Bioenergetic reprogramming of articular chondrocytes by exposure to exogenous and endogenous reactive oxygen species and its role in the anabolic response to low oxygen

H. K. Heywood* and D. A. Lee
School of Engineering and Materials Science, Queen Mary University of London, UK

Abstract

Monolayer culture is integral to many cell-based cartilage repair strategies, but chondrocytes lose regenerative potential with increasing duration in vitro. This coincides with elevated reactive oxygen species (ROS) levels and a bioenergetic transformation characterized by increasing mitochondrial function. This study investigates ROS as stimuli for bioenergetic reprogramming and the effect of antioxidants on the propensity of chondrocytes to regenerate a cartilaginous matrix. Articular chondrocytes were cultured in monolayer under a 2% O2 atmosphere. Oxidative stress was increased using 50 μM H2O2 or a 20% O2 culture atmosphere, or decreased using the antioxidant N-acetyl-cysteine (NAC). Mitochondrial function was characterized using 200 nM Mitotracker green and an oxygen biosensor. After two population doublings ± NAC, chondrocytes were encapsulated in alginate beads (1 × 10^7 cells/ml) for an additional 10 days before DMB assay of glycosaminoglycan content. The beads were cultured under both 20% O2 and the more physiological 5% O2 condition. Chondrocytes expanded in 20% O2 exhibited elevated mitochondrial mass and functional capacity, which was partially mimicked by the exogenous ROS, H2O2. Oligomycin treatment revealed that the increased oxygen consumption was coupled to oxidative phosphorylation. NAC limited these markers of bioenergetic reprogramming during culture-expansion with no significant effect on subsequent GAG production under 20% O2. However, NAC treatment in monolayer abolished the hypoxic induction of GAG in alginate beads. This supports the hypothesis of a causal relationship between exposure to ROS and acquired mitochondrial function in chondrocytes. Additionally, mitochondrial function may be required for the hypoxic induction of GAG synthesis by chondrocytes. © 2015 The Authors. Journal of Tissue Engineering and Regenerative Medicine Published by John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords chondrocyte; regenerative medicine; reactive oxygen species; mitochondria; antioxidant; N-acetyl cysteine; hypoxia

1. Introduction

Monolayer culture is integral to many cell-based cartilage repair strategies, but chondrocytes lose regenerative potential with increasing duration in vitro. It is well established that during monolayer expansion chondrocytes lose their characteristic morphology and that their expression of key extracellular matrix macromolecules, including sulphated glycosaminoglycans, is diminished (Benya and Shaffer, 1982; Dell’Accio et al., 2001; Giovannini et al., 2010). Chondrocytes also undergo a bioenergetic transformation in culture, switching from an almost exclusively glycolytic energy metabolism towards increasing dependence on mitochondrial oxidative phosphorylation (Champagne et al., 1987; Heywood and Lee, 2008, 2010;
Mignotte et al., 1991). There is evidence to suggest that this bioenergetic reprogramming is not readily reversible (Boubriak et al., 2009), potentially persisting on re-implantation to the joint. Thus, it may be important to understand its cause and consequence to chondrocyte function. This study investigates the stimuli for such bioenergetic reprogramming and its effect on the ability of chondrocytes to regenerate a cartilaginous matrix.

It is well established that chondrocytes exhibit exceptionally low oxygen consumption rates compared to the majority of mammalian cell types (Stockwell, 1991). For example, chondrocyte oxygen consumption rates are reported to be around 1–6 fm/mg/h (Bowie et al., 1941; Heywood et al., 2010; Heywood and Lee, 2008; Rosenthal et al., 1941), compared to approximately 100 fm/mg/h in MSCs (Pattappa et al., 2011) and 325 fm/mg/h in hepatocytes (Balis et al., 1999). Thus, primary chondrocytes derive the majority of their ATP from glycolysis, with estimates attributing just 1–10% of cellular ATP production to mitochondrial oxidative phosphorylation (Heywood et al., 2010, 2014; Heywood and Lee, 2008). However, our laboratory has identified that oxygen consumption increases markedly in chondrocytes that have been cultured in monolayer compared to immediately following isolation (Heywood et al., 2010, 2014; Heywood and Lee, 2008). These studies have confirmed that the additional oxygen consumption in monolayer-expanded cells is coupled to ATP generation via mitochondrial oxidative phosphorylation and is associated with increased mitochondrial mass (Heywood et al., 2010). Indeed, earlier work by Mignotte and others demonstrated a 13-fold increase in cellular mitochondrial DNA (mtDNA) relative to total DNA following 6 days of monolayer culture (Champagne et al., 1987; Mignotte et al., 1991).

In addition to the generation of ATP, mitochondria have other roles, including the generation of reactive oxygen species (ROS) as an essential signalling mechanism (Bell et al., 2007a; Cillero-Pastor et al., 2008; Lee et al., 2000; Milner et al., 2007). There is some evidence to show that sublethal doses of endogenous or exogenous H2O2 are sufficient to stimulate mitochondrial biogenesis in a number of cell types (Lee and Wei, 2005; Lee et al., 2000, 2002). As such, it has been proposed that ROS act as a stimulus for bioenergetic reprogramming (Lee and Wei, 2005; Venditti et al., 2014). A marked increase in ROS generation and markers of associated oxidative stress are observed by the chondrocytes on transfer into monolayer culture (Heywood and Lee, 2008, 2010; Heywood et al., 2014). However, any causal relationship between elevated ROS in vitro and the bioenergetic reprogramming of chondrocytes in monolayer culture has not been examined. Accordingly, this study examines whether: (a) exogenous ROS are sufficient to promote bioenergetic reprogramming in chondrocytes, thereby increasing mitochondrial mass and function; (b) bioenergetic reprogramming can be downregulated by treatment with the pro-antioxidant, N-acetyl cysteine (NAC); and (c) NAC treatment during monolayer culture affects the subsequent regeneration of a cartilaginous matrix by chondrocytes, with implications for cartilage repair strategies.

2. Materials and methods

2.1. Cell source

The metacarpophalangeal joints of 18–24 month-old cattle were opened under sterile conditions. Full-depth cartilage tissue slices were removed from the proximal joint surface, using a scalpel. Chondrocytes were isolated from the cartilage extracellular matrix by sequential incubation at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) with addition of 5.7 mg/ml pronase for 1 h, followed by 100 U/ml collagenase for 14 h, as described previously (Heywood and Lee, 2008). The freshly isolated chondrocytes were seeded into 175 cm² tissue-culture flasks at a density of 2.2 × 10⁴ cells/cm² and cultured with 40 ml medium/flask at 37 °C in a humidified/5% v/v CO2 incubator atmosphere. Media during culture consisted of DMEM supplemented with 10% v/v fetal calf serum (FCS), 2 mm l-glutamine, 20 mm HEPES, 88 U/ml penicillin and 88 mg/ml streptomycin. All reagents were from Sigma-Aldrich (Poole, UK). Cells from individual donor animals were maintained separately during culture. The joints were obtained as waste material from a commercial abattoir; ethical approval for animals in research was not applicable.

2.2. Cell culture

To determine whether exposure to exogenous oxidants can promote an oxidative bioenergetic transformation in culture, cells were cultured under a 2% v/v O2 atmosphere and compared to cells where the level of oxidative stress was increased by either addition of the exogenous oxidant hydrogen peroxide (H2O2) or increasing the O2 atmosphere to 20% v/v (Heywood and Lee, 2010). An aliquot of H2O2 was added to selected flasks to provide a final concentration of 50 μM, and replenished at feeding intervals. The oxygen atmosphere was controlled using an X Vivo Biospherix (New York) system, with integrated incubators and workspace to maintain a continuous level of O2 in the atmosphere during culture and any manipulation. After 9 days, the cells were recovered from the flasks by incubation in trypsin–EDTA solution and resuspended in fresh media. The cell yield was determined by haemocytometer. Aliquots of the cell suspension were used for determination of their bioenergetic phenotype, as defined by mitochondrial mass and key parameters of mitochondrial function, i.e. oxidative phosphorylation, respiratory capacity and ROS, as described below.

To determine whether reducing oxidative stress inhibits bioenergetic reprogramming, freshly isolated cells were seeded in flasks and cultured under a 20% O2 atmosphere in the presence or absence of the pro-antioxidant N-acetyl cysteine (NAC). NAC removes cellular H2O2, a by-product of superoxide dismutation, by augmenting the activity of the endogenous glutathione antioxidant system (Brand et al., 2004). The probe carboxy-dichlorodihydrofluorescein (H2DCF; Molecular Probes)
becomes fluorescent on oxidation by ROS. A dose–response study in the presence of H$_2$DCF revealed that 2 mM NAC reduced cellular ROS levels to <10% of untreated control values, whilst exogenous H$_2$O$_2$ increased ROS levels, as expected (Figure 1A, B). Viability was maintained in the selected doses of 50 μM H$_2$O$_2$ and 2 mM NAC, indicated by strong cytoplasmic staining with calcein-AM (green) and few ethidium homodimer-1 (red)-positive nuclei (5 μM; Invitrogen) (Figure 1C). During culture in the presence or absence of NAC, cell proliferation level was monitored by light microscopy and manual cell counting. After a matched level of two population doublings, the culture-expanded cells were recovered to a suspension for analysis of their bioenergetic phenotype, or encapsulation into alginate to assess their capacity for cartilage matrix regeneration, described below.

2.3. Mitochondrial mass and reactive oxygen species

The cells were suspended at 1 × 10$^6$ cells/ml and incubated for 1 h at 37 °C in complete DMEM with 200 nM Mitotracker Green (Invitrogen). The cells were washed in warm Hanks’ buffer and resuspended at 1 × 10$^6$ cells/ml in fresh Hanks’ buffer containing 5 μM dihydroethidium (DHE; Invitrogen). 100 μl aliquots were added to a 384-well assay plate and the fluorescence monitored over 20 min, using 488 nm excitation and 520 nm and 590 nm emission wavelengths to detect mitochondrial mass and ROS, respectively (BMG Optima fluorimeter). An unstained cell suspension controlled for background fluorescence. Addition of xanthine/xanthine oxidase (100 μM/10 μM/ml) was used as a positive control for superoxide generation. DHE solution without cells was used as a negative control to confirm that auto-oxidation of the dye was negligible.

2.4. Oxidative phosphorylation and respiratory capacity

A fluorescence-based oxygen biosensor (BD Biosciences, UK) was used to monitor the dissolved oxygen concentration in cell suspensions over time, as reported previously (Heywood et al., 2006a, 2010). Briefly, chondrocyte suspensions were prepared in fresh DMEM with cell densities of 2 × 10$^6$ cells/ml. 320 μl aliquots of the cell suspensions were loaded into the wells of the 96-well oxygen biosensor, which was sealed with adhesive film and maintained at 37 °C. The rate of oxygen consumption in the wells was calculated from the gradient of the oxygen–time curve and normalized to total cell number. Oligomycin inhibits the mitochondrial F$_1$F$_0$ ATP synthase; thus, the proportion of cellular oxygen consumption that was eliminated by the addition of 2 mg/ml oligomycin (Sigma-Aldrich) is a measure of oxidative phosphorylation. The addition of 3 μM CCCP (Sigma-Aldrich) uncouples the mitochondrial electron transport chain from

![Figure 1](https://wileyonlinelibrary.com)
ATP demand, revealing changes in the maximal respiratory capacity of the chondrocyte mitochondria.

2.5. The effect of NAC treatment on subsequent cartilage matrix regeneration

After two population doublings in monolayer culture, control and NAC-treated chondrocytes (above) were encapsulated in 2% w/v alginate, beads as described (Lee et al., 2003), with a density of 10 × 10^6 cells/ml. The mean volume of the beads was 0.022 ± 0.004 ml (assuming a density of 1 g/ml). The beads were transferred into a 24-well plate, with three beads and 2 ml medium/well, and cultured for an additional 10 days in fresh medium (± continued NAC treatment), with medium exchange on alternate days. During culture, half the alginate bead samples were maintained under an oxygen atmosphere containing 20% v/v O₂, whilst the other half was maintained under a 5% v/v O₂ atmosphere, which more closely represents conditions in the joint. The beads were digested in saline sodium citrate buffer with 2.8 U/ml papain and analysed using the DMB assay for the cartilage matrix constituent, sulphated glycosaminoglycan (GAG) with modifications for alginate samples (described fully in Enobakhare et al., 1996).

2.6. Statistics

Data presented represent mean and standard error of the mean (SE) from 8 to 18 measurements, derived from three independent experiments, where cells from each donor animal were maintained separately. Statistical comparisons were performed using two-tailed t-test with Bonferroni correction for multiple comparisons as appropriate. Data for mitochondrial mass were first normalized to represent fold increase from day 0 values.

3. Results

Transfer to the in vitro environment is associated with increased ROS levels (Heywood and Lee, 2008; Heywood et al., 2014). Here we directly interrogated the role of ROS as a stimulus for bioenergetic reprogramming of chondrocytes.

3.1. Exposure to exogenous oxidants promotes an oxidative energy metabolism in chondrocytes

This study aimed to determine whether addition of exogenous ROS was sufficient to promote bioenergetic reprogramming in freshly isolated articular chondrocytes in monolayer. Cells exposed to exogenous ROS by increasing the O₂ atmosphere to 20% v/v exhibited significantly increased (P < 0.001) mitochondrial mass after 9 days of culture, compared to cells cultured in monolayer under a 2% v/v O₂ atmosphere (Figure 2A). Cells exposed to the exogenous ROS, H₂O₂, had a similar trend in
mitochondrial mass, but this did not reach statistical significance ($P = 0.06$). Greater mitochondrial mass was associated with proportionally increased rates of the mitochondrial functional parameters, oxidative phosphorylation (Figure 2B) and respiratory capacity (Figure 2C). Detailed analysis of the effect of exogenous ROS on chondrocyte mitochondrial metabolism is presented in Figure 3. These data confirm that monolayer cultured cells exhibit a significant ($P < 0.05$) increase in oxygen consumption relative to freshly isolated cells (day 0 values, inset), rising from 6 fm/cell/h to $>20$ fm/cell/h. Both $\text{H}_2\text{O}_2$- and $20\% \text{ O}_2$-treated cells exhibited a further significant increase in mitochondrial oxidative phosphorylation compared to the 2% O$_2$ condition ($P < 0.05$ and $P < 0.001$, respectively). Culture at $20\% \text{ O}_2$ also significantly increased maximal respiratory capacity recorded in the presence of CCCP ($P < 0.001$), consistent with increased mitochondrial capacity and higher levels of cellular superoxide, as detected by 10 $\mu$M dihydroethidium (Figure 4).

### 3.2. Antioxidant treatment down-modulates the oxidative metabolic transformation

This study examined whether reducing endogenous ROS could block the oxidative metabolic transformation observed when primary chondrocytes are cultured in vitro. Treatment with 2 mM NAC significantly enhanced the rate of proliferation of the cells during expansion in monolayer culture, resulting in a lower population-doubling time (Figure 5A). Accordingly, measurement of metabolic parameters was performed at a matched proliferation

![Figure 3](image-url)  
**Figure 3.** Assessment of oxygen consumption, compartmentalized to indicate mitochondrial functional parameters, for chondrocytes following exposure to exogenous ROS (50 $\mu$M H$_2$O$_2$ or 20% O$_2$) during monolayer culture for 9 days, compared to cells cultured at 2% O$_2$ and freshly isolated cells (inset). Data represent mean ± SE of eight measurements derived from three experiments.

![Figure 4](image-url)  
**Figure 4.** The cellular generation of superoxide, determined by monitoring the rate of increase in dihydriothidium fluorescence over 20 min by chondrocytes following exposure to exogenous ROS (50 $\mu$M H$_2$O$_2$ or 20% O$_2$) during monolayer culture for 9 days, compared to cells cultured at 2% O$_2$ and freshly isolated cells. Cell-free and xanthine/xanthine oxidase incubations were included as negative and positive assay controls, respectively. Data represent mean and SE of 8–15 measurements from three independent experiments and are illustrated relative to values obtained for freshly isolated cells.

![Figure 5](image-url)  
**Figure 5.** (A) The duration of monolayer culture required for freshly isolated chondrocytes to reach two population doublings for cells cultured at 20% O$_2$ in the presence or absence of 2 mM NAC; data represent mean and SD of three measurements, each representing cells from an individual donor animal. (B) Oxygen consumption, compartmentalized to indicate mitochondrial functional parameters for chondrocytes following monolayer expansion at 20% O$_2$ to two population doublings in the presence or absence of 2 mM NAC, compared to freshly isolated cells (inset); data represent mean ± SE of eight measurements derived from three experiments.
level of two population doublings. Treatment with 2 mM NAC significantly reduced the oxidative metabolic transformation during culture (Figure 5B). Oxidative phosphorylation was significantly reduced ($P < 0.0001$), resulting in lower total oxygen consumption rates. The NAC-treated cells also exhibited reduced respiratory capacity, determined from CCCP-stimulated respiration ($P < 0.0001$), which is consistent with lower mitochondrial mass.

### 3.3. The effect of N-acetyl cysteine treatment on the subsequent regenerative activity of articular chondrocytes in 3D culture

The ability of monolayer-cultured chondrocytes to regenerate a cartilaginous matrix once re-implanted into a cartilage defect is vital to the success of cell-based repair strategies. In this study, monolayer-expanded chondrocytes were encapsulated within alginate beads in order to assess the effect of NAC treatment during population expansion on the subsequent propensity of the cells, when embedded in a 3D culture environment, to synthesize sulphated GAG, a key component of the cartilaginous extracellular matrix. Alginate beads seeded with untreated cells accumulated 23% more GAG under 5% O$_2$ compared to 20% O$_2$ (Figure 6A). Antioxidant treatment during culture–expansion had no significant effect on subsequent GAG accumulation in alginate beads cultured under 20% O$_2$. However, NAC treatment during monolayer culture resulted in reduced GAG levels in beads cultured under 5% O$_2$ compared to their untreated counterparts. Thus, it appeared that prior treatment in monolayer with NAC may impair the subsequent hypoxic induction of GAG in the alginate beads. This observation persisted even when cells treated in monolayer with NAC were washed before culturing in control media during the alginate phase (Figure 6A).

This phenomenon was investigated further by examining the effect of adding NAC treatment to reduce ROS during the alginate culture phase only. Here, the cells retained a hypoxic response, demonstrated as increased GAG accumulation during culture at 5% O$_2$ (Figure 6B). This suggests that the previously observed effect of NAC to block this response may have been acquired only during the monolayer phase, concurrent with the suppressive effect of NAC on the acquisition of mitochondrial function in monolayer. To explore the role of acquired mitochondrial function for the induction of GAG further, additional cell samples were treated with 0.5 mM dichloroacetate (DCA). As a pyruvate dehydrogenase activator, dichloroacetate rapidly stimulates mitochondrial function (Michelakis et al., 2008). Dichloroacetate treatment significantly increased GAG accumulation in alginate beads by >40% compared to untreated control cells (Figure 6B), which could not be increased further by reducing the oxygen level to 5% v/v. Thus, mitochondria mediate the anabolic response of culture-expanded chondrocytes to reduced oxygen levels.

### 4. Discussion

Many cell-based cartilage repair strategies utilize an in vitro culture phase for population expansion. There is evidence indicating that the acquisition of a more aerobic energy metabolism in vitro may not be readily reversed on re-introduction to 3D culture (Boubriak et al., 2009), and so it is important to understand both the mechanisms and potential consequences of such bioenergetic reprogramming to tissue repair. This study supports the hypothesis that bioenergetic reprogramming of chondrocytes in monolayer can be modulated by ROS, which are a natural by-product of mitochondrial activity.
Up to 5% of total O₂ consumption is channelled into superoxide production in the mitochondrial electron transport chain (Bovers et al., 1972; Turrens, 2003). Superoxide is predominantly generated at complexes I and III of the electron transport chain and is subsequently subject to dismutation into hydrogen peroxide (H₂O₂), which may then be removed by the glutathione antioxidant system (Brand et al., 2004). The aerobic environment during monolayer culture in vitro is anticipated to increase the production of superoxide in chondrocyte mitochondria, by the mass action of oxygen on the electron transport chain (Bovers and Chance, 1973; Turrens, 2003). As such, it has been proposed that ROS act as a potential stimulus of bioenergetic reprogramming which may participate in a positive feedback cycle, promoting increased oxygen consumption and further increases in ROS.

Monolayer culture under 20% O₂ increased parameters of mitochondrial function, including mitochondrial mass, oxidative phosphorylation and maximal respiratory capacity, consistent with the literature (Champagne et al., 1987; Heywood and Lee, 2008, 2010; Mignotte et al., 1991). This effect was substantially reduced by culture at 2% O₂. Treatment of cells cultured under 20% O₂ with the pro-antioxidant NAC substantially reduced alteration in mitochondrial function to levels similar to those observed in cells cultured at 2% O₂. Moreover the effect of culture at 20% O₂ could be partially mimicked by treatment of cells at 2% O₂ with the exogenous ROS, H₂O₂. These findings are consistent with observations from other cells types (Lee et al., 2000; Lee and Wei, 2005), but is notable because in vivo chondrocytes have an exceptionally low mitochondrial density (Brighton et al., 1984; Champagne et al., 1987).

A key finding of this study is that treatment with NAC helped to maintain a metabolic phenotype more comparable to that of primary chondrocytes by reducing the shift towards an aerobic energy metabolism. Moreover, cells cultured in the presence of NAC required significantly less time to reach two population doublings. Accelerated proliferation kinetics is potentially beneficial to cell-based cartilage therapies, such as aurologous chondrocyte implantation, which typically require that chondrocytes are cultured in vitro until three or four population doublings are achieved. Accelerating this phase could provide significant savings to the overall treatment times and procedure costs. However, any such benefits will only be realized if the propensity to regenerate a cartilaginous matrix on re-implantation to the joint is not impaired by prior NAC treatment.

The ability of monolayer-expanded chondrocytes to regenerate a cartilaginous matrix was assessed on reintroduction to 3D culture conditions. Only the GAG accumulating in the beads was determined here, representing the GAG that ultimately contributes to new tissue formation. It is possible that an altered proportion of GAG loss to the media between treatment groups may occur under some circumstances. However, we note that previous studies which have examined GAG retention found that the proportion of GAG lost to the culture medium is unaffected by treatment of normal chondrocytes with NAC, hypoxia or glucose level (Collins et al., 2015; Markway et al., 2013; Heywood et al., 2006b).

Once encapsulated into alginate beads and cultured under a 20% O₂ atmosphere, chondrocytes expanded in monolayer in the presence of NAC accumulated similar quantities of the cartilage extracellular matrix constituent GAG, compared to untreated cells. This is consistent with the absence of any significant effect of NAC treatment on the expression of cartilage phenotype markers, SOX9, type II collagen and aggrecan, immediately prior to alginate encapsulation (see supporting information, Figure S1A and Table S1). Induction of hypertrophy is also associated with altered capacity for articular cartilage repair and may be induced by elevated ROS levels (Kishimoto et al., 2010; Morita et al., 2007). However, no significant difference in the expression of hypertrophic markers, collagen type X and Activin receptor-like kinase-1 (Dell’Accio et al., 2001; Markway et al., 2013; van den Berg, 2011) were observed after expansion with NAC (see supporting information, Figure S1B). Accordingly, GAG accumulation under normoxic conditions was neither augmented nor fundamentally impaired by NAC treatment during the earlier expansion phase. However, conditions in the joint are expected to be 1–10% O₂ (Grishmaw and Mason, 2000), and reducing the O₂ concentration within this range is reported to increase chondrocyte matrix synthesis, including GAG accumulation (Domm et al., 2002; Lafont et al., 2008; Li et al., 2014; Murphy and Polak, 2004; Murphy and Sambanis, 2001). This effect was observed in the current study, with an enhancement of GAG accumulation for chondrocytes expanded in monolayer at 20% O₂ when oxygen in the incubator atmosphere was reduced to 5% v/v during the subsequent culture within alginate beads (Figure 6). The presence of NAC during monolayer expansion abolished this anabolic response to hypoxia during subsequent alginate culture. This highlights a potential detrimental effect on the regenerative behaviour of cells expanded with NAC on re-implantation into a cartilage defect under low oxygen conditions. Interestingly, the anabolic response to hypoxia was retained when NAC was provided only during the alginate culture phase but was absent during monolayer expansion.

Mitochondrial function is important for hypoxia-signalling mechanisms in mammalian cells. Although the precise mechanisms are unclear, it has been reported that ROS produced under hypoxia by the mitochondrial electron transport chain are involved in the stabilization of the hypoxic inducible factor-1α subunit (HIF1-α) protein (Ball et al., 2012; Bell et al., 2007a, 2007b), a master-regulator of hypoxic signalling. However, others report that altered respiratory activity of the mitochondria modulates the cellular hypoxic response by acting as an oxygen sink, which decreases the local cellular oxygen concentration below a critical threshold (Chua et al., 2010; Li et al., 2014). The observation that NAC treatment...
only blocked the hypoxic induction of GAG when applied during the monolayer phase, and not the alginate phase alone, supports the second hypothesis. This was further supported by the augmentation of GAG accumulation under 20% O₂ conditions by the mitochondrial activator dichloroacetate (Figure 6). Together, these data raise the intriguing concept that the acquired mitochondrial function may play an important role in mediating the anabolic response of culture-expanded chondrocytes to reduced oxygen levels.

In summary, the current study supports the hypothesis of a causal relationship between exposure to ROS and bioenergetic reprogramming in articular chondrocytes. Additionally, mitochondrial function may be required for the hypoxic induction of GAG synthesis by cultured chondrocytes. This reveals that bioenergetic reprogramming in vitro may influence chondrocyte function following re-implantation in vivo. The antioxidant NAC inhibited the acquisition of mitochondrial function in monolayer. Although these chondrocytes required less time to reach a designated proliferation level, this potential benefit to cell-based cartilage therapies is likely to be outweighed by the concurrent impairment of cartilage matrix regeneration that was observed on re-implantation to a hypoxic environment such as the joint.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

This study was supported by the Dunhill Medical Trust (Grant No. R339/0214), the Wellcome Trust (Grant No. 080440/Z/06/Z) and Engineering and Physical Sciences Research Council (EPSRC; Platform Grant No. EP/E046975/1).

References

Balis UJ, Behnia K, Dwarkanath B, et al. 1999; Oxygen consumption characteristics of porcine hepatocytes. Metab Eng 1: 49–62.

Ball KA, Nelson AW, Foster DG, et al. 2012; Nitrile oxide produced by cytotoxic c oxidase helps stabilize HIF-1α in hypoxic mammalian cells. Biochem Biophys Res Commun 420: 727–32.

Bell EL, Klimova TA, Eisenbart J, et al. 2007a; The Qo site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production. J Cell Biol 177: 1029–36.

Bell EL, Klimova TA, Eisenbart J, et al. 2007b; Mitochondrial reactive oxygen species trigger hypoxia-inducible factor-1α protein in hypoxia occurs independently of mitochondrial reactive oxygen species production. J Biol Chem 285: 31727–84.

Collins JA, Moots RJ, Clegg PD, et al. 2015; Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Biochem Biophys Res Commun 437: 222–27.

Champagne AM, Benel L, Ronot X, et al. 1987; Rhodamine 123 uptake and mitochondrial DNA content in rabbit articular chondrocytes evolve differently upon transfer from cartilage to culture conditions. Exp Cell Res 171: 404–10.

Chua VL, Dufour E, Dassa EP, et al. 2010; Stabilization of hypoxia-inducible factor-1α protein in hypoxia occurs independently of mitochondrial reactive oxygen species production. J Biol Chem 285: 31727–84.

Heywood HK, Bader DL, Lee DA 2006a; Rate of oxygen consumption by isolated articular chondrocytes is sensitive to medium glucose concentration. J Cell Physiol 206: 402–10.

Heywood HK, Bader DL, Lee DA 2006b; Glucose concentration and medium volume influence cell viability and glycosaminoglycan synthesis in chondrocyte-seeded alginate constructs. Tissue Eng 12: 3487–96.

Heywood HK, Knight MM, Lee DA 2010; Both superficial and deep zone articular chondrocyte subpopulations exhibit the Crabtree effect but have different basal oxygen consumption rates. J Cell Physiol 223: 630–9.

Heywood HK, Lee DA 2008; Monolayer expansion induces an oxidative metabolism and ROS in chondrocytes. Biochem Biophys Res Commun 373: 224–9.

Heywood HK, Lee DA 2010; Low oxygen reduces the modulation to an oxidative phenotype in monolayer-expanded chondrocytes. J Cell Physiol 224: 228–53.

Heywood HK, Nalesso G, Lee DA, et al. 2014; Culture expansion in low-glucose conditions preserves chondrocyte differentiation and enhances their subsequent capacity to form cartilage tissue in three-dimensional culture. BioRes Open Access 3: 9–18.

Kishimoto H, Akagi M, Zushi S, et al. 2010; Induction of hypertrophic chondrocyte-like phenotypes by oxidized LDL in cultured bovine articular chondrocyte through increase in oxidative stress. Osteoarthr Cartilage 18: 1284–90.

Lafont JE, Talma S, Hofgaard C, et al. 2008; Hypoxia promotes the differentiated human articular chondrocyte phenotype through SOX9-dependent and - independent pathways. J Biol Chem 283: 4778–86.

Lee DA, Reiser T, Bader DL 2003; Expansion of chondrocytes for tissue engineering in alginate beads enhances chondrocyte...
phenotype compared to conventional monolayer techniques. Acta Orthop Scand 74: 6–15.

Lee HC, Wei YH 2005; Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. Int J Biochem Cell Biol 37: 822–34.

Lee HC, Yin PH, Chi CW, et al. 2002; Increase in mitochondrial mass in human fibroblasts under oxidative stress and during replicative cell senescence. J Biomed Sci 9: 517–26.

Lee HC, Yin PH, Lu CY, et al. 2000; Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. Biochem J 348: 425–32.

Li S, Oreffo RO, Sengers BG, et al. 2014; The effect of oxygen tension on human articular chondrocyte matrix synthesis: integration of experimental and computational approaches. Biotechnol Bioeng 111: 1876–85.

Markway BD, Cho H, Johnstone B 2013; Hypoxia promotes redifferentiation and suppresses markers of hypertrophy and degeneration in both healthy and osteoarthritic chondrocytes. Arthritis Res Ther 15: R92.

Michelakis ED, Webster L, Mackey JR 2008; Dichloroacetate (DCA) as a potential metabolic targeting therapy for cancer. Br J Cancer 99: 989–94.

Mignotte F, Champagne AM, Froger-Gaillard B, et al. 1991; Mitochondrial biogenesis in rabbit articular chondrocytes transferred to culture. Biol Cell 71: 67–72.

Milner PI, Wilkins RJ, Gibson JS 2007; The role of mitochondrial reactive oxygen species in pH regulation in articular chondrocytes. Osteoarthr Cartilage 15: 735–42.

Morita K, Miyamoto T, Fujita N, et al. 2007; Reactive oxygen species induce chondrocyte hypertrophy in endochondral ossification. J Exp Med 204: 1613–23.

Murphy CL, Polak JM 2004; Control of human articular chondrocyte differentiation by reduced oxygen tension. J Cell Physiol 199: 451–9.

Murphy CL, Sambanis A 2001; Effect of oxygen tension and alginate encapsulation on restoration of the differentiated phenotype of passed chondrocytes. Tissue Eng 7: 791–803.

Patappa G, Heywood HK, de Bruijn JD, et al. 2011; The metabolism of human mesenchymal stem cells during proliferation and differentiation. J Cell Physiol 226: 2562–70.

Rosenthal O, Bowie MA, Wagoner G 1941; Studies in the metabolism of articular cartilage. I. Respiration and glycolysis of cartilage in relation to its age. J Cell Physiol 17: 221–33.

Stockwell RA 1991; Morphometry of cytoplasmic components of mammalian articular chondrocytes and corneal keratocytes: species and zonal variations of mitochondria in relation to nutrition. J Anat 175: 251–61.

Turrens JF 2003; Mitochondrial formation of reactive oxygen species. J Physiol 552: 335–44.

van den Berg WB 2011; Osteoarthritis year 2010 in review: pathomechanisms. Osteoarthr Cartilage 19: 338–41.

Venditti P, Napolitano G, Barone D, et al. 2014; Vitamin E supplementation modifies adaptive responses to training in rat skeletal muscle. Free Radic Res 48: 1179–89.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.

Figure S1. Gene expression (A) considered representative of cartilage phenotype (SOX9, collagen type II and aggrecan) or (B) associated with cartilage hypertrophy (collagen type X and Activin receptor-like kinase receptor-1) were assessed. Total mRNA of monolayer cells cultured for 2 population doublings under 20% O2 in the presence or absence of 2 mM NAC was extracted and prepared for qPCR analysis of gene expression as described in (Heywood et al., 2014). No significant differences were observed (paired t-test) between control and NAC cultured cells with either phenotypic or hypertrophic markers. Data represents the mean ± SD of 3 cell donors, tested in duplicate and additional replicate qPCR reactions. Primer sequences are given in Table S1. Gene expression was assessed using a standard curve prepared by serial dilution of cDNA of freshly isolated chondrocytes with normalization to β2 microglobulin housekeeping control. COL10A1 and ALK-1 are expected to have lower expression in freshly isolated cells and therefore the standards were prepared by dilution series of a preliminary PCR amplification.

Table S1. Sequences of primers used for qPCR to examine expression of phenotypic and hypertrophic genes in chondrocytes.