It must be noted that not all ROS induced DNA damage has been investigated in this study. It is also possible the NO donor is not the direct cause of the DNA damage, but might be via secondary mediators.

A limitation of the recovery study is that some oxidative DNA damage may have occurred in the recovery experiments as a result of cell culture, possibly preventing recovery by 5 days (Martin et al., 2004). Culture of chondrocytes at reduced oxygen tension can reduce oxidative DNA damage (Martin et al., 2004) and alter the production of reactive oxygen species (Cernanec et al., 2002; Cernanec et al., 2007; Mathy-Hartert et al., 2005). However, our controls were under the same culture conditions as the experimental groups, and our experimental groups had significantly more DNA damage.

There is some variation in the levels of DNA damage in the different groups of non-OA chondrocytes. Our previous work identifies an initial high level of endogenous NO production immediately after excision from the joint that stabilizes to a lower baseline level after 3 days (Fermor et al., 2001). In the OA and oxidative DNA studies, neither the non-OA nor OA chondrocytes were placed in cell culture prior to DNA analysis. However, it was necessary to use cell culture in the recovery experiments to culture chondrocytes for 3 days,

which stabilized the baseline level of NO prior to exposing the non-OA chondrocytes to the NO donors. The differences in culture conditions and timing of experiments could possibly alter the baseline level of DNA damage in non-OA chondrocytes, resulting in a higher OTM for chondrocytes that did not undergo cell culture. The improved quantification of OTM using ImageJ and CASP is able to detect larger OTMs than was possible in earlier studies (Davies et al., 2008). Additionally, the levels of DNA breaks per chondrocyte were calculated from the x-ray calibration curve under certain experimental conditions. Therefore, these data are only representative estimates of the numbers of breaks per chondrocyte.

In summary, our study demonstrates that oxidative DNA damage in OA increases with the severity of OA and chondrocyte DNA damage induced by nitric oxide and peroxynitrite in non-OA chondrocytes does not undergo complete endogenous repair. The increased levels of DNA damage and the reduction of endogenous DNA repair may hinder the ability of the chondrocytes to maintain cartilage health, thus contributing to the pathogenesis of OA.

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Figure 1.

Levels of nuclear DNA damage in pig articular cartilage associated with different histological grades of OA, as measured by the modified Mankin Score, (a) Representative histology, (b) representative comets and (c) Olive Tail Moment (OTM). Mean $\pm$ SEM, $N \geq 3$ (Grade 0, $N = 3$; Grades 1-2, $N = 9$; Grades 3-5, $N = 4$; Grades 6-7, $N = 7$), $n = 50/\text{group}$. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 2. Determination of oxidative nuclear DNA damage in OA. DNA repair enzymes Endonuclease III (EndoIII), formamidopyrimidine (Fpg), or oxoguanine glycosylase-1 (Ogg1) incubated with OA nuclei in the modified comet assay determined the presence of oxidative DNA damage in OA. Mean $\pm$ SEM, N = 3, n = 50/group. *** = $p < 0.001$.

Figure 3. Calibration of DNA damage levels with x-ray damage. Non-OA porcine articular chondrocytes were exposed to 0-12 Gy of x-ray irradiation and the levels of DNA damage were measured by OTM. $OTM = 1.4996 \text{ Gy} + 2.738$, $r^2 = 0.985$. Mean $\pm$ SEM, $N = 3$, $n = 50$ /group.

Figure 4.

Levels of increased DNA damage and incomplete endogenous DNA repair in articular chondrocytes in alginate treated with either NOC-18 or SIN-1 for 24 hrs, followed by 5 days recovery without NO donors in the media. Mean $\pm$ SEM, N = 3, n = 50/group. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 1

Calculation of percentage of nuclei with DNA damage, number of DNA strand breaks/nuclei, and number of breaks per 1000kB of DNA compared with severity of OA, as described by the modified Mankin Grade.

Grade	% chondrocytes with DNA damage	Number of DNA strand Breaks per chondrocyte	Number of Breaks/ 1000 kB of DNA
1-2	59.9 ± 8.2	3465 ± 233 ***	0.65 ± 0.04 ***
3-5	86.9 ± 1.8 **	3257 ± 218 **	0.61 ± 0.04 **
6-7	88.5 ± 5.7 ***	9352 ± 519 ***	1.7 ± 0.1 ***

Mean ± SEM

**
= p<0.01

= p<0.001, compared with control.