OXIDATIVE STRESS INHIBITS THE PROLIFERATION, INDUCES PREMATURE SENESCENCE AND PROMOTES A CATABOLIC PHENOTYPE IN HUMAN NUCLEUS PULPOSUS INTERVERTEBRAL DISC CELLS

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Abstract

Aged and degenerated intervertebral discs are characterised by a significant increase in the number of senescent cells, which may be associated with the deterioration of this tissue due to their catabolic phenotype. On the other hand, carboxymethyl-lysine has been found to be accumulated with ageing in the proteins of the disc, evidencing the existence of oxidative stress in this tissue. Accordingly, here we investigated the effect of oxidative stress on the physiology of human nucleus pulposus cells. Hydrogen peroxide (H_2O_2) at subcytotoxic concentrations transiently increased the intracellular levels of reactive oxygen species, activated the p38 MAPK, ERKs, JNKs and Akt signalling pathways and induced the nuclear translocation of NF-κB and Nrf2. It also provoked DNA damage and triggered a DNA repair response by activating the ATM-Chk2-p53-$p21^{WAF1}$-pRb pathway, ultimately resulting in a G1 cell cycle delay and the decrease of cells' proliferation. Prolonged exposure to H_2O_2 led to premature cellular senescence, as characterised by the inhibition of proliferation, the enhanced senescence-associated β galactosidase staining and the over-expression of known molecular markers, without though a significant decrease in the chromosome telomere length. H_2O_2-senescent cells were found to possess a catabolic phenotype, mainly characterised by the up-regulation of extracellular matrix-degrading enzymes (MMP-1, -2, -9 and ADAMTS-5) and the down-regulation of their inhibitors (TIMPs), as well as of several proteoglycans, including aggrecan, the major component of the nucleus pulposus. The senescent phenotype could be reversed by N-acetyl-L-cysteine, supporting the use of antioxidants for the improvement of disc physiology and the deceleration of disc degeneration.

Keywords: oxidative stress, H_2O_2, intervertebral disc nucleus pulposus cells, MAPKs, Akt, Nrf2, NF-κB, ROS, ATM, senescence.

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Introduction

Low back pain affects the majority of the population worldwide. The high prevalence of the disease leads to a great financial burden imposed to the health care system for prevention and treatment, while the indirect costs due to absence from work or reduced productivity are also considerable (Katz, 2006). Given its socioeconomic impact, it is of importance to understand the causing factors of this spinal disorder. Even though its aetiology is not yet fully elucidated, it has often been associated with intervertebral disc degeneration (Luoma *et al.*, 2000; Urban and Roberts, 2003).

Intervertebral discs lie between vertebrae and mainly consist of extracellular matrix (ECM), in which a low number of cells are embedded. Two types of intervertebral disc cells are distributed in the two distinct regions of the tissue, *i.e.* fibroblast-like cells in the outer filamentous annulus fibrosus and chondrocyte-like cells in the inner gel-like nucleus pulposus. The intervertebral disc is characterised by low cellularity due to the prevailing stressful environmental conditions that strictly hold down cell population in a milieu where the provision of nutrients is restricted. However, despite their low numbers, cells are very important for the preservation of a dynamic equilibrium between synthesis and degradation of the disc's ECM, a fundamental characteristic of a healthy tissue. Disturbance of this equilibrium caused by the combination of decreased biosynthesis of ECM structural components and increased degrading enzymatic activity is typical for disc degeneration (Le Maitre *et al.*, 2004; Le Maitre *et al.*, 2006; Le Maitre *et al.*, 2007b; Roberts *et al.*, 2006b). Recently, the presence of a high number of senescent cells has been shown in the intervertebral disc (Freemont, 2009; Gruber *et al.*, 2007; Le Maitre *et al.*, 2007a; Roberts *et al.*, 2006a). Nevertheless, the stimuli leading to this senescent phenotype have not yet been fully elucidated (Kletsas, 2009).

It has been reported that reactive oxygen species (ROS) are produced in the form of hydrogen peroxide (H_2O_2) in nucleus pulposus cells *in vivo* (Kim *et al.*, 2009). In addition, advanced glycation end products (AGEs), such as the carboxymethyl-lysine (CML) formed by the oxidation-derived carboxymethyl modification of lysine (Nerlich *et al.*, 1997) are known to accumulate in proteins of the intervertebral disc (Sivan *et al.*, 2006; Vo *et al.*, 2013) and their concentration has been shown to raise with advancing age and/or disc degeneration, especially in the nucleus pulposus (Nerlich *et al.*, 2007). On the other hand, increased levels of ROS have been incriminated with the

induction of premature cellular senescence in many cell types (Lu and Finkel, 2008; Passos and von Zglinicki, 2006; Toussaint *et al*., 2000).

Even though oxidative stress can regulate proliferation and provoke premature senescence in various cell types, little is known about the role of this type of stress in intervertebral disc cells. For that reason and given the documented presence of H_2O_2 in the disc *in vivo*, and especially in the nucleus pulposus, here we assessed the effect of this oxidative agent on the proliferation and induction of senescence in human nucleus pulposus intervertebral disc cells *in vitro*. Additionally, the gene expression profile and the putative catabolic phenotype of the resulting after H_2O_2 treatment senescent cells were examined.

Materials and Methods

Isolation of human nucleus pulposus intervertebral disc cells and cell culture conditions

Human intervertebral disc cells used in this study were from a pre-existing cell bank of our laboratory established by Pratsinis *et al*. (Pratsinis *et al.*, 2012). All cells were isolated from tissues acquired from consenting patients subjected to an orthopaedic operation. In brief, the nucleus pulposus was separated from the annulus fibrosus during surgery based on the different morphology of the two regions. After chopping under aseptic conditions and collagenase digestion, the released cells were recovered by centrifugation. The characterisation of nucleus pulposus and annulus fibrosus cells was performed based on the specific aggrecan and collagen II (markers for nucleus pulposus cells) and collagen I (marker for annulus fibrosus cells) gene expression, respectively (data not shown). Cultures were maintained in DMEM containing antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Biochrom AG, Berlin, Germany) and 20 % (v/v) FBS (Gibco BRL, Invitrogen, Paisley, UK) and subcultured when confluent with a trypsin/citrate (0.25 %/0.30 %, w/v) solution.

Estimation of reactive oxygen species' production

The 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) assay was used for the measurement of ROS production (Mavrogonatou *et al.*, 2010) in nucleus pulposus cells treated with H_2O_2. Cells were plated in quadruplicates in 96-well plates with DMEM containing 20 % (v/v) FBS until ~ 90 % confluence. H_2O_2 (Sigma, St. Louis, MO, USA) was added in concentrations from 0 to 1 mM along with 10 µM of DCFH-DA (Sigma). The kinetics of ROS production up to 72 h (as a % ratio of the untreated control) was monitored using the Infinite 200 Tecan microtiter-plate photometer (Tecan Trading AG, Switzerland).

Estimation of cell viability, proliferation and cell cycle progression

The effect of different concentrations of H_2O_2 (0-2 mM) on the viability of human nucleus pulposus cells was investigated using the MTT assay, as previously described (Mavrogonatou *et al.*, 2014). Briefly, cells were plated in quadruplicates in DMEM containing 20 % (v/v) FBS in 96-well plates until ~ 90 % confluence before the addition of H_2O_2 for 72 h. Cell viability was estimated using the ratio of OD_{550} of H_2O_2-treated cells to the untreated controls.

The proliferation of nucleus pulposus cells exposed to H_2O_2 treatment was assessed with the DNA synthesis assay, as reported earlier (Mavrogonatou and Kletsas, 2009). In detail, cells were grown in 48-well plates in DMEM supplemented with 20 % (v/v) FBS until 80-90 % confluence and 0-2 mM H_2O_2 along with 0.1 µCi/mL [methyl-^{3}H]-thymidine (Amersham Biosciences,

Table 1. Real-time PCR primers

Target gene	Forward Primer	Reverse Primer
$p16^{INK4}$	TAGTTACGGTCGGAGGCCGAT	GCACGGGTCGGGTGAGAG
$p21^{WAF1}$	CTGGAGACTCTCAGGGTCGAA	CCAGGACTGCAGGGTTCCT
Catalase	CCTTTCTGTTGAAGATGCGGCG	GGCGGTGAGTGTCAGGATAG
SOD	TGCAGTCTGAAGAGCTATCTC	TGAACGTCACCGAGGAGAAGT
COX2	CCTGTGCCTGATGATTGC	CTGATGCGTGAAGTGCTG
ADAMTS-4	AGAGACAAAGATCCAGGAAAGG	GGCTGAGGACCGTAAAGG
ADAMTS-5	TGTCCTGCCAGCGGATGT	ACGGAATTACTGTACGGCCTACA
MMP-1	CCTTCTACCCGGAAGTTGAG	TCCGTGTAGCACATTCTGTC
MMP-2	AAGAACCAGATCACATACAGGATCA	GTATCCATCGCCATGCTCC
MMP-9	GCACGACGTCTTCCAGTACC	TCAACTCACTCCGGGAACTC
MMP-13	TTGAGCTGGACTCATTGTCG	GGAGCCTCTCAGTCATGGAG
TIMP-1	AAGGCTCTGAAAAGGGCTTC	GAAAGATGGGAGTCGGAACA
TIMP-2	CGACTGGTCCAGCTCTGAC	ACCCACAACCATGTCTAAAAGG
TIMP-3	GCAGATAGACTCAAGGTGTGTGAAA	TCCCTCACTCTTACATGCAGACA
ACAN	AGAAGAGAGCCAAACAGC	TTGTGGTTGCCTCTGAAT
Biglycan	TCTGTCACACCCACCTACAGC	AGGGGAGATCTCTTTGGGCAC
Decorin	CCTGATGACCGCGACTTCGAG	TTTGGCACTTTGTCCAGACCC
Versican	ACGGGATTGAAGACACACAAG	AGCCTCAAAATTCAGTGTGTA
GAPDH	GAGTCCACTGGCGTCTTC	GCATTGCTGATGATCTTGAGG

Buckinghamshire, UK) were added for 24 h. After fixation of the cells and DNA solubilisation, tritiated thymidine incorporation into DNA was estimated by scintillation counting.

Cell cycle analysis was performed in cells fixed with 50 % (v/v) ethanol after staining with propidium iodide (Sigma), as previously described (Mavrogonatou and Kletsas, 2009). A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was used for the experiments, while the analysis was made with Cell Quest (Becton Dickinson) and Modfit (Verity Software House, Topsham, ME, USA) software.

Western blot analysis

Protein samples for SDS electrophoresis were collected as reported before in a Laemmli sample buffer containing protease and phosphatase inhibitors (Sigma) (Mavrogonatou and Kletsas, 2009). Western blot analysis was performed using the following antibodies: anti-PARP, anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-SAPK/JNK, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-Chk2 (Thr68), anti-phopsho-p53 (Ser15) (all purchased from Cell Signalling Technology, Hertfordshire, UK), anti-phospho-ERK1/2 (Thr202/Tyr204), panERK, anti-$p21^{WAF1}$ (all supplied by BD Transduction Laboratories, Bedford, MA, USA), anti-phospho-ATM (Ser1981), anti-p53 (both supplied by Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ATM (Merck Millipore, Billerica, MA, USA), anti-$p16^{INK4a}$ and anti-pRb (both purchased from BD Pharmingen, San Diego, CA, USA). Anti-α-tubulin, as well as the secondary horseradish peroxidase-conjugated antibodies were obtained from Sigma. All primary antibodies were used in a 1:1000 dilution, except for anti-$p16^{INK4a}$ (1:400) and anti-pRb (1:250). The enhanced chemiluminescence (ECL) reagent used for visualisation of the results was from Amersham Biosciences.

Immunofluorescence

For the immunofluorescence experiments assessing the phosphorylation of H2A.X on Ser139 and the localisation of the p65 subunit of NF-κB and of Nrf2, cells were grown on glass coverslips before treatment. Fixation and labelling were performed as described previously (Mavrogonatou and Kletsas, 2012) using antibodies against phospho-H2A.X (Ser139) (Upstate Biotechnology, Lake Placid, NY, USA), the p65 subunit of NF-κB and Nrf2 and a FITC-conjugated IgG (all from Santa Cruz Biotechnology). Counter-staining was performed with 2 μg/mL 4′, 6-diamidino-2-phenylindole (DAPI) dihydrochloride (Sigma).

The proliferative potential of cells was estimated after dual labelling with 5-bromo-2′-deoxyuridine (BrdU) and DAPI, as previously reported (Pratsinis and Kletsas, 2007) with slight modifications. In brief, cells were permeabilised with 0.2 % Triton X-100 in PBS after fixation, incubated with 2 N HCl, labelled with an anti-BrdU-FITC antibody (Roche Applied Science, Mannheim, Germany) for 16 h and counter-stained with 2 μg/mL DAPI.

In all cases, labelled cells were visualised using a Zeiss AxioPlan 2 microscope (Carl Zeiss, Jena, Germany). For the quantification of BrdU incorporation, approximately a total of 200 cells from at least two different fields were counted.

Evaluation of H_2O_2-induced premature senescence

For the evaluation of H_2O_2-induced premature senescence in human nucleus pulposus intervertebral disc cells, several protocols were tested (data not shown). The protocol that was ultimately applied included four consecutive exposures of confluent cultures to 500 μM H_2O_2 in DMEM supplemented with 10 % (v/v) FBS, with a 3 d interval between two sequential exposures to H_2O_2. The final plating of the cells for the assessment of markers of senescence was made three days after the last exposure to H_2O_2. Whenever necessary, cells were pre-incubated with 2 mM N-acetyl-L-cysteine (NAC, Sigma) for 16 h before medium change to DMEM supplemented with 10 % (v/v) FBS containing H_2O_2 or DMEM supplemented with 10 % (v/v) FBS containing H_2O_2 and NAC.

Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal staining was performed as reported before (Konstantonis *et al.*, 2013). Briefly, cells were grown on glass coverslips and fixed with 3 % (v/v) formaldehyde in PBS. Fixed cultures were incubated with the SA-β-gal staining solution (40 mM citric acid /sodium phosphate pH 6.0, 150 mM NaCl and 2 mM $MgCl_2$ containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/mL X-Gal) for 24 h at 37 °C. Samples were observed under a Zeiss Axioplan 2 phase contrast microscope.

Telomere length assay

The measurement of the cells' telomere length was performed using the TeloTAGGG telomere length assay kit (Roche Applied Science) according to the manufacturer's instructions. In brief, 2 μg of genomic DNA extracted with the NucleoSpin Tissue XS kit (Macherey-Nagel, Düren, Germany) was digested with *Hinf*I and *Rsa*I and separated by agarose gel electrophoresis. Capillary southern transfer of the digested DNA to the positively charged nylon membrane Porablot NY plus (Macherey-Nagel) was done with 20× SSC buffer, while transferred DNA was fixed by baking at 120 °C. The membrane was hybridised with the telomere-specific digoxigenin (DIG)-labelled hybridisation probe and then incubated with an anti-DIG antibody. The generated luminescence signal after incubation with the substrate solution was captured by exposure of the membrane to an X-ray film. Densitometric analysis of the scanned X-ray film after overlaying each sample lane with a grid and subtracting the background was performed with the ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD). The mean terminal restriction fragments (TRF) length has been calculated using the formula:

$$meanTRF = \frac{\Sigma(ODi)}{\Sigma(\frac{ODi}{Li})}$$

where ODi is the chemiluminescent signal and Li is the length of the TRF at position i (Harley *et al.*, 1990).

Real-time PCR analysis

RNA extraction was done using the Macherey-Nagel NucleoSpin RNA kit following the manufacturer's instructions including an on-column DNase treatment step to remove any residual DNA. First-strand cDNA synthesis and real-time PCR were performed using the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan) and the KAPA SYBR universal fast master mix (KAPA Biosystems, Woburn, MA), respectively as reported earlier (Mavrogonatou *et al*., 2014). Primers used are listed in Table 1. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Mean Ct values of genes under investigation were normalised to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that served as the reference gene.

Statistical analysis

Experiments were conducted at least three times. Similar results were obtained in all assays using cells deriving from different donors. Data were tested for normality before the assessment of statistical significant differences using the parametric Student's *t* test or the non-parametric Mann-Whitney test with Statgraphics Centurion software (Manugistics Inc., Dallas, USA). Values presented are the means ± standard deviations. Differences were considered statistically significant when $p < 0.05$.

Results

Effect of H_2O_2 on the intracellular ROS production, viability and proliferation of human nucleus pulposus cells

In order to evaluate the extent of oxidative stress provoked by H_2O_2 in human nucleus pulposus cells, we assessed the differences in the intracellular ROS levels after treatment with several H_2O_2 concentrations using the DCFH-DA assay. Even the lowest concentration of H_2O_2 used (0.05 mM) led to a rapid significant increase in the production of ROS, while the concentration of 1 mM resulted in a > 3,500-fold increase of the intracellular ROS levels (Fig. 1). In all cases, H_2O_2-induced ROS production considerably increased at ~ 30 min of treatment and decreased to a great extent after 48-72 h. H_2O_2 slightly affected cell viability in concentrations up to 0.8 mM (no more than 20 % cell death in comparison to the untreated control) and only at concentrations ≥ 1 mM it abruptly decreased the viability of nucleus pulposus cells to ~ 10 % compared to the untreated control (Fig. 2A). H_2O_2-mediated cell death was due to apoptosis, as estimated by the formation of typical apoptotic bodies, as well as by the cleavage of poly (ADP-ribose) polymerase (PARP), a renowned marker for apoptosis (Fig. 2B). Finally, H_2O_2 was also found to drastically inhibit cell proliferation, as estimated by tritiated thymidine incorporation (Fig. 2C). Interestingly, although the H_2O_2 LD_{50} was found to be very near 1 mM for nucleus pulposus cells, the respective IC_{50} for cell proliferation proved to be much lower (between 125 and 250 μM). Based on these results, the selected concentration of H_2O_2 for the rest of our experiments was 500 μM, the highest H_2O_2 concentration with practically no cytotoxic effect after an exposure of the cells for 72 h.

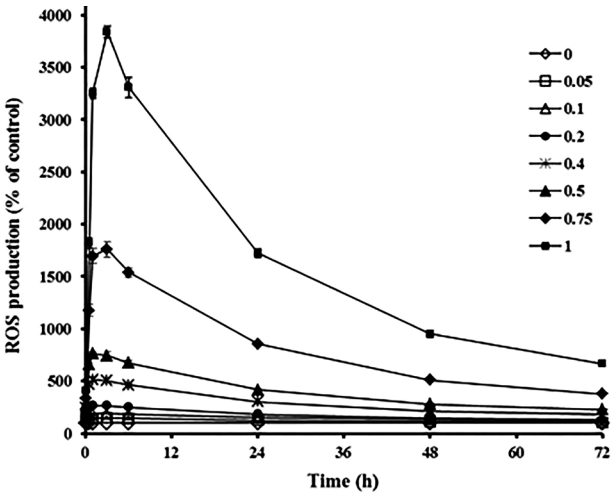

Fig. 1. Estimation of intracellular ROS levels in H_2O_2-treated human nucleus pulposus intervertebral disc cells. Cells were plated in a 96-well plate until ~ 90 % confluence. H_2O_2 was added in the designated concentrations along with DCFH-DA (10 μM) and measurements were recorded at several time points up to 72 h. Intracellular ROS levels were calculated by dividing the values of treated with H_2O_2 cells to those of untreated cells and are presented as a % ratio of the control. Results are expressed as mean values ± standard deviations. A representative experiment out of three similar ones performed in quadruplicates is presented here.

Mode of action of H_2O_2-induced oxidative stress in nucleus pulposus cells

We then attempted to explore the mechanisms underlying H_2O_2 action in our cell model. For that reason, we first assessed the phosphorylation status of the three members of the MAPK superfamily (p38 MAPK, ERKs and JNKs), as well as of Akt, which are all associated with cellular responses to stress and the regulation of cell proliferation and viability. All MAPKs and Akt were found to be rapidly and transiently phosphorylated by H_2O_2 (Fig. 3).

In addition, we investigated the putative genotoxic effect of oxidative stress on intervertebral disc cells by assessing the formation of phosphorylated H2A.X foci in the nuclei of the cells after treatment with H_2O_2. Indeed, exposure to 500 μM H_2O_2 resulted in the phosphorylation of H2A.X on Ser139 and the subsequent accumulation of the histone in the sites of DNA damage (Fig. 4A). The presence of H2A.X foci was obvious from 30 min to 24 h *post*-treatment. Nucleus pulposus cells responded towards the genotoxic outcomes of H_2O_2 by inducing a DNA repair mechanism, including the activation of ataxia telangiectasia mutated (ATM) kinase, which in turn led to the phosphorylation of Chk2 and p53 (Fig. 4B). In accordance to the observed reduction of proliferation mentioned above, the entire p53-$p21^{WAF1}$-pRb axis was found to be activated in nucleus pulposus cells by H_2O_2

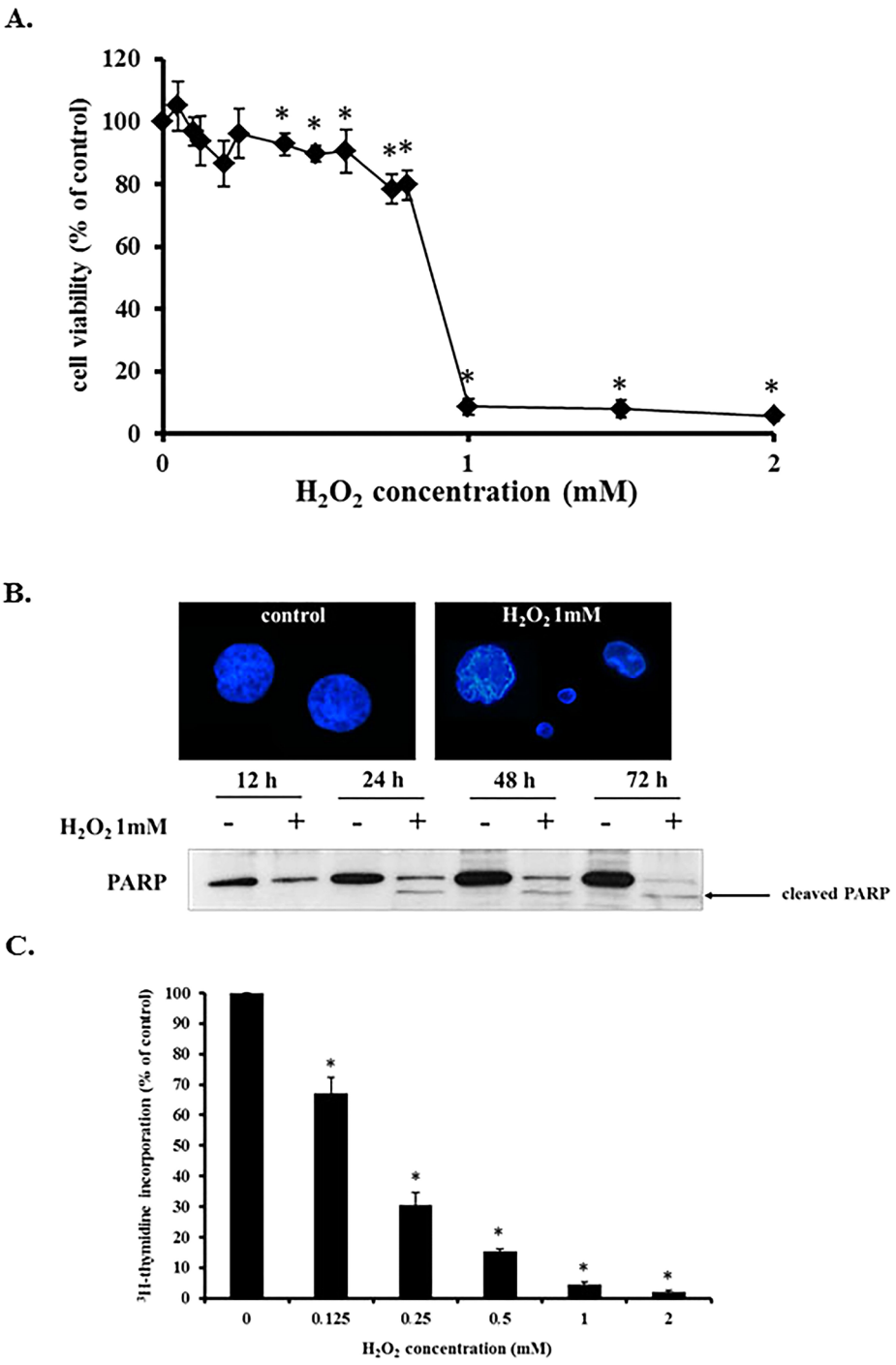

Fig. 2. Effect of H_2O_2 on the viability and proliferation of human nucleus pulposus cells. (**A**) Cells were grown in DMEM supplemented with 20 % (v/v) FBS and were exposed to several H_2O_2 concentrations (0-2 mM) for 72 h. Then MTT was added, cells were solubilised and optical density was measured at 550 nm. (**B**) Cells were exposed to 1 mM H_2O_2 for 72 or 12-72 h before fixation and DAPI labelling or protein extraction and western blot analysis, respectively. In the first case, samples were visualised under a fluorescence microscope, while in the second case samples were analysed using an antibody for PARP. (**C**) Cells were plated in DMEM supplemented with 20 % (v/v) FBS before the addition of 0-2 mM H_2O_2 along with 0.1 μCi/mL [methyl-^{3}H]-thymidine for 24 h. After fixation of the cells and DNA solubilisation, tritiated thymidine incorporation into DNA was estimated by scintillation counting. In (**A**) and (**C**) mean values ± standard deviations from three experiments are presented, while in (**B**) representative pictures are depicted. Statistically significant differences in comparison to the control (Student's *t*-test, $p < 0.05$) are shown by asterisks.

(Fig. 4B), leading to a cell cycle arrest at the G0/G1 phase, accompanied by a decrease in the percentage of the cells accumulating at the G2/M phase (Fig. 4C).

Finally, we examined the subcellular localisation of the transcription factors NF-κB and Nrf2 in nucleus pulposus cells exposed to 500 μM H_2O_2. We observed a nuclear translocation of both NF-κB p65 subunit and Nrf2 in H_2O_2-treated cells within 2 h of exposure (Fig. 5), indicating the activation of these transcription factors as a response to oxidative stress.

Long-term exposure to H_2O_2 induces premature senescence in human nucleus pulposus cells

We then followed by investigating the long-term effect of H_2O_2 on nucleus pulposus cells. We measured BrdU incorporation, which indicates the percentage of the putatively proliferating cells in a given population, as well as the SA-β-gal staining, a classical marker of senescence. We observed a dose-dependent relationship of both parameters with the number of exposures to H_2O_2 and a terminally senescent phenotype of the cells after the application of four consecutive exposures to 500 μM of H_2O_2 (Table 2 and Fig. 6). These senescent cells exhibited a 2 ± 1 % BrdU incorporation and a 80 ± 3 % positive SA-β-gal staining in comparison to the untreated control (in which the respective percentages were 67 ± 3 % and 15 ± 3 %, respectively) (Fig. 6A). It is worth mentioning that sequential exposures of human nucleus pulposus cells to low H_2O_2 concentrations (1-100 μM) also resulted in a dose-dependent but more modest reduction of BrdU

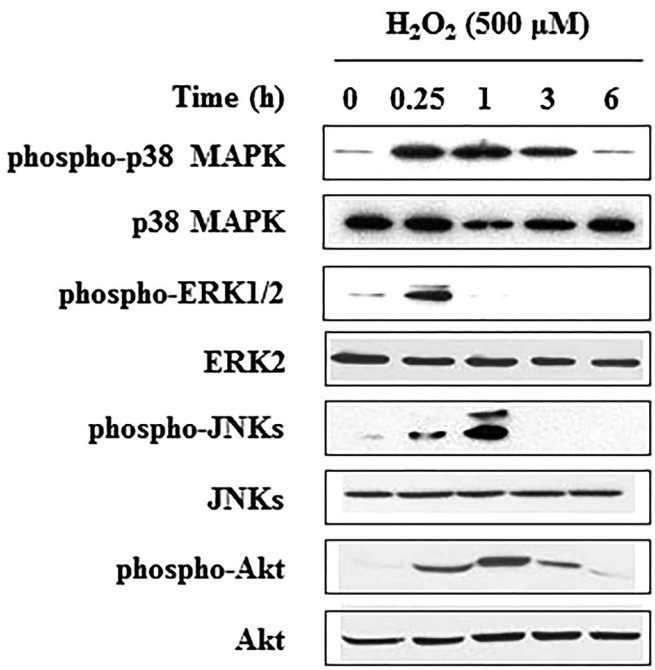

Fig. 3. Phosphorylation of MAPKs and Akt in H_2O_2-treated human nucleus pulposus intervertebral disc cells. Cells were grown in 60-mm dishes in DMEM supplemented with 20 % (v/v) FBS until ~ 90 % confluence before the addition of 500 μM H_2O_2 for the specified time periods. Western blot analysis was performed for the phosphorylated forms of p38 MAPK, ERKs, JNKs and Akt (Ser473). Blots for the non-phosphorylated forms of the kinases are presented to verify equal loading. Four experiments were conducted and representative blots are shown.

Table 2. Effect of serial exposures to H_2O_2 on the induction of senescence in human nucleus pulposus intervertebral disc cells

Number of exposures to H_2O_2	BrdU incorporation (%)	SA-β-gal-positive cells (%)
0	67 ± 3	15 ± 3
1	43.5 ± 5	20 ± 1
2	22.5 ± 4	34 ± 6
3	1 ± 1	45 ± 14
4	2 ± 1	80 ± 3

incorporation and increase in the percentage of SA-β-gal positively stained cells (data not shown).

Subsequently, we investigated the expression of known biochemical markers of senescence in order to characterise H_2O_2-senescent nucleus pulposus cells at the molecular level. In accordance to our findings concerning BrdU incorporation and SA-β-gal staining mentioned above, H_2O_2-senescent cells had higher expression levels of p53, $p16^{INK4a}$ and $p21^{WAF1}$ than control cells, while only the hypophosphorylated form of pRb was present (Fig. 6B), consistent with the observed ceased proliferation. Interestingly, when the mean TRF was measured (Fig. 6C), we found a small but non-significant decrease (p = 0.74) in H_2O_2-treated cells (*meanTRF* = 9.53 ± 1.36 Kb) in comparison to the control cells (*meanTRF* = 9.95 ± 0.09 Kb). As can be seen in Fig. 7A, incubation of nucleus pulposus cells with 2 mM NAC decreased significantly the H_2O_2-induced ROS levels. When we serially exposed cells to H_2O_2, the presence of 2 mM NAC was able to limit the percentage of senescent cells to a high degree (Fig. 7B), thus validating the ROS-mediated nature of the H_2O_2-induced senescence in nucleus pulposus cells.

Prematurely senescent nucleus pulposus cells by H_2O_2 express a catabolic phenotype

In an attempt to better characterise the H_2O_2-senescent nucleus pulposus cells, we investigated the expression profile of several genes that were either markers of senescence and oxidative stress response or encoded proteins regulating ECM production and degradation in the intervertebral disc. Real-time PCR analysis revealed the up-regulation of senescent markers, ROS-induced and ECM-degrading enzymes and the down-regulation of ECM structural components (Fig. 8). In detail, the mRNA levels of $p16^{INK4a}$ and $p21^{WAF1}$ were shown to increase ~ 3- and 5.5-fold, respectively in accordance to the senescent phenotype of H_2O_2-treated cells. In the set of genes encoding enzymes related to oxidative stress that were examined, two of them [superoxide dismutase (SOD) and cyclooxygenase 2 (COX2)] were found to present increased expression levels (~ 2-fold), while one (catalase) was down-regulated. Finally, the produced catabolic phenotype of H_2O_2-senescent nucleus pulposus cells was supported by the up-regulation of several ECM-degrading enzymes at the same time that genes encoding components of the disc ECM had lower mRNA levels than the control cells. More specifically, ADAMTS-5, MMP-1,

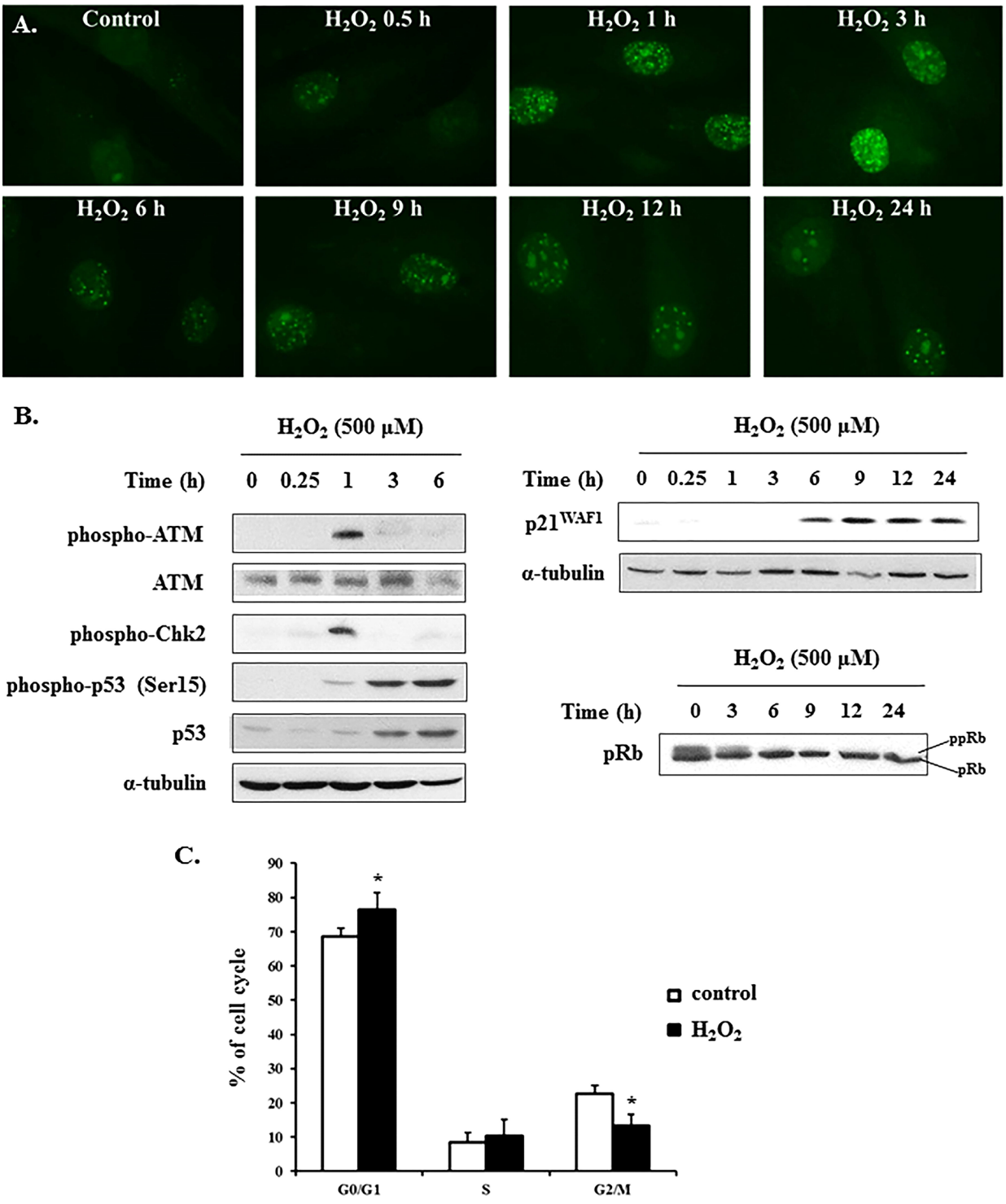

Fig. 4. H_2O_2 is genotoxic and triggers a DNA repair response to human nucleus pulposus cells. (**A**) Cells were plated on glass coverslips and treated with 500 µM H_2O_2 for the time points shown. After fixation, samples were labelled with an antibody for the phosphorylated form of histone H2A.X on Ser139 followed by an incubation step with an anti-rabbit FITC-conjugated secondary antibody. Representative microscopy images are shown here. (**B**) Cells were treated with H_2O_2 and protein extraction was performed at the designated time points. Samples were analysed by western blotting for the phosphorylated form of ATM on Ser1981, the phosphorylated form of Chk2 on Thr68, the phosphorylated form of p53 on Ser15, $p21^{WAF1}$ and pRb. Western blot analysis for α-tubulin served as the loading control. Experiments were repeated there times and representative blots are depicted. (**C**) Cells were treated with 500 µM H_2O_2 for 24 h before fixation. Fixed cells were stained with propidium iodide and analysed by flow cytometry. Values presented in the graph are means ± standard deviations from three experiments. Asterisks denote statistically significant differences in comparison to the control (Mann-Whitney test, $p < 0.05$).

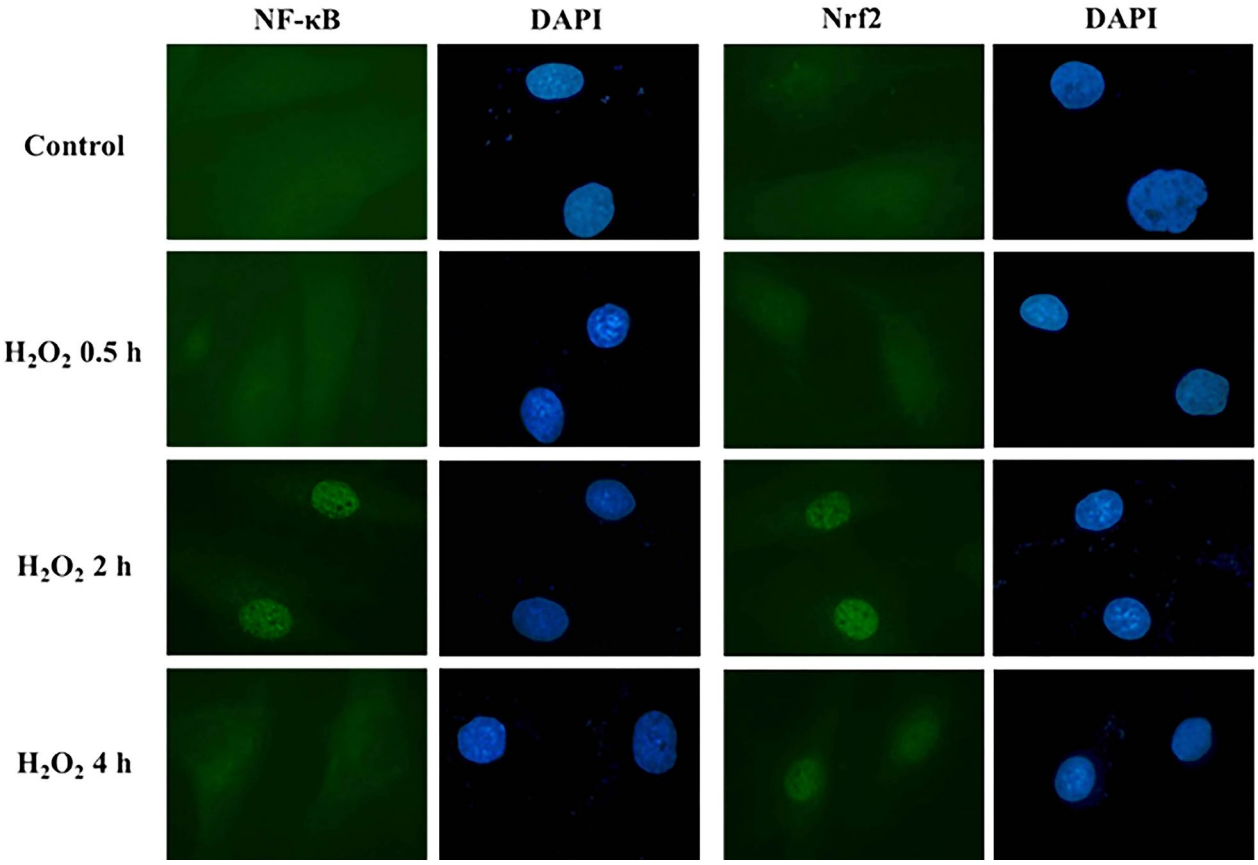

Fig. 5. Nuclear translocation of NF-κB p65 subunit and Nrf2 after exposure of human nucleus pulposus cells to H_2O_2. Cells were grown on glass coverslips and they were then exposed to 500 μM H_2O_2 for the designated time periods. Samples were fixed and labelled using antibodies against the p65 subunit of NF-kB and Nrf2 and counter-stained with DAPI. The pictures are representative of three independent experiments.

MMP-2 and MMP-9 were significantly up-regulated in H_2O_2-senescent nucleus pulposus cells. On the other hand, all intervertebral disc proteoglycans tested here – aggrecan (the major ECM constituent of the nucleus pulposus), biglycan, decorin and versican – were found to be down-regulated along with an observed decrease in the mRNA levels of TIMP-1, -2 and -3.

Discussion

The intervertebral disc is characterised by the appearance of signs of degeneration relatively early in life, compared to the majority of the other tissues in the body. This is most probably due to the effect of the several intense stresses the disc is exposed to. Even though one of the major features of the normal intervertebral disc is the absence of vascularisation – leading to relatively hypoxic conditions – disc cells still use oxidative phosphorylation for their energy production, with ROS being the main by-product (Vo *et al*., 2013). In addition, ROS can be produced in response to cytokines such as interleukins (ILs), TNF-α or TGF-β, known to be expressed in the intervertebral disc, or to various DNA-damaging insults, such as ionising radiation and genotoxic drugs. On the other hand, the lack of vascularisation may result in the accumulation of these waste products and the continuous damage of cellular macromolecules. Taking these into account, several *in vivo* studies have assessed and verified the presence of oxidative stress and the increased concentration of oxidation products in aged intervertebral discs (Hou *et al.*, 2014; Nerlich *et al*., 2007; Nerlich *et al*., 1997). Oxidative stress in the disc is manifested in the form of H_2O_2, as shown in human nucleus pulposus specimens by Kim *et al*. (2009). Therefore, here we investigated the effect of exogenously supplied H_2O_2 on the viability, proliferation and senescence of nucleus pulposus cells, as well as the underlying molecular mechanisms of the observed phenomena.

We showed that H_2O_2 considerably increased intracellular ROS levels in disc cells in a dose-dependent manner. In all H_2O_2 concentrations tested, ROS levels peaked early after stimulation and declined thereafter. This decrease most probably stemmed from the activation of antioxidant mechanisms, including Nrf2 activation, evidenced by its translocation into the nucleus. Cytotoxicity studies using the MTT assay indicated that the viability of the cells remained close to the control levels at H_2O_2 concentrations up to 600 μM, after which it declined sharply.

The nucleus pulposus of the intact intervertebral disc is characterised by a very low rate of cell proliferation (Johnson and Roberts, 2003) and an extremely low cell

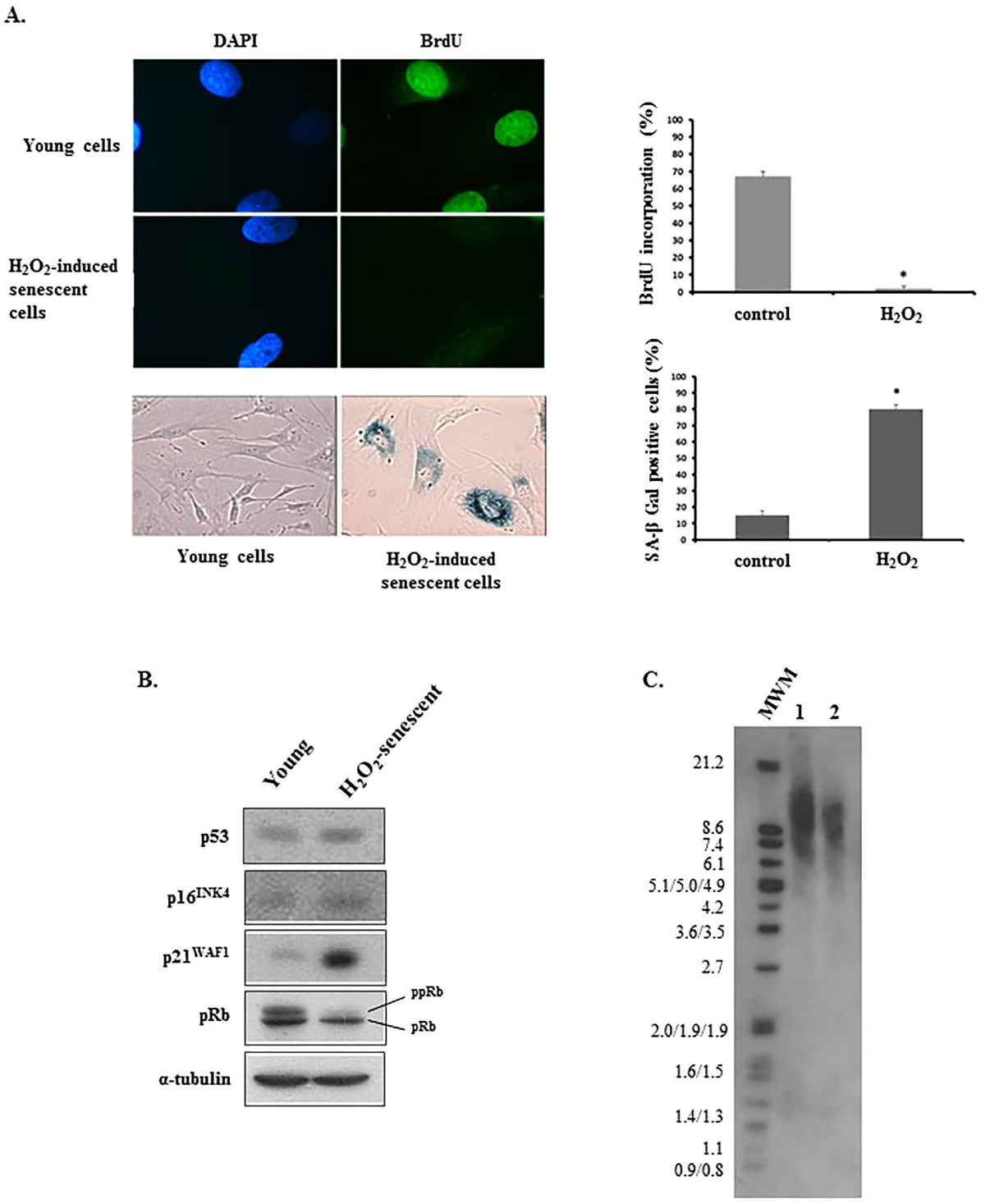

Fig. 6. Repeated exposure of human nucleus pulposus cells to H_2O_2 leads to premature senescence. (**A**) Cells were plated on glass coverslips after four consecutive exposures to 500 μM H_2O_2. For the estimation of BrdU incorporation, BrdU was added at a concentration of 50 μM for 48 h before fixation and labelling with an anti-BrdU-FITC antibody, while for SA-β-gal staining cells were directly fixed. Samples were visualised and cells were counted under a fluorescence or phase contrast microscope, respectively. In the graphs mean values ± standard deviations are presented deriving from three independently conducted experiments. Asterisks represent statistically significant differences in comparison to the control (Student's *t*-test, $p < 0.05$). (**B**) Cells were plated on 60-mm dishes after four serial exposures to 500 μM H_2O_2 and total protein extracts were collected. Western blotting was performed for the total levels of p53, $p16^{INK4a}$, $p21^{WAF1}$ and pRb. The expression levels of α-tubulin were examined as a loading control. Blots presented are selected among three experiments. (**C**) Control and H_2O_2-senescent cells were plated on 100-mm dishes before genomic extraction and southern blotting for the determination of the *meanTRF*. The gel presented is representative of three independent repetitions. Lane 1: Control, Lane 2: Cells exposed four consecutive times to 500 μM H_2O_2 as described in Materials and Methods.

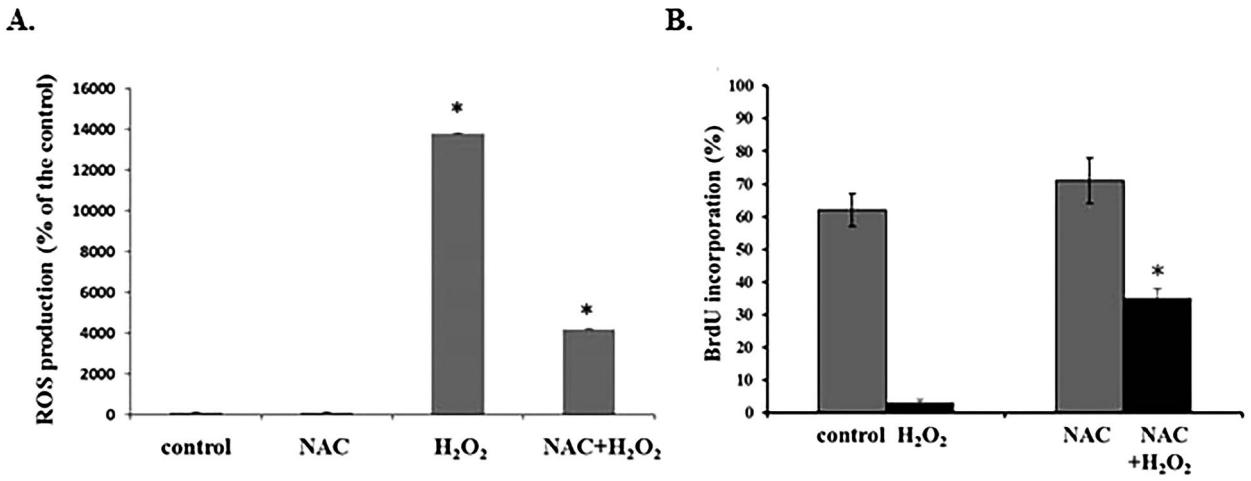

Fig. 7. The H_2O_2-induced premature senescence of human nucleus pulposus cells is ROS-mediated. (**A**) Cells were grown in 96-well plates until confluence and they were then incubated with 2 mM NAC for 16 h. H_2O_2 (500 μM) was added along with 10 μM DCFH-DA for 15 min and measurements were recorded. (**B**) Cells on glass coverslips were subjected to the H_2O_2-induced premature senescence protocol in the presence or not of 2 mM NAC and BrdU incorporation was estimated in the fixed samples. Data presented in the graphs are the mean values ± standard deviations of three separate experiments and asterisks pinpoint statistically significant differences in comparison to the untreated control (**A**) or to the H_2O_2-treated sample in the absence of NAC (**B**) (Student's *t*-test, $p < 0.05$).

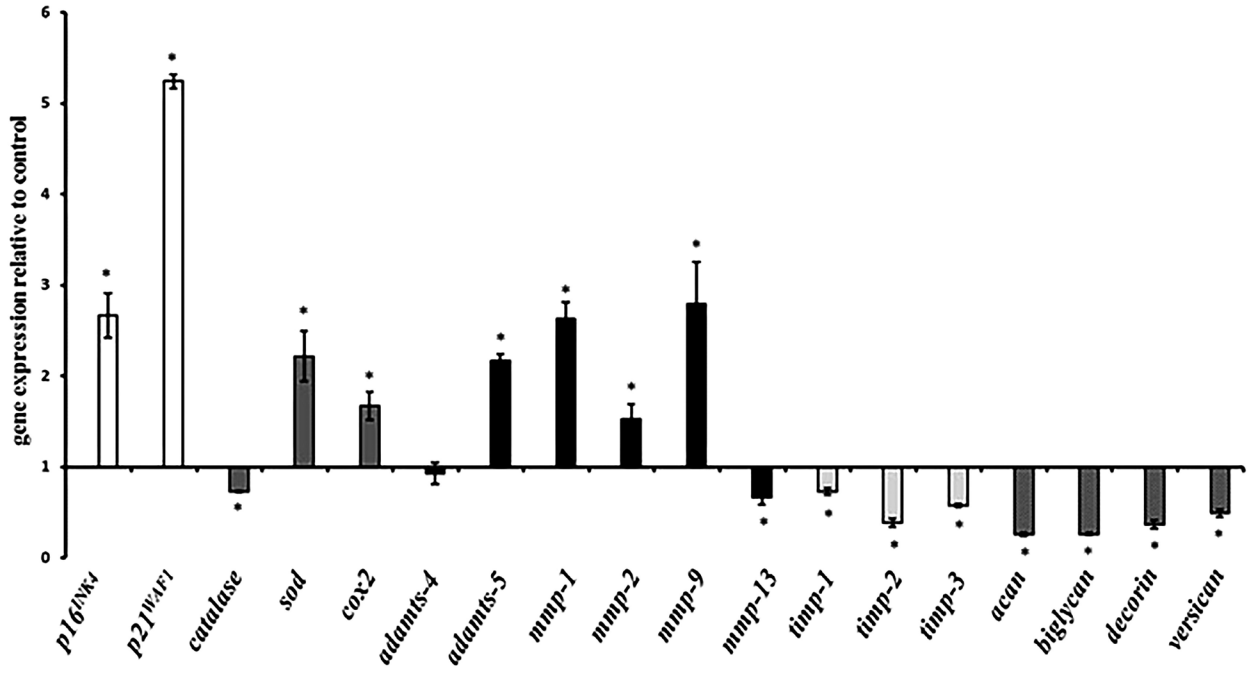

Fig. 8. H_2O_2-senescent nucleus pulposus cells show a catabolic phenotype. Cells were exposed to 500 μM H_2O_2 four consecutive times and they were then plated in 100-mm dishes. Total RNA was extracted and mRNA levels of the designated genes were measured by quantitative RT-PCR. Mean Ct values of target genes in each sample were normalised to that of GAPDH. Ratios of the expression levels of the senescent cells to those of the untreated control are presented as mean values ± standard deviations of three independent experiments. Statistically significant differences (Student's *t*-test, $p < 0.05$) are marked by asterisks.

density, most probably due to the exceptionally adverse nutritional conditions in this non-vascularised tissue (Urban *et al.*, 2004). The repression of this cell population at an extremely low number in the intact tissue can be the combined outcome of many parameters, such as the nutritional status, the particular ECM components, the lack of exogenous growth factors, as well as the multiple stresses these cells are exposed to. We demonstrated here that oxidative stress (in the form of H_2O_2) can drastically inhibit intervertebral disc cell proliferation in a dose-dependent manner. Similarly, we have shown that another major stress, *i.e.* hyperosmolality, also inhibits nucleus pulposus cells' proliferation and decreases the proliferative effect of several growth factors, such as PDGF or IGF-I

(Mavrogonatou and Kletsas, 2009; Mavrogonatou and Kletsas, 2010). The above are in favour of the hypothesis that the presence of exogenous stresses, including oxidative stress, in the normal disc may serve in holding the cell population down to a certain level, thus, among others, ensuring adequate nutrition and cell viability. For the rest of our experiments we selected the highest H_2O_2 concentration with practically no cytotoxic effect (500 μM). However, it is interesting to note that the actual intracellular H_2O_2 concentration in Hela cells has been measured to be ~ 650-fold lower than the initial concentration exogenously supplied (Huang and Sikes, 2014), which means that the observed phenomena in human nucleus pulposus cells could have been triggered by an intracellular H_2O_2 concentration < 1 μM.

An immediate effect of H_2O_2 was found to be the activation of PI3K/Akt and of all three MAPK signalling pathways, *i.e.* p38 MAPK, ERK and JNK, a typical cellular response to exogenous stresses. All pathways were activated within 15 min after stimulation with H_2O_2, while they followed different kinetics of dephosphorylation. According to previous reports, all pathways mentioned above are triggered in intervertebral disc cells by several stimuli, such as inflammatory cytokines, growth factors, hypoxia or hyperosmotic conditions, and they have different functions in cell and tissue physiology (Studer *et al.*, 2008; Wuertz *et al.*, 2012). ERK and Akt pathways have been reported to be stimulated by oxidative stress in other cell systems, as well (*e.g.* trabecular meshwork cells), in which their inhibition results in decreased cell viability (Awai-Kasaoka *et al.*, 2013), most probably due to the activation of Nrf2, as an antioxidant defence (Bak *et al.*, 2012; Park *et al.*, 2013).

Oxidative stress was shown here to have a direct genotoxic effect, revealed by the formation of γH2A.X foci in the nuclei of disc cells, as soon as 30 min after treatment with H_2O_2. The accumulation of the foci peaked at approx. 3 h and then started to decline, possibly due to the activation of DNA repair mechanisms. However, even 24 h later several foci remained. Double strand break (DSB) formation, as indicated by the accumulation of γH2A.X foci, was followed by the so-called DNA damage response (DDR), characterised by the activation of the ATM-Chk2-p53-$p21^{WAF1}$-pRb pathway, that led to the inhibition of cell proliferation by blocking the G1-S transition of the cell cycle. This finding is in accordance with the G1 phase arrest observed in other cell types after exposure to H_2O_2 (Bladier *et al.*, 1997; Deshpande *et al.*, 2002; Park, 2013). ATM activation is in general considered as a direct effect of DSB formation through the action of the Mre11-Rad50-Nbs1 (MRN) DNA repair complex, thus orchestrating the DNA repair response. However, it has been proposed that ATM activation by DSBs is inhibited in the presence of H_2O_2, as the latter blocks the ability of the MRN to bind to damaged DNA; in this case, ATM is activated by direct oxidation (Guo *et al.*, 2010a; Guo *et al.*, 2010b). On the other hand, p38 MAPK (also activated by H_2O_2) can directly activate the ATM downstream target p53, as well as $p16^{INK4a}$, and consequently block cell proliferation (Han and Sun, 2007). All the above indicate that the anti-proliferative effect of oxidative stress on nucleus pulposus cells results from a cross-talk of several signalling pathways.

Exposure of intervertebral disc cells to H_2O_2 led to the activation of the transcription factor NF-κB, as demonstrated by the nuclear translocation of its p65 subunit. NF-kB is typically activated by stress and cellular damage (Wuertz *et al*., 2012), under oxidative (Bubici *et al.*, 2006) and genotoxic (Wu *et al.*, 2006) stresses, leading to the transcription of inflammatory genes (Pedruzzi *et al.*, 2012). Interestingly and in agreement with our data, NF-κB has been reported to be activated in adult intervertebral disc samples in close correlation with CML accumulation [the latter being a marker of oxidative stress (Nerlich *et al*., 2007)], thus supporting its role in intervertebral disc degeneration. On the other hand, we also observed the nuclear translocation of Nrf2, a basic leucine zipper transcription factor that regulates the expression of antioxidant proteins, as a response to H_2O_2 in order to protect cells against oxidative damage. Based on several reports, we can hypothesise that this translocation stems from the activation of the pathway(s) found to be activated by oxidative stress, such as the MAPKs (de Bittencourt Pasquali *et al.*, 2013; Owuor and Kong, 2002), Akt (Sykiotis *et al.*, 2011) or even ATM (Li *et al.*, 2004). The role of the simultaneous activation of these antagonistic pathways in disc physiology in response to stress requires further investigation.

Normal cells have a limited lifespan when cultured *in vitro*. After a certain number of population doublings, characteristic for each individual cell strain, the cells enter a state termed senescence, characterised by their inability to proliferate. This type of senescence, called replicative senescence (RS), is the result of the gradual shortening of telomeres, the end of the chromosomes. At a critical point this shortening is perceived by the cells as a DNA damage, thus triggering a classical DDR, characterised by the activation of the ATM-Chk2-p53-$p21^{WAF1}$ axis that leads to the hypophosphorylation of the pRb protein and consequently to the inability of the cells to undergo the G1-S transition in the cell cycle (Campisi and d'Adda di Fagagna, 2007). Alternatively, cells when exposed repeatedly to subcytotoxic doses of several genotoxic stresses, such as UV or ionising radiation, genotoxic drugs or oxidative stress (Papadopoulou and Kletsas, 2011; Roninson, 2002; Toussaint *et al.*, 2000) or even after the overexpression of certain oncogenes (Bartkova *et al.*, 2006; Serrano *et al.*, 1997) can undergo a type of senescence termed stress-induced premature senescence (SIPS). Interestingly, the activation of a DDR is a central motif in most types of senescence. Roberts *et al*. (2006a) have shown for the first time that senescent cells accumulate in the aged and degenerated discs, and this finding has been further verified by several laboratories (Gruber *et al*., 2007; Kim *et al*., 2009; Le Maitre *et al*., 2007a). As it is well known that the intervertebral disc is characterised by a very low proliferative rate (Johnson and Roberts, 2003), it is more probable that senescence in this tissue is of the SIPS type due to exogenous stresses (Kletsas, 2009). Here, we found that exposure to repeated subcytotoxic doses of H_2O_2 provoked premature senescence to human disc cells, shown

by their inability to synthesise DNA and by the increased number of cells positive to SA-β-gal staining. This effect increased gradually with the number of the exposures to H_2O_2 and after four consecutive doses more than 95 % of the cells could be driven to senescence. Interestingly, we observed an increased number of senescent cells even as a response to much lower H_2O_2 concentrations, but after a higher number of exposures. These H_2O_2-senescent disc cells overexpressed classical senescence markers, such as p53, the cell cycle inhibitors $p21^{WAF1}$ and $p16^{INK4a}$ and the hypophosphorylated pRb. It has been reported by von Zglinicki *et al*. that oxidative stress leads to telomere shortening in human fibroblasts (von Zglinicki *et al.*, 2000). When we measured the length of telomeres in H_2O_2-senescent intervertebral disc cells we did not find any statistically significant decrease. Our observations are in line with those of Chen *et al*. claiming that the senescent phenotype is uncoupled with telomere shortening in H_2O_2-treated fibroblasts (Chen *et al.*, 2001). However, these authors hypothesised that cell replication is needed for telomere shortening in response to oxidative stress (Chen *et al*., 2001). As our experiments have been conducted in confluent (non-proliferating) cultures, we cannot exclude the possibility that a combination of H_2O_2-treatment and cell replications would lead to shorter telomeres in nucleus pulposus cells, as well.

Beyond their inability to proliferate, representing a major obstacle to tissue repair processes, senescent cells are also characterised by a catabolic phenotype. Several studies, performed mainly in fibroblasts, have reported that senescent cells overexpress matrix degrading enzymes, inflammatory cytokines and other inflammatory molecules, and it has been proposed that due to this catabolic and inflammatory phenotype they could negatively affect tissue homeostasis (Campisi and d'Adda di Fagagna, 2007). Accordingly, we investigated the gene expression profile of nucleus pulposus cells that became senescent after continuous exposures to H_2O_2. It should be mentioned that the differences observed at the H_2O_2-induced terminally senescent stage were not vast. This finding was not surprising, given that cells may have become adapted after a long residence to the stressful environment, ultimately exhibiting a lower that then initial response, but constant residual differential gene expression. As expected, we found an increase in the expression of two classical senescence markers, the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p16^{INK4a}$, in senescent cells compared to young ones. We also observed a decrease in catalase and an increase in SOD expression, partly in agreement with data reported for senescent skin fibroblasts (Kaneko *et al.*, 2001). The expression of COX2 was also found up-regulated. This is in accordance to the results of Zdanov *et al*. (2009) who reported similar results in fibroblasts; these authors have also demonstrated that this up-regulation is mediated by p53, shown here to be up-regulated in senescent cells after exposure to oxidative stress.

Nucleus pulposus cells are considered critical regulators of the ECM in this tissue, composed mainly of proteoglycans, the most prominent being aggrecan (Roughley *et al.*, 2002). Gene expression analysis of senescent nucleus pulposus cells indicated an intense down-regulation of aggrecan, as well as of other proteoglycans, such as biglycan, decorin and versican. In addition to this anti-anabolic phenotype, catabolic markers were also induced. More specifically, MMP-1, MMP-2, MMP-9 and ADAMTS-5 were up-regulated, while ADAMTS-4 was found to be unchanged and MMP-13 down-regulated. Although in general TIMP expression parallels that of MMPs, we found that TIMP-1, TIMP-2, and TIMP-3 – known to inhibit both MMPs, as well as ADAMTSs – were also down-regulated in senescent intervertebral disc cells, indicating a generalised catabolic phenotype. Numerous *in vivo* studies have reported similar results on the up-regulation of several MMPs and of ADAMTS-5, or the down-regulation of aggrecan and decorin (Le Maitre *et al*., 2004; Pockert *et al.*, 2009; Roberts *et al.*, 2000; Singh *et al.*, 2009; Sivan *et al.*, 2014). However, these *in vivo* studies have demonstrated an increase of TIMP-1 and TIMP-2, biglycan and ADAMTS-4, in contrast to our results, suggesting that the expression of these genes *in vivo* may be regulated not only by the senescent status of disc cells, but also by other factors of their local microenvironment. On the other hand, *in vitro* and organotypic studies have shown that oxidative stress, provoked in an environment with increased O_2, leads to an increase of ADAMTS-5, MMP-1 and MMP-3 (Nasto *et al.*, 2013). The above indicate that oxidative stress-mediated premature cellular senescence may affect nucleus pulposus homeostasis due to the catabolism of its ECM components, thus compromising the structural integrity of this tissue.

In conclusion, our data indicate that oxidative stress, typically met in the aged intervertebral disc, can inhibit disc cells' proliferation and provoke their premature senescence. The catabolic phenotype of these senescent cells may contribute to the degeneration of the tissue. Based on these findings, further studies are needed to establish the putative application of antioxidants as treatments that could efficiently decelerate intervertebral disc degeneration.

Acknowledgements

This work was supported by the European Union FP7 (''GENODISC'' project, HEALTH-F2-2008-201626).

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Discussion with Reviewers

Reviewer I: Does the NP cell senescence model induced by H_2O_2 treatment correspond to the situation in degenerated intervertebral disc?
Authors: It is known that the intervertebral disc offers a harsh environment to its containing cells due to the peculiar physicochemical conditions prevailing in this tissue (high osmolality as a result of the extracellular matrix composition and the mechanical forces the tissue is subjected to, low oxygen concentration and limited nutrients' availability due to the absence of vascularisation, low pH due to the almost compulsory anaerobic metabolism of the cells and oxidative stress provoked by the accumulation of catabolic by-products).

The presence of senescent cells in degenerated intervertebral discs has been reported (Freemont, 2009; Gruber *et al.,* 2007; Le Maitre *et al*., 2007a; Roberts *et al*., 2006a), thus the induction of senescence in an *in vitro* nucleus pulposus cell culture could correspond to the situation of the degenerated disc. However, we believe that senescent phenotype *in vivo* is the combined result of all stressful conditions the cells are exposed to and not of an individual factor, such as reactive oxygen species. In this respect, H_2O_2 was used in our experiments just as a physiologically relevant means to trigger senescence, since this oxidative agent has been shown to exist in the tissue *in vivo* (Kim *et al*., 2009).

Reviewer II: How does the simulation of oxidative stress by hydrogen peroxide application correspond to oxidative stress in the nucleus pulposus of intervertebral discs? Does the applied protocol simulate physiological levels for normal or degenerated discs? Would the authors expect different results with cells of the annulus fibrosus?
Authors: The existence of oxidative stress in the intervertebral disc *in vivo* has been established by the accumulation of carboxymethyl-lysine in the proteins especially of the nucleus pulposus. In addition, the presence of H_2O_2 in human nucleus pulposus specimens has been demonstrated (Kim *et al*., 2009), allowing us to assume that nucleus pulposus cells are confronted with this particular oxidative agent *in vivo*. The H_2O_2 content in human nucleus pulposus specimens has been found to be around 0.25 µmol *per* 20 mg of tissue with no differences among different degeneration grades (Kim *et al*., 2009). H_2O_2 is also produced by chondrocytes *in vitro* (Tiku *et al*., 1990). In consideration of these previously reported data on nucleus pulposus cells and specimens, we selected to perform our experiments in nucleus pulposus cells. We believe that our *in vitro* model could simulate one aspect of senescence in the degenerated disc (among a variety of factors that may in combination contribute to the appearance of senescent cells *in vivo*), since we demonstrated that H_2O_2-senescent nucleus pulposus cells are characterised by a catabolic gene expression profile typical of the aged or degenerated disc. Based on our experience with intervertebral disc cells in our laboratory and taking into account their similar responses towards a number of physiological insults, we would not expect a different behaviour of annulus fibrosus cells from that of nucleus pulposus cells after exposure to H_2O_2.

Reviewer III: These results were measured with cells in monolayer culture and normoxia. Monolayer is a very unnatural state for these cells. Do the authors have any evidence that the exposure with H_2O_2 would be possible in 3D alginate bead culture or pellet culture and these effects could be even more enhanced, for instance under hypoxia?
Authors: We share the Reviewer's opinion that 3D cultures can better simulate the *in vivo* conditions of nucleus pulposus cells in the tissue. In fact, there is an ongoing research in our laboratory on the responses of these cells towards various physiological conditions using 3D cultures in alginate beads. Our preliminary unpublished results indicate a similar behaviour of the cells exposed to H_2O_2 under normoxic and hypoxic conditions when cultured in monolayers, while many similarities seem to exist between 2D and 3D alginate bead cultures in a hypoxic environment.

Reviewer IIII: In this study, human IVD cells from degenerated tissue were used. How might the results change if healthy human IVD cells or bovine IVD cells were used and why?
Authors: We share the Reviewer's concern about possible different behaviours of IVD cells deriving from a healthy/degenerated tissue or from different species. However, we strongly believe that the general cellular responses towards oxidative stress observed in this study would be similar if we had used healthy human or bovine IVD cells. What we could expect to be different would probably be the range of cytotoxic H_2O_2 concentrations. We feel, for example, that bovine IVD cells (which – based on our laboratory experience – exhibit a much higher proliferation rate than human pathologic IVD cells) may have been more sensitive to H_2O_2 treatment. Otherwise, we would expect activation of the same biochemical pathways.

Editor's Note: Scientific Editor in charge of the paper: Mauro Alini.