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The effects of direct current stimulation on isolated chondrocytes seeded in 3D agarose constructs

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Abstract. Endogenous electrical activity has been detected in articular cartilage. It has previously been suggested that the associated electrical currents and potentials are important to the mechanotransduction processes in cartilage. The present study investigates the effects of direct current on cell proliferation and matrix synthesis, using the well established 3D chondrocyte – agarose model system.

Bovine chondrocytes isolated from metacarpalphalangeal joints were seeded in agarose constructs and exposed to a current density of 4 mA/cm^2 for 6 h, a magnitude and period which was shown to maintain cell viability. The influence of the optimized electric stimulus was assessed by protein incorporation and mRNA measurements, using radiolabels and real-time QPCR, respectively.

Results indicated no systematic influences of electrical current on protein synthesis, cell proliferation and mRNA expression levels. These data suggest that both the mode of stimulation and the model system are critical for the *in vitro* modulation of chondrocyte metabolism.

Keywords: Electric stimulation, chondrocyte, proteoglycan, collagen

1. Introduction

The solid constituents of articular cartilage consist primarily of proteoglycan and collagen II molecules, which account for its load bearing capacity. The extracellular matrix is maintained by a relatively low cell density. The chondrocytes are sustained by the diffusion of nutrients and metabolites derived from the synovial fluid, which is actively transported across the articular surfaces during normal joint activities. The resulting compressive stresses are transferred to the embedded chondrocytes and cause physical, electrical and chemical changes within the tissue [24]. It is established that these changes in the tissue dynamics can influence cellular response. Accordingly, chondrocytes have been shown to respond to exogenous physical stimuli such as hydrostatic pressure, fluid flow, osmotic changes, dynamic compression, ultrasound and electric stimulation [13,17,23,28,32,42,52,55]. In particular, the response to dynamic compression through increased matrix elaboration is well documented and the mechanical–electrical coupling is proposed to directly influence chondrocytic processes [6,20].

Historically, research and clinical use of electrical stimulation for skeletal tissue augmentation and/or repair have been largely restricted to studies involving bony tissues. These studies inevitably examined the formation of cartilage, which preceded bone formation in non-union fracture, using a range of

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electrical stimulation regimens [4,9,10,37,44]. However, with the increasing incidence of joint disease resulting from the ever-ageing population, there is considerable focus on repair strategies for articular cartilage.

Electrical stimulation encompasses a wide range of approaches, including the use of directly applied DC and AC voltages, and induced currents via capacitance coupling and electromagnetic fields. Relevant studies include those in which exposure of electrical current was reported to stimulate proliferation and cause increases in the turnover of matrix molecules by chondrocytes [4,8,18,35,38,44,45,47,49–51]. Indeed, articular cartilage defects have reportedly been filled by hyaline cartilage following direct current exposure to defect sites using implanted electrodes [5,37]. There is evidence that the increase in matrix turnover may be attributed to the down-regulation in the production of matrix degrading enzymes [15]. Furthermore, it has been demonstrated that chondrocytes in monolayer when electrically stimulated demonstrate both galvanotactic and galvanotropic responses [12,39]. Accordingly, the influence of electric current exposure on cellular mechanisms, in particular signal transduction processes, responsible for the up-regulation and/or down-regulation of matrix molecules including degradative enzymes have been investigated [43,48]. However, variations in cell culture model systems and methods of current exposure prevent direct comparisons between different studies.

This study uses the well established agarose model in an adaptable electrical stimulation system to systematically investigate the effect of direct current exposure on the viability of embedded chondrocytes. Thereafter, we tested the hypothesis that the optimised current exposure would enhance chondrocyte proliferation, up-regulate glycosaminoglycan synthesis, aggrecan and collagen II expression.

2. Materials and methods

2.1. Preparation of chondrocyte-agarose constructs

Chondrocytes were isolated aseptically from the metacarpalphalangeal joint of skeletally mature (18–24 month old) steers and seeded in agarose using a well established method [14,33,34]. Briefly, full depth cartilage from the proximal surface of the joint was extracted aseptically and sequentially digested at 37°C for 1 h in Dulbecco's Modified Eagles Medium supplemented with 16% (v/v) foetal calf serum (DMEM + 16% FCS), 2 μM L-glutamine, 0.85 μM L-ascorbate, penicillin (5 μg/ml), streptomycin (5 μg/ml), 20 mM HEPES and 700 units/ml of pronase (Type E, BDH Ltd., Poole, UK) followed by 16 hours in DMEM + 16% FCS supplemented 100 units/ml of collagenase (Type XI-A, Sigma-Aldrich, Poole, UK). The explant digest was passed through a cell sieve and the resulting cell suspension was washed twice with DMEM + 16% FCS. Thereafter, cell number was determined using a haemocytometer and cell viability assessed, using the trypan blue exclusion method. The cell suspension was subsequently seeded in 4% ultra-low gelling agarose (type IX, Sigma-Aldrich, Poole, UK) at 4×10^6 cells/ml and cast in Perspex moulds to produce $5 \times 5 \times 5$ mm^3 cubic constructs. Following gelation, each construct was equilibrated in DMEM + 16% FCS at 37°C and 5% CO_2 for 24 h prior to experimentation. Cells pooled from the isolation of 4–6 joints were employed in each experiment.

2.2. Influence of direct current density on cell viability

Equilibrated constructs were introduced into an electric stimulation tank along with 1.4 ml (for each construct) of DMEM + 16% FCS. The tank was designed such that it accommodated four constructs

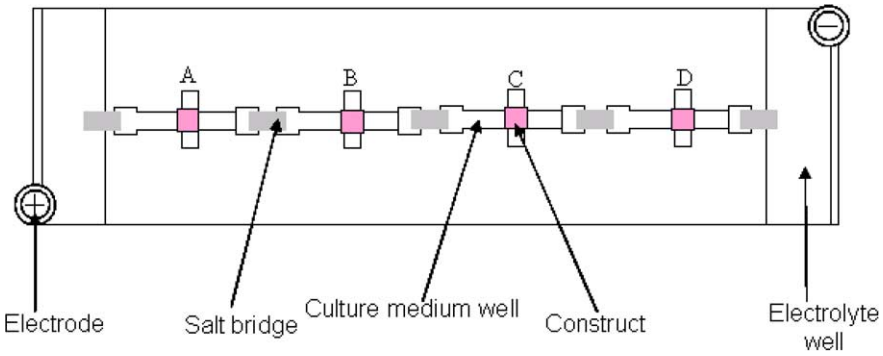

Fig. 1. A schematic of the electric stimulation tank incorporating four constructs connected in series by 3% agarose salt bridges.

connected in series by 3% agarose salt bridges, as shown schematically in Fig. 1. Constructs were subjected to current densities of 2, 4 and 24 mA/cm^2 for 12 h. Thereafter, recovered constructs were profiled for viability by staining each construct with Calcein-AM (Molecular Probes, Cambridge, UK) and Ethidium homodimer-2 (Molecular Probes, Cambridge, UK) following an established method [27]. To review briefly, constructs were bisected and incubated at 37°C for 40–45 min in 0.6 ml DMEM + 16% FCS supplemented with 5 μM each of Calcein-AM and Ethidium homodimer. Thereafter, each construct was mounted with its cut surface on a cover slip and visualised under a fluorescence microscope (Eclipse, Nikon, Kingston-upon-Thames, UK) with a xenon lamp fluorescent light source. Viability was recorded by a systematic sampling of the construct using a standard sampling area of 10 × 5 mm^2, as determined by the width and half the depth of a 1 cm^2 eye piece graticule viewed under a ×20 objective. Sampling was performed continuously from one face of the specimen to the other. The number of live (bright/green) and dead (dark/red) cells were counted in each sampling area and subsequently estimated as values of percentage viability (Fig. 2). A minimum of 3 replicate counts per sampling depth per construct were recorded.

2.3. Influence of direct current on GAG and DNA synthesis

Constructs equilibrated for 24 h were randomly allocated into stimulated or control groups. Stimulated constructs were subjected to a current density of 4 mA/cm^2 for 6 h, while controls remained unstimulated for the same period. Each construct was bathed in 1.4 ml of DMEM + 16% FCS supplemented with 10 μCi/ml $^{35}SO_4$ and 1 μCi/ml [^{3}H] thymidine and incubated at 37°C and 5% CO_2 for the duration of the current exposure. Following stimulation, both constructs and bathing media were recovered, weighed and digested for biochemical analysis as detailed previously [34]. Constructs were digested in 560 units/ml papain and 1000 units/ml agarase (both Sigma-Aldrich, Poole, UK). Incorporation of $^{35}SO_4$ into newly synthesised glycosaminoglycans was determined in both medium and the agarose digest by Alcian blue precipitation [34] onto filters using the multiscreen system (Millipore, Watford, UK). Corresponding [^{3}H] thymidine incorporation was measured in the agarose digests by 10% trichloroacetic acid precipitation onto filters using the multiscreen system (Millipore, Watford, UK). Total DNA was determined using the Hoescht 33258 method [3].

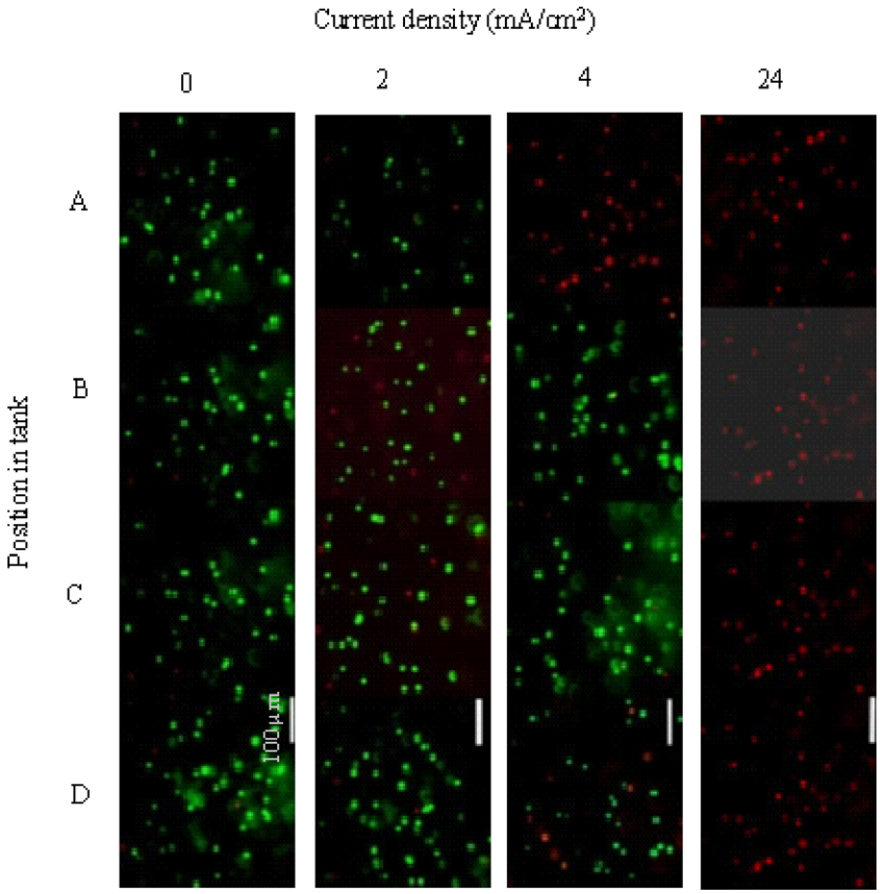

Fig. 2. Live-dead micrographs of chondrocytes in agarose following exposure to 0, 2, 4 and 24 mA/cm^2 for 12 h versus the location of constructs within the stimulation tank, labeled A to D. Cells viable after stimulation are stained bright (green) and non viable cells dark (red). Scale bars indicate 100 μm. (The colors are visible in the on-line version of the article.)

2.4. Influence of direct current density on aggrecan and collagen II mRNA production

Stimulated and control constructs were recovered following the 6 h stimulation at 4 mA/cm^2, were weighed, snap frozen in liquid nitrogen and subsequently stored at −80°C until analysed. Each construct was dissolved in 750 μl of buffer QG (Qiagen, Crawley, UK) at 42°C for 10 min. Following dissolution, 125 μl of 100% isopropanol was added. Total RNA was subsequently extracted using the RNeasy mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The extracted RNA was cleared of genomic DNA contamination using DNA-*free*™ (Ambion, Applied Biosystems, Warrington, UK) and the quantity of total RNA was determined. 200 ng of the total RNA was reverse transcribed with StrataScript® RT/RNase block enzyme mixture with oligo(dT) primers according to the manufacturer's protocol (cDNA synthesis kit, Stratagene, La Jolla, CA, USA).

Real-time quantitative PCR assays were performed in 25 μl reaction mixtures which included forward and reverse primers of aggrecan, collagen II and GAPDH (the housekeeping gene), their respective molecular beacons (Table 1, all supplied by Sigma-Genosys, Haverhill, Suffolk, UK) SureStart® Taq DNA polymerase, Brilliant® QPCR master mix (both Stratagene, La Jolla, CA, USA) and 2 μl of reverse transcribed cDNA, using a thermocycler (MX3000, Stratagene, La Jolla, CA, USA).

Table 1
Primer sequences for collagen II, aggrecan and GAPDH genes

Target	PCR primer sequence 5′–3′	Genbank accession number	Source
Collagen II	AAACCCGAACCCAGAACC (f) AAGTCGAACTGTGAGAGG (r) 6CGCGATGCGTCAGGTCAGGTCAGCCATATCGCG	X02420	Sigma-Genosys Ltd., Poole, UK
Aggrecan	TGGTGTTTGTGACTCTGAGG (f) GATGAAGTAGCAGGGGATGG (r) 6CGCGATCCACTCAGCGATTGTCAGGTTCTGAGATCGC	U76615	Sigma-Genosys Ltd., Poole, UK
GAPDH	TTCAACGGCACAGTCAAG (f) CTCAGCACCAGCATCACC (r) 8CGCGATCCACCATCTTCCAGGAGCGAGATCCGATCGCG	U85042	Sigma-Genosys Ltd., Poole, UK

GAPDH was validated (data not shown) and chosen because its expression did not significantly change following electric stimulation [11,54]. Relative quantities of mRNA expression of the target genes were normalised to GAPDH. Quantitative data was derived by substituting the Ct (threshold cycles) values, obtained from the Real-time PCR, into the formula derived by Pfaffl [36,46].

$$\text{Ratio} = \frac{(\text{E}_{\text{target}})^{\Delta Ct\ \text{target(control}-\text{treated)}}}{(\text{E}_{\text{housekeeping}})^{\Delta Ct\ \text{housekeeping(control}-\text{treated)}}}, \tag{1}$$

where: $\Delta Ct = Ct_{\text{control}} - Ct_{\text{treated}}$ (The difference in threshold values between control and stimulated samples). The efficiencies of the target and housekeeping genes were determined from standard curves produced from Ct values obtained by the PCR amplification of serial dilutions of the cDNA ranging from 0.00001 to 1.0 μl from an unstimulated control

$$\text{Efficiency } (E) = 10^{-\left(\frac{1}{\text{gradient of standard curve}}\right)}. \tag{2}$$

Efficiencies of 97%, 94% and 99% were obtained for aggrecan, collagen II and GAPDH respectively.

2.5. Statistical analysis

Data from electrically stimulated and control constructs were analysed and compared using a one way ANOVA with *post hoc* Bonferroni-corrected t-test. A confidence level of 5% was considered to be statistically significant ($p < 0.05$).

3. Results

3.1. The influence of current density on cell viability

Exposure to electrical stimulation for a period of 12 h resulted in a decrease in viability both with an increase in magnitude of current density up to 24 mA/cm^2 and, noticeably, with proximity to the positive electrode (position A in Fig. 1). Indeed, exposure to 4 mA/cm^2 for 12 h resulted in viability loss from

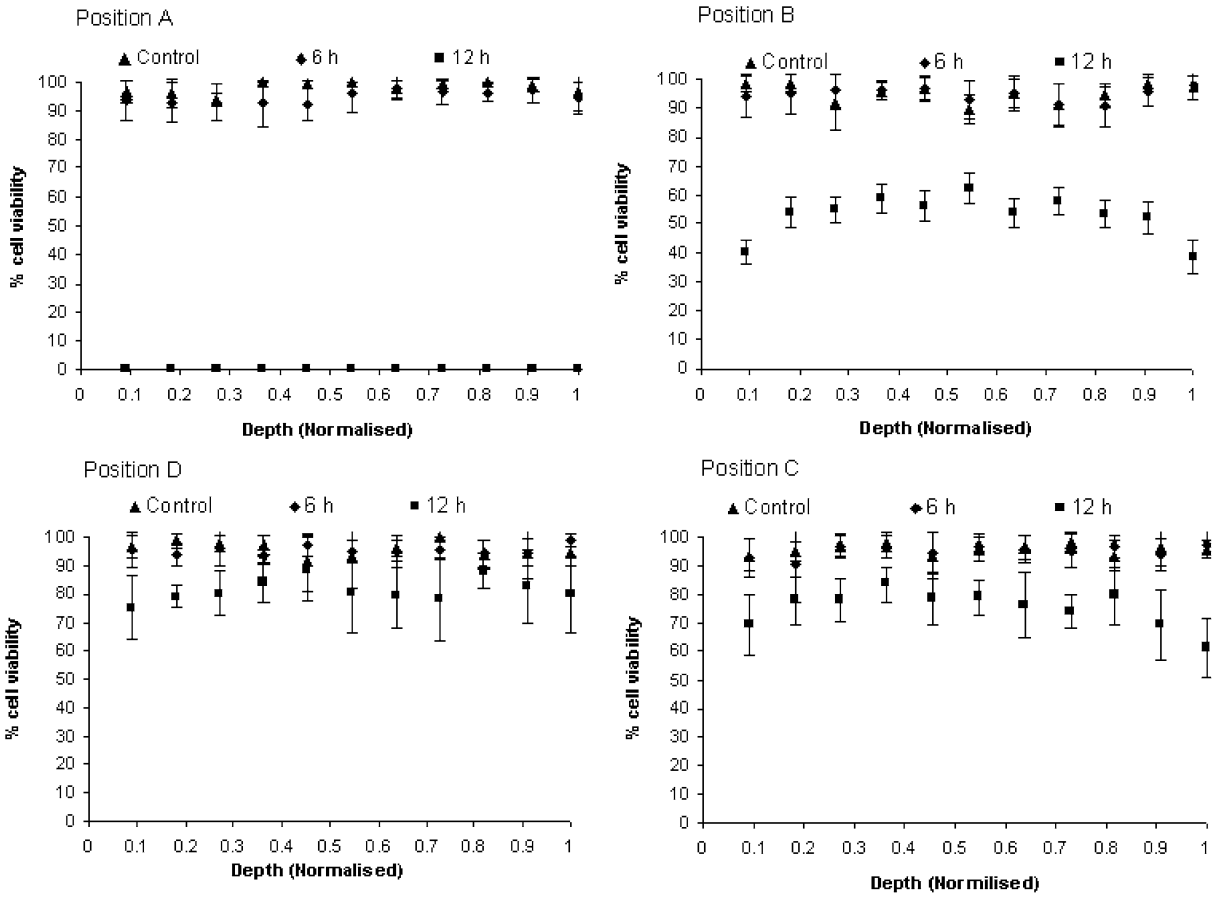

Fig. 3. Temporal effect of applied current density on the viability of chondrocytes seeded in agarose constructs and exposed to 4 mA/cm^2 for 6 and 12 h. The 4 graphs represent the viability profiles of stimulated and control constructs with respect to their position in the stimulation tank. Position A is closest to the positive electrode and position D closest to the negative electrode. Data presented represent the mean ± SD of 3 replicate experiments.

82% at position D, to 0% at position A (Fig. 2). By contrast, mean viability values greater than 90% were maintained in controls and constructs stimulated at 2 mA/cm^2. Subsequent reduction in the duration of exposure at 4 mA/cm^2 from 12 to 6 h resulted in a viability of greater than 90% viability at all locations within the stimulation tank (Fig. 3).

3.2. The influence of direct current on GAG synthesis

Absolute DNA amounts ranged between 5.9 and 6.4 μg in four replicate experiments across the four locations within the tank. No significant differences between stimulated and control groups were evident ($p > 0.05$).

The data for $^{35}SO_4$ incorporation, normalised to absolute DNA levels, obtained from the 6 h exposure of chondrocyte-seeded agarose constructs to 4 mA/cm^2 demonstrated considerable variability between the four replicate experiments (Fig. 4(i)–(iv)). For example, although Experiment 4 indicated a small up-regulation of $^{35}SO_4$ incorporation in stimulated constructs compared to controls in all locations within

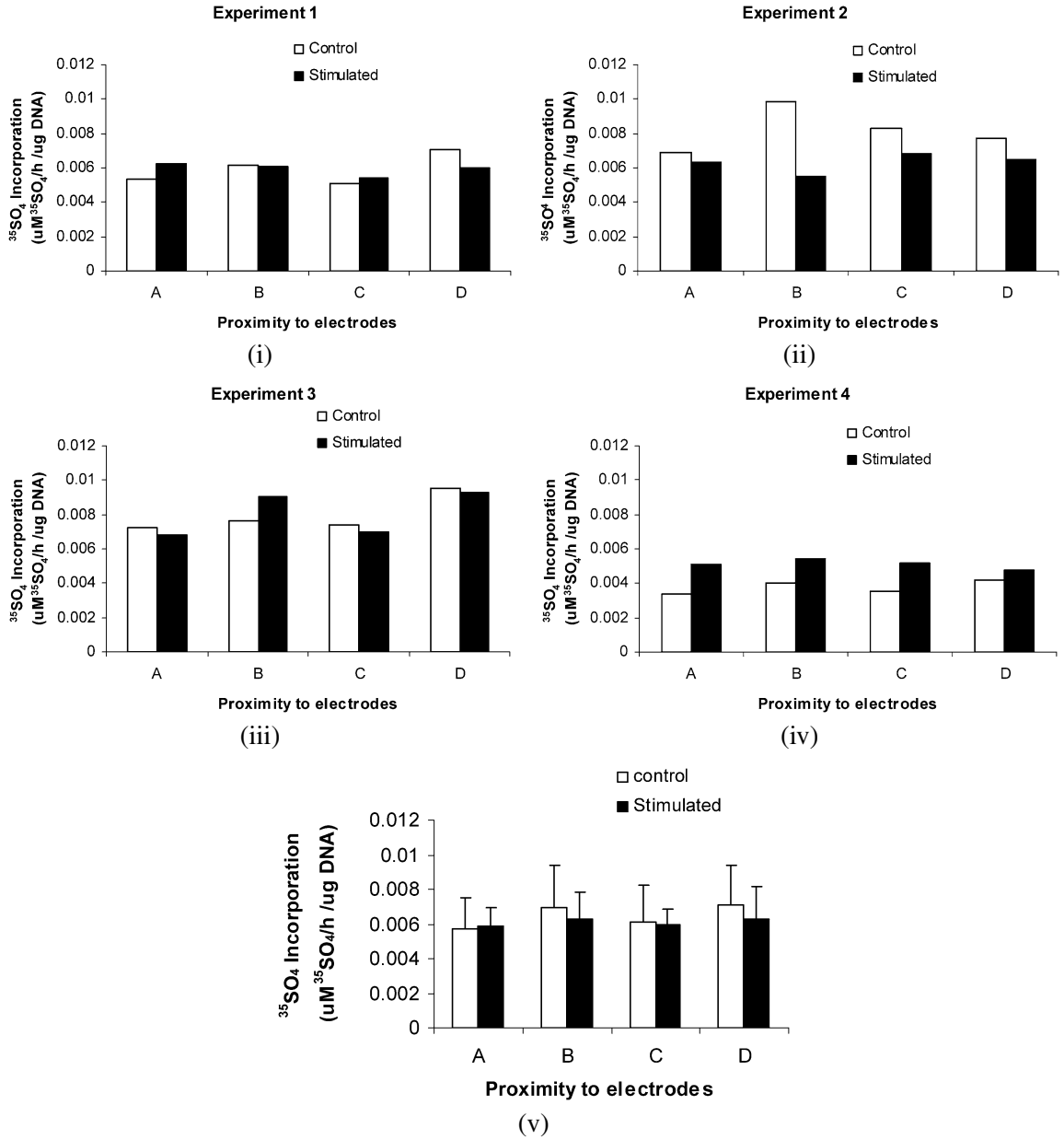

Fig. 4. The influence of direct current on $^{35}SO_4$ incorporation. Graphs represent constructs exposed either to a current density of 4 mA/cm^2 for 6 h, or are unstimulated controls. Figures (i)–(iv) represent the incorporation data from the 4 separate experiments. Position A is closest to the positive electrode and position D closest to the negative electrode. Figure (v) represents the mean ± SD of the pooled data from the 4 replicate experiments.

the tank, a reverse trend was observed in Experiment 2. When the data were pooled, mean $^{35}SO_4$ incorporation rates ranged from 0.0057 ± 0.002 μMSO$_4$/h/μg DNA to 0.0071 ± 0.002 μMSO$_4$/h/μg DNA in both stimulated and control constructs (Fig. 4(v)). The pooled data clearly revealed no systematic trends between either of the groups or with location of the constructs in the chamber ($p > 0.05$ in all cases).

3.3. The influence of direct current on DNA synthesis

The corresponding data for [^{3}H]-thymidine incorporation, normalised to absolute DNA levels, is illustrated for the four individual experiments (Fig. 5(i)–(iv)). Although the majority of cases suggested an up-regulation [^{3}H]-thymidine incorporation in the stimulated constructs, as noted in Experiment 4, there was still variability both between experiments and with location in the stimulation chamber. When

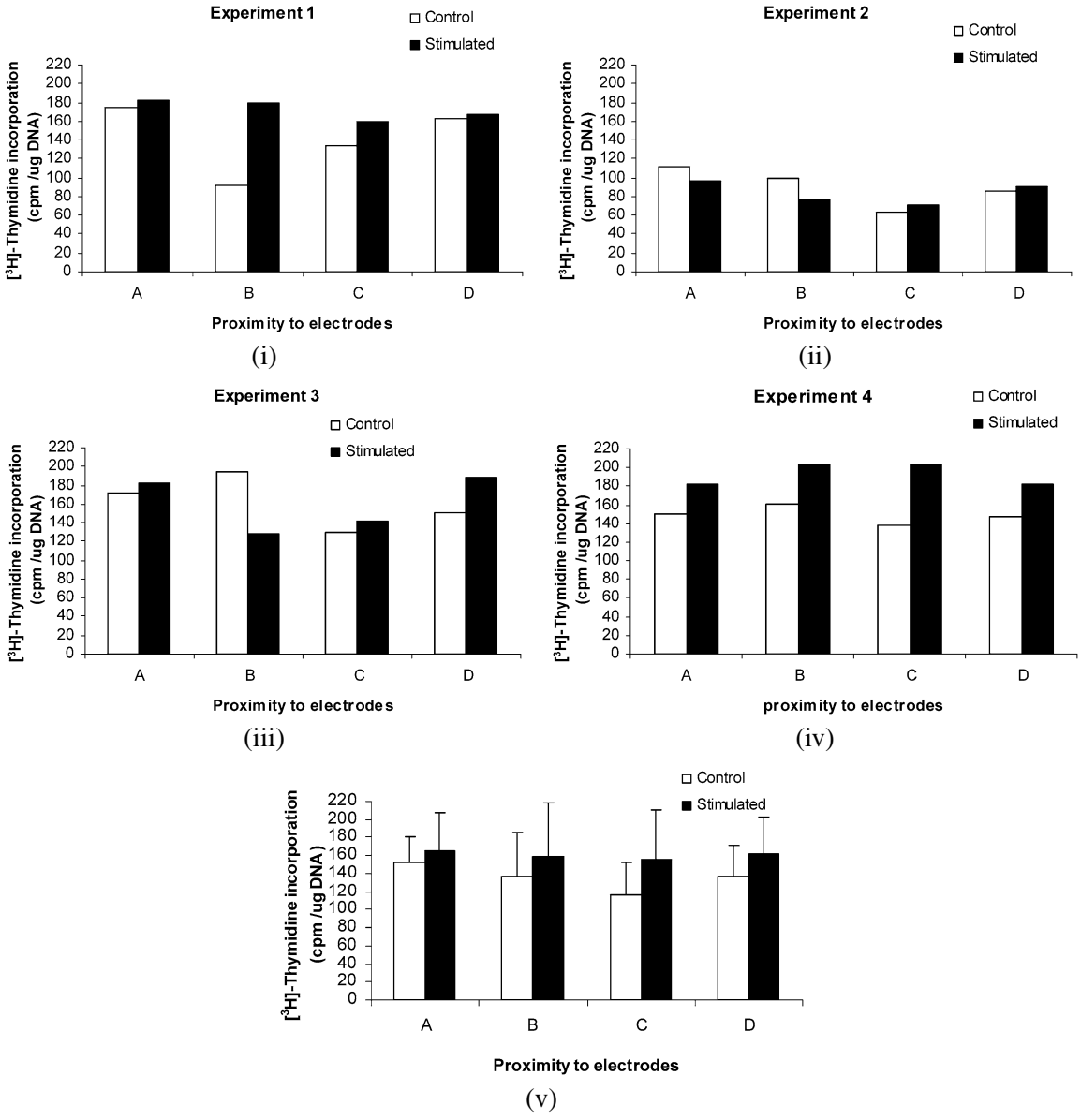

Fig. 5. The influence of direct current on [^{3}H]-thymidine incorporation. Graphs represent constructs exposed either to a current density of 4 mA/cm^2 for 6 h, or are unstimulated controls. Figures (i)–(iv) represent the incorporation data from the 4 separate experiments. Position A is closest to the positive electrode and position D closest to the negative electrode. Figure (v) represents the mean $\pm$ SD of the pooled data from the 4 replicate experiments.

the data were pooled, mean [^{3}H]-thymidine incorporation rates ranged from 116.6 ± 35.3 cpm/μg DNA and 161.2 ± 42.9 cpm/μg DNA in both stimulated and control constructs (Fig. 5(v)). Although the mean values were greater in the stimulated constructs for each of the locations, none of the differences were statistically significant ($p > 0.05$ in all cases).

3.4. The influence of direct current on aggrecan mRNA expression

Fold values for aggrecan gene expression were determined using Ct values obtained from the PCR amplification of unstimulated control and corresponding stimulated cDNA samples from Eq. (1). The results of five replicate experiments are illustrated in Fig. 6(i)–(v). It is evident that there were no systematic trends and considerable variability between experiments. For example, in Experiment 2 there was a consistent up-regulation in aggrecan expression reaching fold values of up to 3.0, whereas in Experiment 2 there was a consistent down-regulation with normalised values as low as 0.30. In addition some within-experiment variability was also revealed, for example in Experiment 5 (Fig. 6(v)).

3.5. The influence of direct current on collagen II mRNA expression

Fold values for collagen II expression were also determined from the Ct values obtained from the PCR amplifications. The results of the five replicate experiments are illustrated in Fig. 7. Similar to aggrecan expression, it is evident that there is considerable variability both between and within the five replicate experiments. Although there was clear up-regulation in collagen II gene expression in the four locations associated with Experiment 3, with folds attaining a value of 4.47, this consistent trend was not evident in any of the other 4 experiments, where normalised values were regularly (7 out of 20 cases) less than unity.

4. Discussion

In the present study a stimulation tank was developed, which allowed the application of current directly to chondrocytes within the well established agarose model system, to examine the metabolic effects of an exogenous applied current. Conditions within the stimulation tank were optimized by monitoring the effect of varying current densities and duration of exposure on the viability of the embedded chondrocytes (Figs 2 and 3). The results revealed that a current density of 2 mA/cm^2 applied for 12 h sufficiently maintained the viability of the cells. However, when the magnitude of the applied current density was increased to 4 mA/cm^2, a loss in viability from 82% at position D, to 0% at position A was observed (Fig. 2). Reducing the duration of exposure at 4 mA/cm^2 to 6 h resulted in a minimal loss of viability and hence an elimination of the associated viability gradient across the tank. The optimization study also indicated an influence of medium pH. Indeed, the pH change in culture medium from 7.40 to 2.25, associated with the application of direct current (data not shown), could account for the loss in viability. With the existing control system, it proved difficult to maintain a constant current density at a low level of 2 mA/cm^2. Accordingly, it was decided to select a current density of 4 mA/cm^2 applied for 6 hours for all subsequent experiments. At this setting the medium was maintained at pH 7.4 and there was no corrosion detected at the electrodes or production of HCl during the electrical stimulation.

Two approaches were adopted to examine the effects of current density on cellular activity. In the first, alterations in chondrocyte proliferation and proteoglycan synthesis were determined by the measured uptake of [^{3}H]-thymidine and $^{35}SO_4$ radioisotopes [14,31,34], respectively. The second approach,

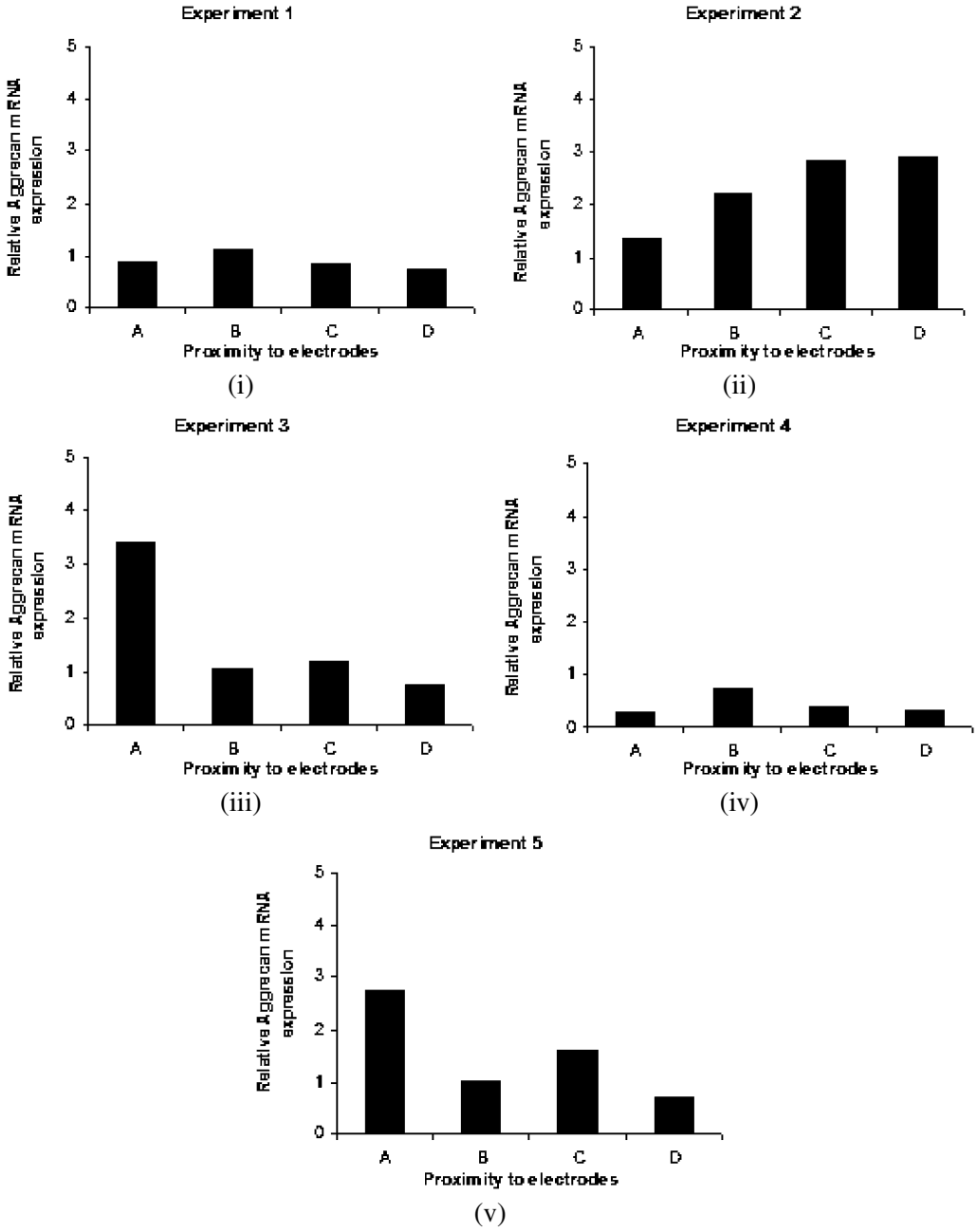

Fig. 6. The influence of current density on aggrecan gene expression. Constructs were stimulated at 4 mA/cm^2 for 6 h following a 24 h equilibration period. Data presented are derived from 5 replicate experiments and are displayed as relative aggrecan mRNA expression (stimulated/control) versus the location of samples within the stimulation chamber. Position A was closest to the positive electrode and position D was closest to the negative electrode.

focused on the processes preceding protein synthesis, as estimated by the relative changes in mRNA expression levels of aggrecan and collagen II. This approach enabled an assessment of the early effects of the applied current. The methodology involving the use of buffer QG, simplified the extraction process

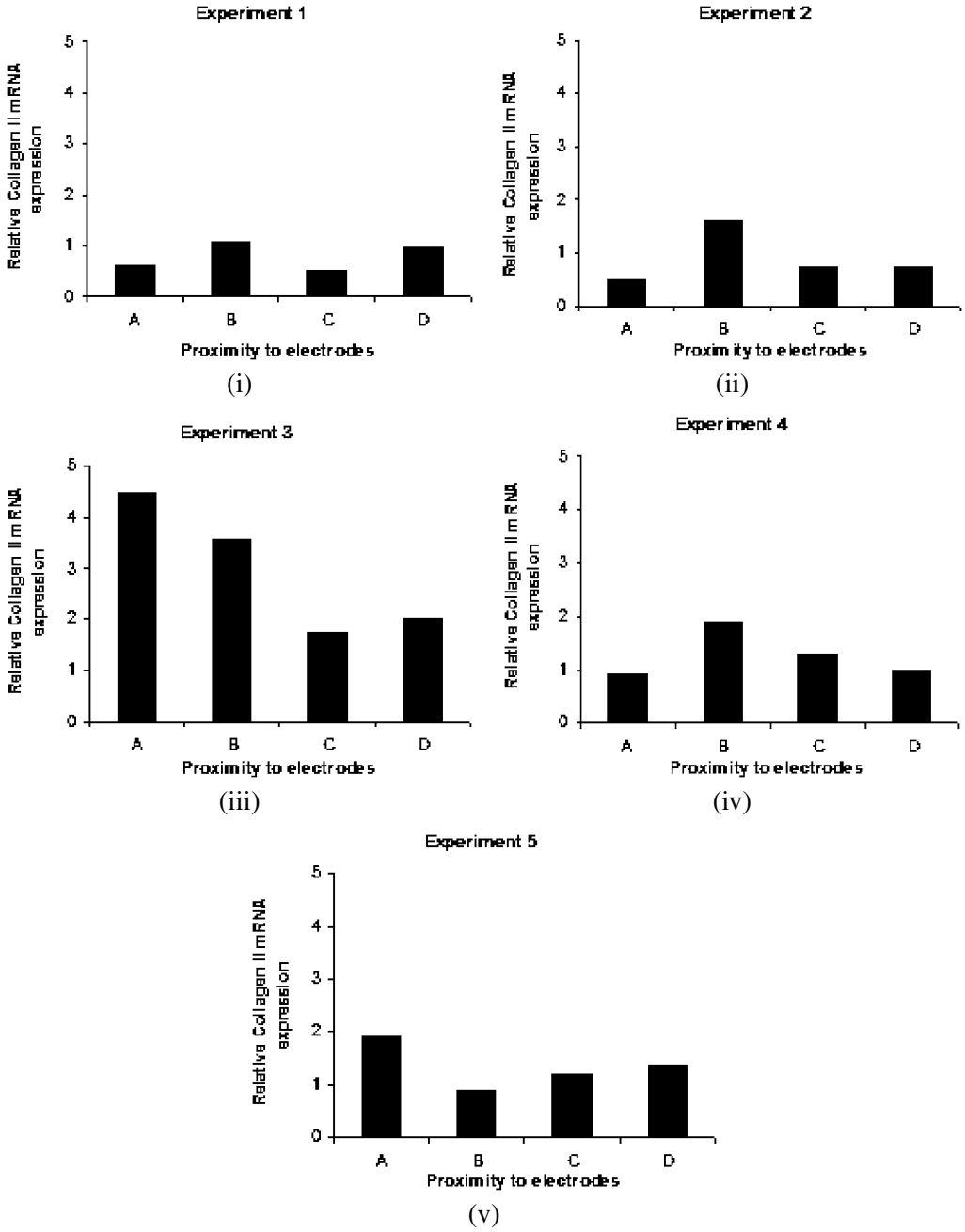

Fig. 7. The influence of current density on collagen II gene expression. Constructs were stimulated at 4 mA/cm^2 for 6 h, following a 24 h equilibration period. Data presented are derived from 5 replicate experiments and are displayed as relative collagen II mRNA expression (stimulated/control) versus the location of samples within the stimulation chamber. Position A was closest to the positive electrode and D was closest to the negative electrode.

and reduced the extraction time and the risk of contamination. Furthermore, it eliminated the use of potentially dangerous chemicals, such as trizol and chloroform [25,29,40]. Expression levels were nor-

malised to GAPDH, a common reference gene [1,2,22,26,54] whose expression levels were reported to be unaffected by electric stimulation [11,13,45,55]. This was confirmed using the present system (data not shown).

Examination of the influence of the applied electric stimulus on matrix synthesis revealed considerable variability in the levels of $^{35}SO_4$ incorporation between the four experiments, using separate batches of chondrocytes. However, pooled $^{35}SO_4$ incorporation data revealed a small reduction in stimulated constructs compared to controls (Fig. 4), although the differences were not statistically significant ($p > 0.05$). These findings indicate that the direct current did not influence $^{35}SO_4$ uptake by chondrocytes in a 3D agarose system. They are in accordance with some studies [49,51], but in contrast to other studies in which $^{35}SO_4$ uptake was markedly enhanced by electrical stimulation [4,37,38].

The relative expression levels of aggrecan mRNA varied considerably both between and within experiments (Fig. 6) with only 40% of stimulated samples showing increased expression levels relative to controls. This finding suggests that the applied current may stimulate aggrecan mRNA production, but its ability to do this is influenced by the sensitivity of the cells and the time for which they are exposed. Intermittent exposure to an electrical stimulus for a period of 7 days was reported to up-regulate aggrecan mRNA expression [11]. By contrast aggrecan mRNA expression was reported to be upregulated in the initial 2 hour period following stimulation in the form of static mechanical strain [19]. The choice of 6 hour electrical stimulation was selected for practical considerations. The transient nature of gene expression culminates in a temporal profile consisting of peaks in expression levels followed by rapid decreases caused by the rapid degradation of transcribed mRNA molecules. Thus the time at which the expression level of a gene is measured is critical. It is highly conceivable that peak levels of aggrecan mRNA expression corresponded to an earlier time point than the 6 hour period used in the present study. A similar argument may be appropriate for collagen II mRNA expression, whose relative expression levels varied between and within experiments (Fig. 7). There was no correlation between the expression levels of aggrecan and collagen II genes. In addition, the expression levels of these genes were independent of the sample proximity to either electrode. A further factor worthy of consideration is the serum concentration in culture medium, which has been previously reported to influence both aggrecan and collagen II expression [26].

The applied current density did not alter the absolute DNA amounts from control levels in all replicate experiments. However, there was again considerable variability in [^{3}H]-thymidine incorporation rates between experiments. Pooled data for these experiments revealed marginal increases in stimulated constructs compared to controls (Fig. 5), although the differences were not statistically significant. This finding adds to the complexity inferred from previous studies in which chondrocyte proliferation was reported to be either up-regulated [18] or inhibited by electric stimulation [4,8,10].

With electrical stimulation, the effective field distribution will vary depending on the nature of the culture system. This is the case for chondrocytes cultured within explants, in pellet form or surrounded by matrix, and the field will certainly be different again in the present model system incorporating isolated chondrocytes in agarose constructs. The type of applied current and the mode of application would also influence the measured response. Thus, alternating currents at varying frequencies have been applied to chondrocytes by various delivery methods including direct stimulation, capacitive coupling or electromagnetic stimulation [3,6,18,38]. Moreover, it is evident from previous reports that specific but different magnitudes and/or frequencies of electric stimulation have been required for proliferation and matrix synthesis, indicating that the two cell processes are uncoupled [8–10].

It has previously been reported that the response of chondrocytes to applied stimuli is influenced by the age and species of the animal model and the type of hyaline cartilage from which they are sourced

[4,7–9,21,30,41]. In the present study, skeletally mature steers were used. In addition, differences in the morphology and metabolism of surface and deep chondrocytes within a tissue sample are well documented [16]. In the present study, a mixed population of cells from full depth cartilage was used and the ratio of chondrocyte sub-populations in each construct was not controlled. This may represent an additional factor accounting for the differences in measured responses between experiments.

The 3D agarose culture system used in this study permitted the direct measurement of the effects of electric stimulation on chondrocytes in the absence of extracellular matrix and associated molecules. The cells incubated within the stimulation chamber were adequately nourished for the duration of the experiments and any differences observed between control and stimulated constructs were not a direct result due to compromised viability. It may be concluded that a direct current density of 4 mA/cm^2 applied for 6 h, did not adequately distinguish the cells exposed to it. It is, however, clear that the influence of electric stimulation on chondrocytes seeded within agarose constructs is dependent on the nascent metabolic activity of the cell population exposed. Further investigations, involving manipulations of the stimulation protocol, are required to determine the appropriate durations and type of electrical exposure which may elicit positive metabolic responses in the agarose model system.

Acknowledgements

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