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Vulnerability to ROS-induced cell death in ageing articular cartilage: The role of antioxidant enzyme activity

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Summary

Objectives: To test the hypothesis that age-related loss of chondrocytes in cartilage is associated with impaired reactive oxygen species (ROS) homeostasis resulting from reduced antioxidant defence.

Methods: *Cell numbers:* The total number of chondrocytes in the articular cartilage of the femoral head of young, mature and old rats was estimated using an unbiased stereological method. *ROS quantification:* Fluorescence intensity in chondrocytes was quantified using the oxygen free radical sensing probe dihydrorhodamine 123 (DHR 123), confocal laser scanning microscopy and densitometric image analysis. In order to delineate the reactive species, explants were pre-treated with *N*-acetylcysteine (NAC) or *N*^ω-nitro-L-arginine methyl ester (L-NAME) prior to ROS quantification. *Induction of intracellular ROS:* Explants were incubated in the redox-cycling drug menadione after which they underwent ROS quantification and cell-viability assay. *Antioxidant enzyme activity:* The activity of catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX) was measured.

Results: *Chondrocyte numbers:* A significant and progressive loss of chondrocytes was observed with ageing. *Cellular ROS levels:* A significant age-related increase in cellular ROS-induced fluorescence was demonstrated. NAC significantly reduced ROS levels in old chondrocytes only. *Induction of intracellular ROS:* Menadione increased cellular ROS levels dose-dependently in young and old chondrocytes, with a greater effect in the latter. Old chondrocytes were more vulnerable to menadione-induced cytotoxicity. *Antioxidant enzymes:* Catalase activity declined significantly in aged cartilage whilst SOD and GPX activities were unaltered.

Conclusions: Substantial loss of chondrocytes occurs in rat articular cartilage which may result from increased vulnerability to elevated intracellular ROS levels, consequent upon a decline in antioxidant defence.

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Key words: Reactive oxygen species, Rat, Ageing, Cell death, Chondrocytes, Catalase, Superoxide dismutase, Glutathione peroxidase, Dihydrorhodamine 123, Stereology.

Introduction

Ageing is known to be an important risk factor for the development of degenerative joint disease and the metabolic and cellular changes occurring in this condition have been extensively investigated¹. Far less attention has been paid to the effects of age on the survival of chondrocytes or to the underlying mechanisms leading to cell death. It is crucial that the status of ageing cartilage and the mechanisms involved in its degradation are understood in order to enhance our ability to manufacture tissue-engineered cartilage from elderly donors. Therefore, we set out to establish the effects of age on chondrocyte survival using an animal model where total numbers of cells per femoral head could be accurately estimated and important

underlying cell survival mechanisms, namely oxidative stress homeostasis could also be examined.

The majority of published data on chondrocyte numbers in ageing cartilage is from studies of large human joints, where it is not practical to estimate total cell numbers at a given site. Estimates have therefore been based on cell density, changes in which may reflect cell redistribution and/or alterations in matrix volume, as well as a shift in overall cell numbers. Results are contradictory with one study reporting unchanged chondrocyte numbers² whilst another found the cell density decreased by as much as 40%³. Stereology^{4,5} allows assumption free or unbiased quantification of objects within a tissue. Such robust counting methods have not been previously employed in cartilage research. The first aim of this study was to use stereology to investigate the effects of age on chondrocyte numbers in rat articular cartilage.

The balance between reactive oxygen species (ROS) production and antioxidant defence determines the extent of cellular oxidative stress. It is widely accepted but not conclusively proven, that the level of oxidative stress increases with age and leads to the damage of macromolecules such as DNA. This forms the basis of the 'free radical theory of ageing'⁶. In cartilage, the proteins within the

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extracellular matrix (ECM) are also likely to be a key target of this stochastic effect. Chondrocytes are capable of producing hydroxyl radical⁷, hydrogen peroxide⁸ and superoxide species⁹. Whilst there is growing support that ROS may contribute to vital cell signalling mechanisms^{10–12}, a large body of evidence has demonstrated the detrimental effects of these highly reactive and labile molecules in cartilage. ROS lead to inhibition of proteoglycan synthesis^{13,14} and have been implicated in the degeneration of the ECM through lipid peroxidation and formation of malondialdehyde and hydroxynonenal adducts¹⁵. There is accumulating data to suggest that excessive levels of ROS are cytotoxic to chondrocytes. Quinone compounds such as menadione, have been widely used to induce oxidative stress in a number of cells and tissues^{16,17}. It is thought that through reduction to a semi-quinone radical, they enter redox cycles with molecular oxygen to produce ROS. The addition of menadione to the medium of cultured rabbit chondrocytes reduced their proliferative capacity dose-dependently¹⁷. This effect was abolished by the addition of catalase, suggesting that the menadione-induced cytotoxicity results from elevated free radical generation. Similarly, superoxide generated by the hypoxanthine–xanthine oxidase (XOD) system leads to the inhibition of growth of articular chondrocytes¹⁸ while intra-articular hydrogen peroxide caused chondrocyte death in the knee joint of mice¹⁹. Nitric oxide (NO) also appears to contribute to age-related impairments of cartilage through the production principally of peroxynitrite²⁰ (formed by a reaction between NO and superoxide anion). Some investigators have reported a direct cytotoxic effect of NO on cultured human chondrocytes²¹ whilst others have suggested that NO mediated chondrocyte cell death requires the generation of additional reactive species²². Our second aim was therefore to examine the effect of age on intracellular ROS levels and then if ROS increased with age, to discover whether enhanced ROS generation might lead to cell death.

Alterations in intracellular ROS levels may result from changes in antioxidant defence systems. The antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) that disproportionate reactive species, play a significant role in protecting cells against radical damage. SOD is an oxidoreductase that serves to dismutate superoxide anions generated during oxidative metabolism, whilst catalase and GPX disproportionate hydrogen peroxide. The importance of these enzymes has been documented in a number of tissues, although little is known about their role in cartilage or the effects of age on their activity. SOD and catalase activity in chondrocytes has been previously documented²³. Our final aim was to assess changes in the activity of the antioxidant enzymes with age.

Methods

ANIMALS AND DIETARY MAINTENANCE

Three month (young), 6 month (mature) and 15–18 month (old) male Sprague Dawley rats, were obtained from a colony maintained at Royal Free and University College Medical School, where they were kept in a controlled environment, housed no more than three per cage, fed and watered *ad libitum* and maintained on a 12:12 h light:dark cycle. All animal procedures were carried out in accordance with the Animals (Scientific procedure) Act, 1986 (European directive 86/609/EEC) and with Home Office approval.

DETERMINATION OF NUMBER OF CHONDROCYTE PER FEMORAL HEAD

Tissue preparation

Four 3, 6 and 15–18 month old rats were sacrificed by asphyxiation with carbon dioxide gas. The femoral head was surgically dissected and examined for obvious evidence of degenerative changes (specimens that exhibited cartilage degeneration were discarded). The femoral heads were fixed overnight in 4% paraformaldehyde at room temperature. The tissue was then decalcified in rapid decalcifier (Cellpath, England) and stored at 4°C in phosphate buffer solution (PBS) + 0.1% sodium azide and 20% sucrose for 24 h prior to sectioning. The samples were placed (with the ligamentum teres facing upwards) in optimum cutting temperature (OCT) embedding medium and immediately immersed in liquid nitrogen. Using a Bright® cryostat the femoral head was sectioned at 30 µm and placed sequentially onto Polysine™ (Menzel-glaser, Germany) glass slides. After 1 h of air drying the sections were rehydrated with PBS and stained with the DNA-binding dye Yo-Yo (1:1000, Molecular Probes, Oregon, US) for 30 min. The sections were then washed twice in PBS and mounted using glass cover slips and anti-fade solution (Citifluor, UK).

STEREOLOGY

Counting of cells in sectioned tissue using stereology (without assumptions regarding size, shape or orientation of objects)^{4,5} requires measurement of the total volume of the tissue (V_T), followed by stereological counting of cells within a measured subdivision of the tissue volume. Finally, a calculation is made extrapolating the cell counts from the counted subdivision to the total volume of tissue.

Volume estimation

The cross-sectional area of the cartilage was evaluated at low magnification ($\times 4$) under a Nikon TE300 microscope. Using digital image analysis software (Kontron-Zeiss, UK) and a purpose-designed macro, the cross-sectional area (A) of every fifth section was measured (except in areas where there was a rapid change in cross-sectional area, e.g., distal part of the head, where every section was measured). Values of A for unmeasured sections were interpolated. Using the Cavalieri principle A was multiplied by the section thickness (T) for each section and then by the total number of sections (S) to give the total volume of articular cartilage (V_T) per femoral head.

Cell density assessment

Image acquisition was performed on a Bio-Rad Radiance 2100 confocal laser scanning microscope linked to a Nikon E800 fluorescence microscope and using a $\times 20$ objective. Preliminary experiments established conditions under which all subsequent imaging was performed. Six to eight fields from every fifth section were randomly sampled. From each field, two single-optical slice images were acquired, separated by 10 µm in the z-plane. The sections were digitally merged using Laserpix software (Bio-Rad, version 4.0) such that cells present in both sections could be clearly seen. An unbiased counting frame was applied in the x–y plane where cells crossing the top and left hand border were excluded from the count while those crossing the other two sides were included. Within this frame, cells present only in the lowermost (reference) optical section were counted,

while cells overlapping into or present only in the upper (see above) section were excluded. This method removes any bias towards counting larger objects. Total numbers of cells counted (N_C) and the total volume of the fields over which the counts were made (V_C) were calculated.

Total cell count

V_T was divided by the volume of cartilage used for cell counting in the confocal microscope (V_C) and multiplied by N_C to obtain the total chondrocyte count (N_T) per femoral head.

ROS LEVELS

Tissue preparation

Hip joints were dissected as before and slices of articular cartilage were aseptically excised from the femoral heads and transferred directly into Dulbecco's Modified Eagle Medium (DMEM) without serum supplement. In preliminary experiments, three ROS-sensitive probes were evaluated. Explants of articular cartilage containing chondrocytes were loaded with either dihydrorhodamine 123 (DHR 123), 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-DCFDA) or 2',7'-dichlorodihydrofluorescein diacetate succinimidyl ester (Oxyburst[®]) (all at 10 μ M, Molecular Probes) for 30 min (T0). The explants were then incubated for a further 4 h in a 5% CO₂ incubator at 37°C. Samples were removed from the medium at hourly intervals, mounted onto slides in anti-fade mountant (Citifluor) and imaged under the confocal microscope.

To delineate the species of ROS generated in chondrocytes, explants were pre-treated for 1 h with the oxygen free radical scavenger *N*-acetylcysteine^{24,25} (NAC) (10 mM, Sigma, UK), or with the nitric oxide synthase inhibitor, *N*³-nitro-L-arginine methyl ester (L-NAME) (100 μ M, Sigma, UK), prior to loading with DHR 123. Samples were imaged after 2 h of incubation with age-matched non-treated explants as controls.

Confocal microscopy

Image acquisition was performed on a Bio-Rad Radiance 2100 confocal laser scanning microscope linked to a Nikon E800 fluorescence microscope and using a $\times 40$, 1.3 NA, oil immersion objective. Preliminary experiments established conditions (aperture, laser power and Kalman filtering) under which all subsequent imaging was performed. In the course of these experiments, a range of samples was tested to ensure that the full range of image intensities fell within the linear response range (0–255 grey levels; 8 bits) of the confocal photomultiplier.

To compensate for day-to-day variation in confocal laser power, a uranyl acetate impregnated slide, which emits light at an intensity proportional to the excitation energy without temporal fading, was used to standardise the imaging conditions. Minor day-to-day variations in image intensity were eliminated by adjusting the laser output gain control. Under these conditions, 6–8 fields from each explant were chosen at random and imaged at a constant depth of 10 μ m from the cartilage surface and digitally stored in preparation for image analysis.

Densitometric image analysis

Following image acquisition, fluorescence intensity of chondrocytes was quantified and expressed as mean grey

value (GV) using image analysis software (Kontron-Zeiss, UK) and a purpose-designed macro. For the majority of samples, segmentation of the images using an automatically generated threshold gave reliable discrimination of chondrocyte cytoplasm from (non-fluorescent) nuclei and the ECM. Following discrimination, mean GV per cell was measured for each field. In the few samples with high ECM fluorescence, selection of chondrocyte cell cytoplasm was not possible using automatic segmentation, therefore the outline of each chondrocyte was traced manually. For each explant, these processes were repeated on 6–8 fields with about 20 cells imaged per field, giving a total of 100–160 cells per sample. Sample means of GV per cell were calculated and used for comparative purposes.

CELL-VIABILITY ASSESSMENT

In order to find out whether cells remained viable *in vitro*, the Live/Dead cytotoxicity assay (Ethidium homodimer-1 and Calcein-AM, Molecular Probes, US) was used²⁶, following the manufacturer's instructions. Ethidium homodimer-1 only enters cells with compromised cell membranes and intercalates with nucleic acids producing red fluorescence. Calcein-AM penetrates the membrane of living cells where cytoplasmic esterases cleave the molecule producing green fluorescence. Thus live cells are depicted green and dead cells red. Images were acquired under the confocal microscope with a $\times 20$ oil objective in order to maximise the number of chondrocytes visualised. The percentage viability of chondrocytes per explant was calculated by manually counting the number of live cells in the *x-y* reference frame as used previously, and dividing by the total number of cells visualised per field.

INDUCTION OF INTRACELLULAR ROS

Cartilage explants from 3 and 15–18 month old rats were treated with the redox-cycling drug, menadione (Sigma, UK) at concentrations of 250, 500 and 1000 μ M for 1 h prior to loading with DHR 123. Cellular fluorescence was then quantified using the above method. A separate group of explants were incubated for 240 min in menadione following which cell viability was assessed using the Live/Dead assay as described before.

ANTIOXIDANT ENZYME ACTIVITY

Tissue preparation

Cartilage explants were prepared from four 3 month and four 15–18 month rats as previously described. The explants were frozen in liquid nitrogen, homogenised using a mikro-dismembrator (B.Braun Biotech, Melsungen, Germany) and incubated for 1 h in lysis buffer (PBS containing; Triton X-100 (0.1%), ethylenediaminetetraacetic acid (500 μ M), sodium dodecylsulfate (0.1% w/v)) at 4°C. Samples were then centrifuged at 3000 *g* for 15 min at 4°C. The supernatant was collected and used for enzyme assays. Protein assay was also carried out following the manufacturer's instructions (Bio-Rad protein assay kit, Hercules, US).

Catalase assay

Catalase activity was determined by a modification of the method described by Cohen *et al.*²⁷ using changes in absorbance of hydrogen peroxide (H₂O₂) at 240 nm. As it is

not possible to define international catalase units, the rate constant of a first-order reaction (k) was used for comparative purposes. Results are expressed as k /mg protein.

SOD assay

Total superoxide dismutase (tSOD) activity was measured by adaptation of the Ransod commercial kit (Randox Laboratories, Crumlin, UK). This method employs xanthine and XOD to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye which was measured using a spectrophotometer. The SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay. Results are expressed as U/mg of protein.

GPX assay

GPX activity was measured by adaptation of the Ransel kit (Randox Laboratories, Crumlin, UK)²⁸. GPX catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH), the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance at 340 nm can be measured spectrophotometrically. Results are expressed as U/mg of protein.

DATA COLLECTION AND STATISTICS

All experiments were repeated at least four times. In ROS quantification experiments, the mean GV at each time point was normalised by expression of the data as a proportion of the value at time zero (T0) of control tissue taken from a 3 month animal and assayed in parallel. Means and standard errors were calculated and used for graphic and descriptive purposes. Comparisons of dependent variables were performed using one-way ANOVA or the Mann–Whitney U test (GraphPad, Prism 3.0). Where significance was shown, data pairs were subjected to *post hoc* testing. Differences of $P < 0.05$ were regarded as significant.

Results

TOTAL NUMBER OF CHONDROCYTES PER FEMORAL HEAD

Cartilage volume

There is a 47% decrease in total cartilage volume between young and mature animals ($P < 0.05$). There is however no further significant decrease in the cartilage volume between mature and old animals [Fig. 1(A)].

Cell numbers

The estimated mean total chondrocyte number per femoral head in each age group is depicted in Fig. 1(B). The number of chondrocytes declines steadily with age. There is a 49% decrease in cell numbers between young and mature ($P < 0.05$) and a further 46% decline between mature and old animals ($P < 0.05$).

CELLULAR ROS LEVELS

In preliminary experiments three fluorescent probes: DHR 123 [Fig. 2(A)] and CM-DCFDA [Fig. 2(B)] and

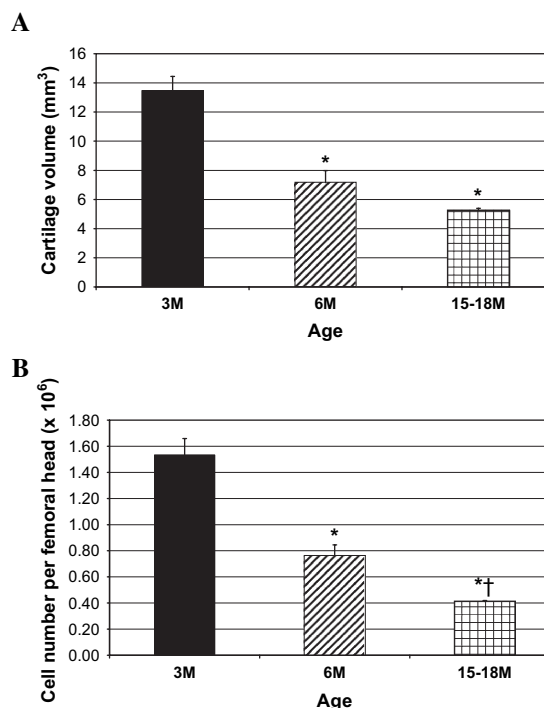


Fig. 1. (A) Mean cartilage volume per femoral head (\pm S.E.M.) of 3, 6 and 15–18 month old rats. There is a significant decrease in cartilage volume between 3 and 6 and 3 and 15–18 months (* $P < 0.05$ compared to 3 m). (B) Mean chondrocyte number (\pm S.E.M.) per femoral head of 3, 6 and 15–18 month old rats. There is a continual loss of chondrocytes with age. (* $P < 0.05$ compared to 3 M; † $P < 0.05$ compared to 6 M.)

Oxyburst[®] were compared for their ability to assay ROS in cartilage explants. The latter probe gave inconsistent loading results and was not tested further. CM-DCFDA was also deemed unsuitable as it exhibited marked leakage of the fluorescent product into the ECM (as demonstrated by the rapid fall in the cellular GV that was accompanied by a sharp rise in ECM GV after 2 h of incubation). In contrast, DHR 123 exhibited superior cellular retention in chondrocytes [Fig. 3(A and B)]. Validation of the suitability of DHR 123 for assaying ROS was achieved by demonstrating that intracellular fluorescence in chondrocytes increased dose-dependently following treatment with menadione and was significantly reduced by incubation with the antioxidant, catalase (data not shown). DHR 123 produced images of high contrast and of intensities that clearly varied depending on age and treatment and that were suitable for quantification by confocal microscopy and densitometric image analysis. Under the conditions employed, the change in DHR 123 fluorescence (expressed as mean GV) was assumed to reflect cellular ROS levels.

Age-changes in cellular ROS

The mean cellular GV plotted against incubation time in culture is depicted in Fig. 4. In all age groups there was a gradual increase in cellular fluorescence intensity with incubation time. Old chondrocytes, however, exhibited significantly higher fluorescence compared to young cells at each time point ($P < 0.05$). Mature chondrocytes also demonstrated greater cellular GV compared to young,

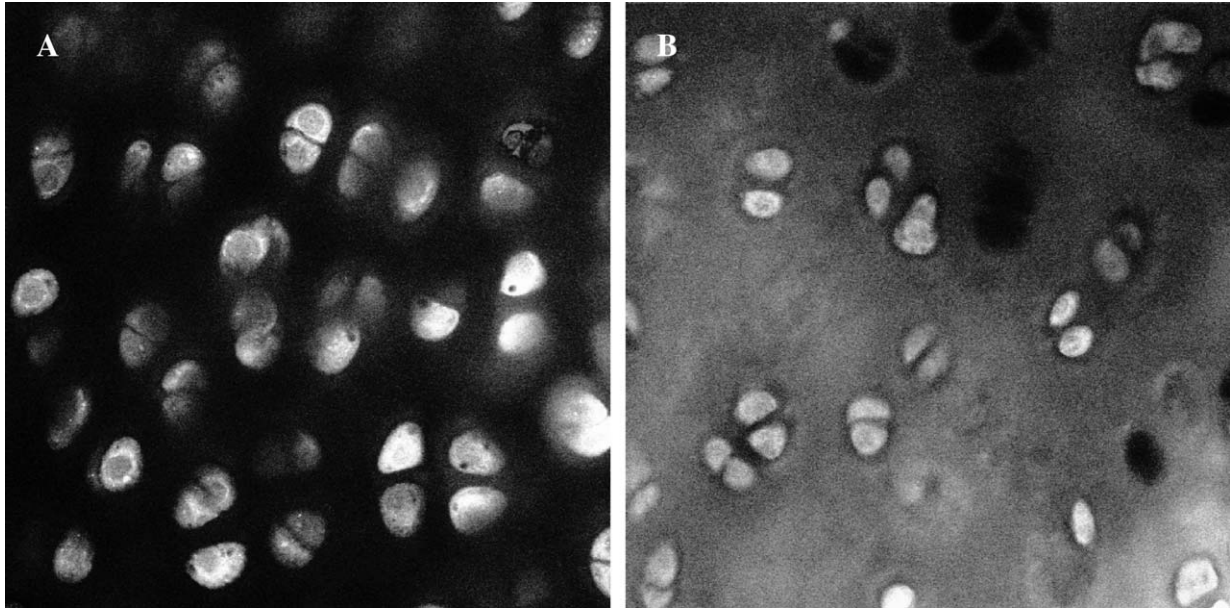


Fig. 2. Confocal images following 120 min of incubation with DHR 123 (A) and CM-DCFDA (B). There is localisation of ROS-induced signal within the cytoplasm of chondrocytes with both probes. However, with CM-DCFDA there are a number of empty lacunae and ECM fluorescence intensity is high, suggesting leakage of oxidised probe from cells into the ECM.

but this difference did not reach statistical significance. Incubation in L-NAME did not significantly alter cellular GV in any age group. In contrast, NAC reduced cellular fluorescence in mature ($P > 0.05$) and old chondrocytes ($P < 0.05$, Fig. 5).

Cell-viability assessment

Assessment with the Live/Dead assay revealed $>95\%$ cell viability following 4 h of incubation with DHR 123 (in all three age groups), showing that increased ROS was not likely to result from *in vitro* induction of cell death.

INDUCTION OF INTRACELLULAR ROS

Cellular ROS levels

Menadione increased cellular fluorescence dose-dependently after 1 h in both young and old chondrocytes. At each dose the percentage increase in cellular GV was substantially greater in the older cells compared to age-matched controls (Fig. 6), indicating an age-associated impairment of intracellular ROS homeostasis.

Cell-viability assessment

The percentage of live cells following 4 h of incubation in menadione is illustrated in Table I. Old chondrocytes were found to be significantly ($P < 0.05$) more vulnerable to menadione-induced cytotoxicity than young cells at higher concentrations (500 and 1000 μM) of the drug.

ANTIOXIDANT ENZYME ACTIVITY

No significant differences in the activity of tSOD and GPX were detected [Fig. 7(A and B)]. However, a significant 57% decrease ($P < 0.05$) in catalase activity was observed between young and old chondrocytes [Fig. 7(C)], suggesting

a substantial age-related loss of antioxidant defence capability in rat articular chondrocytes.

Discussion

Articular cartilage undergoes a number of well established age-related changes such as decrease in size of the proteoglycan chains²⁹ and an increase in surface fibrillation³⁰. It has been more difficult to establish whether these age-related changes are associated with and perhaps caused by loss of chondrocytes. A causal relationship is likely because chondrocytes are central to preservation of the structural integrity of cartilage and their loss can therefore be expected to lead to reduced capacity for repair and maintenance. The present study has clearly demonstrated that in rat articular cartilage, ageing is associated with a significant loss of approximately 50% of the total number of chondrocytes in the femoral head. Whilst the loss of cells between young and mature animals may represent a maturational phenomenon, the ongoing loss of cells between mature and old rats is likely to have a different significance. This observed age-related cell loss is consistent with the significant increase in the percentage of apoptotic cells observed in the joint surfaces of ageing Wistar rats³¹. Age is one of the most important risk factor for the development of osteoarthritis. Loss of chondrocytes may therefore contribute to cartilage degeneration, providing an explanation for the increased probability of developing degenerative joint disease with age in humans. The extrapolation of our findings to human cartilage clearly requires further study.

The cartilage explant model used in this study for quantification of ROS levels, was chosen because of the likelihood that it reflects the *in vivo* situation more closely than dissociated cell culture, i.e., chondrocytes were maintained within the established ECM and the phenotypic changes associated with dedifferentiation in culture were avoided^{32,33}. Although a number of other techniques

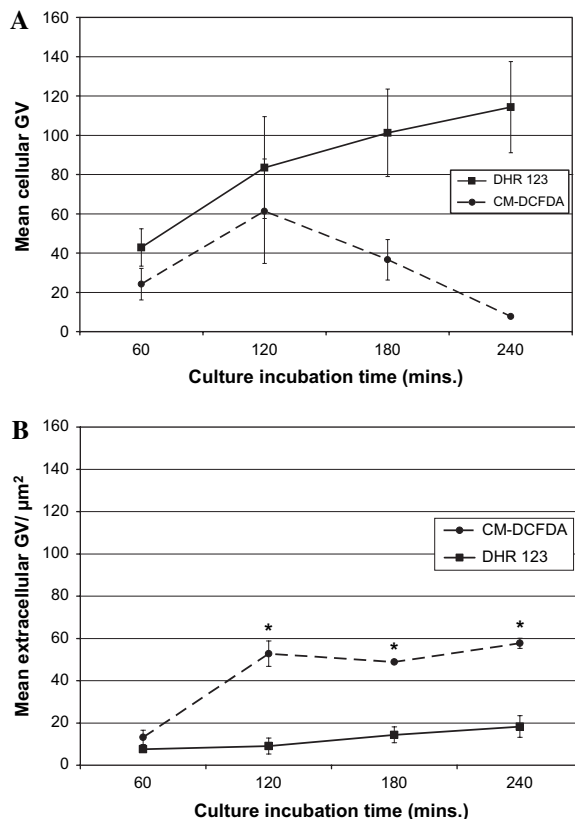


Fig. 3. (A) Mean GV per chondrocyte \pm S.E.M. plotted against incubation time using DHR 123, and CM-DCFDA ($n = 4$). Results are expressed as percent of T0. Peak GV occurs at 4 h with DHR 123. Note that the fluorescence intensity with CM-DCFDA declines rapidly after 2 h of incubation. (B) Mean GV/ μm^2 of ECM plotted against incubation time using DHR 123 and CM-DCFDA ($n = 4$). Results are expressed as percent of T0. With DHR 123 there is no significant increase in ECM GV over 4 h *in vitro*. In contrast, a rapid rise is detected with CM-DCFDA after 1–2 h of incubation ($P < 0.05$ at 120, 180, 240 when compared to 60 min). Combined with rapid decline in intracellular fluorescence see (A), this figure strongly indicates leakage of the oxidised probe into the ECM. (* $P < 0.05$.)

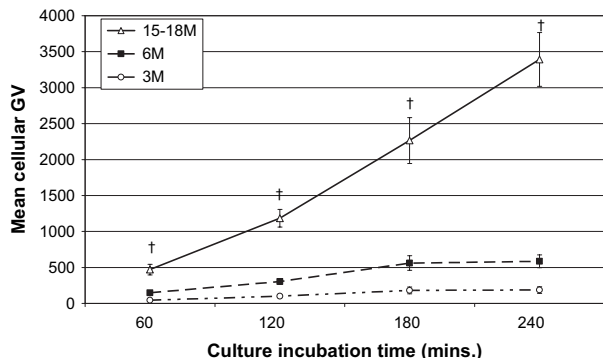


Fig. 4. Mean cellular GV \pm S.E.M. at three age groups, plotted against culture incubation time. Results are expressed as percent of T0 of 3 month animal. There is a steady increase in cellular fluorescence with incubation time at all three age groups. Increase in age is associated with a rise in cellular GV. ($\dagger P < 0.05$ compared to 3 M.)

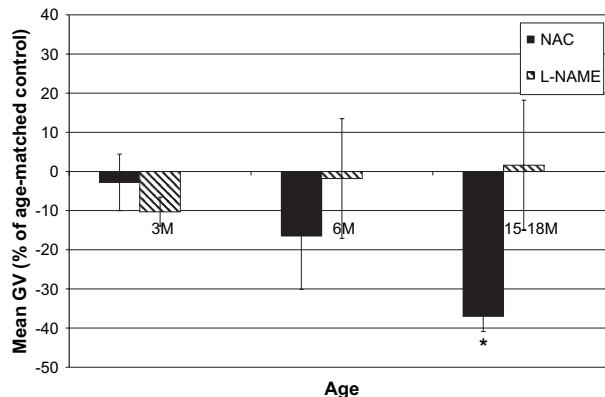


Fig. 5. Mean cellular GV \pm S.E.M. of three age groups at 120 min of incubation, following pre-treatment with NAC and L-NAME. Results are expressed as percent of age-matched control. (* $P < 0.05$ compared to control.)

varying in complexity and sensitivity have been described for ROS quantification³⁴, none have gained universal acceptance. Fluorogenic probes are a convenient and sensitive means of monitoring oxidative activity of living cells³⁵ as ROS promote a dose-dependent increase in the fluorescence of these compounds³⁶. It has been shown that DHR 123 can be oxidised by peroxynitrite³⁷, hydrogen peroxide³⁸ and hydroxyl radicals³⁹ lending support to our claim that changes in the fluorescence intensity of this probe reflect cellular ROS levels. Our data provide further support for the sensitivity and specificity of DHR 123 as an assay tool for intracellular ROS by showing dose-dependent increases in DHR 123 fluorescence intensity in response to the redox-cycling drug, menadione, and decreases with the antioxidant, NAC. Using DHR 123, we have demonstrated that ageing is associated with a significant increase in cellular ROS levels. This increase may be the result of increased proton leakage and/or a decline in antioxidant defence with age. Elevated ROS is likely to provide a key mechanism linking ageing to chondrocyte loss because of the well-established cytotoxic effect of ROS on chondrocytes^{17–19,22} which appears to be dependent on activation of caspases⁴⁰.

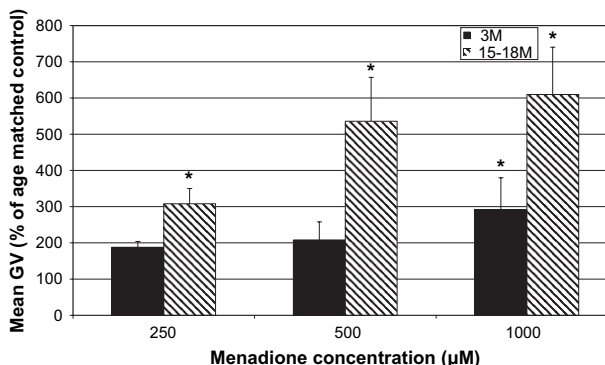


Fig. 6. Mean cellular GV \pm S.E.M. following 60 min of treatment with menadione at 250, 500 and 1000 μM concentrations. Results are expressed as percent of age-matched control. Menadione increases cellular GV dose-dependently at both age groups but the effect is larger in old chondrocytes. (* $P < 0.05$ compared to age-matched control.)

Table I
Percentage of viable cells following 4 h of incubation in menadione (250, 500 and 1000 μ M concentration) using Live/Dead assay

Menadione concentration (μ M)	Cell viability (% live)	
	3 M	15–18 M
250	47	50
500	39	18*
1000	26	4*

* $P < 0.05$ compared to 3 month animal.

Although assaying oxidative activity in living cells is complicated by the likely presence of multiple species of oxygen and nitrogen free radicals, we have attempted to delineate the principal groups of reactive species involved.

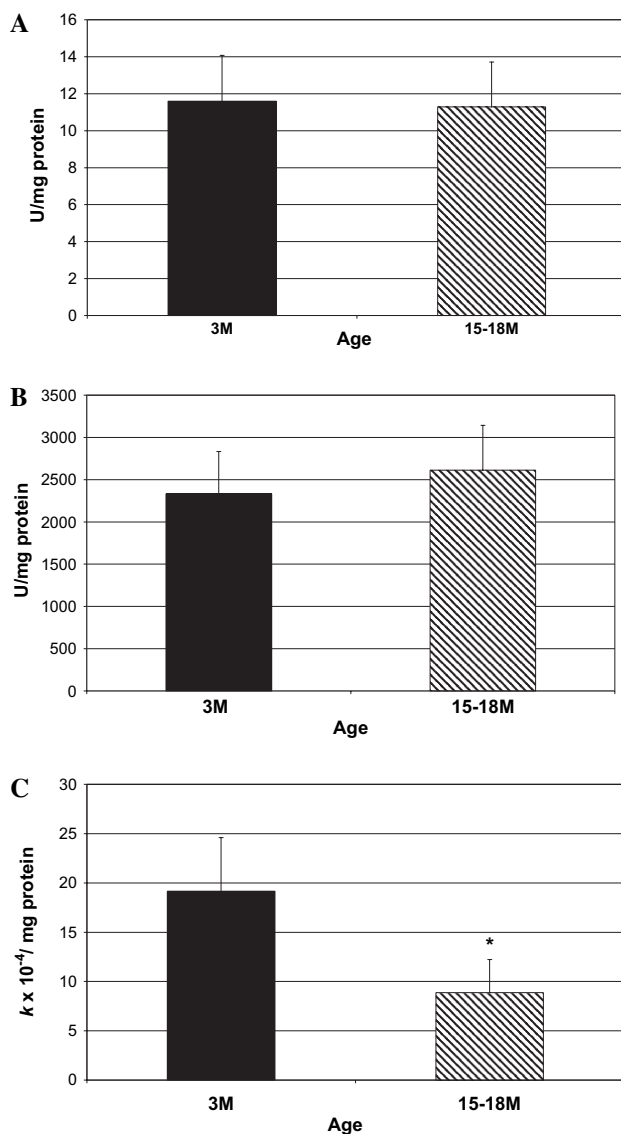


Fig. 7. (A) Mean tSOD activity \pm S.E.M. of cartilage in 3 and 15–18 month old animals. (B) Mean GPX activity \pm S.E.M. of cartilage in 3 and 15–18 month old animals. (C) Mean catalase activity \pm S.E.M. of cartilage in 3 and 15–18 month old animals. The catalase activity is significantly lower in older chondrocytes. (* $P < 0.05$ compared to 3 M.)

The oxidation of DHR 123 is non-specific³⁶ and the addition of a specific blocker seems a suitable way of investigating the free radical species. We observed that the potent nitric oxide synthase inhibitor L-NAME⁴¹ did not alter cellular ROS levels. On the other hand, NAC, an antioxidant^{24,25} that includes amongst its effects promotion of intracellular glutathione⁴², significantly reduced ROS levels in old chondrocytes. Taken collectively, these results suggest that the increase in oxidative stress in older animals is due to superoxide derivatives rather than reactive nitrogen species.

Quinone-induced cytotoxicity has been observed in many tissues and is thought to be largely due to redox cycling^{43,44}. In osteoblasts, the quinone menadione, stimulates production of superoxide and hydrogen peroxide and leads to apoptosis⁴⁵. Our study has revealed a similar pro-oxidant effect in rat chondrocytes. However, more significantly these experiments have revealed that old cells are substantially more vulnerable than young ones to the cytotoxic effects of menadione. Furthermore, they indicate that the failure of ROS homeostatic mechanisms can lead to ROS-induced cell death.

Enhanced intracellular ROS levels can result from increased proton leakage from the respiratory chain or reduced neutralisation by antioxidant defence systems. Catalase is an important antioxidant enzyme that scavenges H_2O_2 and a decrease in its activity will consequently lead to accumulation of H_2O_2 . The significant reduction in the activity of this enzyme demonstrated in our study indicates that the increase in ROS levels with age is due to failure of antioxidant defence mechanisms and suggests that H_2O_2 is the principal species present in excess in older cells. However, we cannot rule out the possibility that changes in the ROS scavenging capacity of the ECM⁴⁶ with age might contribute to the differences observed. There have been conflicting reports on the effect of age on antioxidant enzyme activity^{47–49}. Stimulation of peroxy-nitrite generation in cultured human chondrocytes resulted in decreased glutathione and thioredoxin reductase activity, but had no effect on catalase⁵⁰. Our results are consistent with observations in the ageing rodent lung⁴⁹ but in conflict with findings in rat leydig cells⁴⁷, suggesting that alterations in the activity of antioxidant enzymes with age may be species and tissue specific. The decline in catalase activity also explains why equidoses of the redox-cycling drug, menadione lead to greater increases in cellular ROS levels in older cells. These results suggest that as chondrocytes age they become less competent at maintaining oxidative homeostasis through a specific decline in the activity of catalase. We hypothesise that the inability to control oxidative stress levels leads to activation of apoptotic mechanisms and cell death, as supported by our results showing increased vulnerability with age to menadione-induced cell death.

In conclusion, we have demonstrated a plausible link between an age-related reduction in chondrocyte numbers and an increase in cellular ROS levels in rat articular cartilage. Ageing is associated with significant chondrocyte loss and increased vulnerability to ROS-induced cell death, presumably as a result of attritional effects of prolonged enhanced ROS-induced stress. Our data suggest that a key contributor to elevated intracellular ROS is the antioxidant enzyme catalase, activity of which is specifically and significantly depressed in ageing chondrocytes. We propose that ROS-induced death of chondrocytes predisposes ageing cartilage to degenerative disorders.

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