EFFECT OF PULSED-LOW INTENSITY ULTRASOUND EXPOSURE ON CALF CHONDROCYTES

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1.0 Introduction

Articular cartilage possess little capacity for self-repair, after being damaged by disease or trauma, due to the low mitotic activity of its cells [1-3]. This low mitotic activity can potentially be due to the low metabolic and biosynthetic activities of mature chondrocytes and, lack of chondrogenic cells [4, 5]. Articular cartilage is not similar to any other connective tissue, as it is avascular, aneural and has no lymphatic system. As a consequence, nutrients and waste products are delivered through diffusion process by pumping action that occurs during compression of cartilage [6, 7] thus, it is jeopardise during injury and disease.

In spite of the extensive published studies documenting the response of chondrocytes to PLIUS treatment [2, 3, 8], in this study the exact conditions that have been used to treat 3T3 mouse fibroblasts and human dermal fibroblasts [9, 10] were used to test the effects on bovine chondrocytes.

2.0 Materials and Methods

2.1 Materials

DMEM media from Invitrogen (Paisley, UK), Low viscosity sodium alginate from kelp (brown seaweed) (viscosity = 20-40 cP = 0.02-0.04 Pa.s for a 2% solution at 25°C cat no: 180947, Mw 120 000-190 000, mannuronic/guluronic ratio 1.56 - 0.64), Alcian blue dye, chloramine T, 1,9 dimethylmethylene blue dye (DMB), p-dimethylaminobenzaldehyde (P-DMBA), and a DNAQF DNA Quantification Kit, were purchased from Sigma-Aldrich (Poole, Dorset, UK). Foetal calf serum was purchased from PAA Laboratories (Farnborough, Hampshire, UK). All other materials that not mentioned here were obtained from Sigma-Aldrich.
2.2 Methods

2.2.1 Bovine chondrocytes

Chondrocytes were isolated from bovine articular cartilage (Cardiff School of Bioscience, University of Cardiff). Cells were maintained in DMEM supplemented with bovine serum (10 v/v%), gentamycin (0.1 v/v%), and 50µg/ml ascorbate. Cells were stored in sterile conditions at 37°C and 5% CO₂ and the media was replenished every three days.

2.2.2 Cell encapsulation

At confluence the cells were trypsinised (0.05%) EDTA (0.2 g/l) and resuspended in DMEM. Viable cell numbers were determined by staining with trypan blue in haemocytometer [11]. Sodium alginate solutions (2%) were seeded with cells at 2x10⁶ cells/ml density. The cell suspension was transferred to twelve-well plates and incubated in a bath of sterile CaCl₂ solution for 2 h [12]. The resulting discs of calcium alginate with encapsulated cells were washed three times with sterile PBS (phosphate-buffered saline solution) and later transferred to six-well plate with each well containing supplemented DMEM (5 ml) which was changed in every 3 days.

2.2.3 PLIUS treatment

A Sonopuls 491 (Enraf-Nonius, Rotterdam, Amsterdam) ultrasound source was used. Its transducer was immersed in a water bath filled with deionized water maintained at 37°C [13] and treated with a chemical solution (Sigma Clean Water bath) to keep its sterility. The deionized water was changed every week. The six-well plate containing constructs to be treated was placed on top of the transducer; the control group was maintained in the same conditions without being exposed to ultrasound. The ultrasound stimulation was performed 5 min everyday for 10 days at a frequency of 1 MHz and an intensity of 0.2 W/cm² with a 20% duty cycle. Treated and control samples were analysed, at regular intervals, to determine cell numbers, collagen content and GaG content.

2.2.4 Cell content study

Constructs, that had been cultured up to 10 days, were freeze-dried for 2 days, digested in papain solution (1 ml; 125 µg/ml) [14]. Total cell numbers were determined by a Hoechst assay using a DNAQF DNA Quantitation Kit [15] and measured using a spectrophotometer (Promega glomax, Promega, Southampton, UK) at a wavelength of 460 nm, at ambient temperature. The total cell numbers were obtained from a calibration curve (2.0–12.0 x 10⁶ cells/ml) of calf chondrocyte cells.

2.2.5 Hydroxyproline assay

This assay was used for the quantification of hydroxyproline content using a method previously described [16-18] with some modification. With this method, 50µl of papain digested samples were hydrolyzed in HCl (6 M) at 110°C for 20 h and then kept in the vacuum desiccator overnight to allow evaporation of the remaining HCl. The dried samples were then reconstituted with 200 µl assay buffer [5g/l citric acid (monohydrate), 12 g/l sodium acetate (trihydrate), 3.4g/l sodium hydroxide, and 1.2ml/l glacial acetic acid in distilled water, at pH 6.0 [16]. The reconstituted samples were then mixed with activated charcoal and left in ambient conditions for 30 min. The samples were then centrifuged and 50 µl of the clear samples were then mixed with chloramine T solution (62 mM) and then incubated at room temperature for 15 min to allow oxidation to occur. Then the samples were mixed with 50 µl of p-DMBA solution [18] and incubated at 60°C for 30 min. The absorbance of the samples was measured at 550 nm using a microplate reader (Promega glomax, Promega, Southampton, UK). The hydroxyproline contents of the samples were determined from a regression line of absorbance plotted against concentration (0-1 µg/ml) for trans-4-hydroxy-L-proline.

2.2.6 Glycosaminoglycan (GaG) assay

The GaG content was quantified by a previously described method [19] with a slight modification. Briefly, 40 µl of papain digested samples were added to 250 µl DMB dye. Absorbance was measured at 600 nm using a spectrophotometer (Promega glomax, Promega, Southamton, UK). The GaG content of the samples was determined by calibrating the absorbance measurements using results from a standard solution of whale chondroitin 6 sulfate (0-100 µg/ml).

2.2.7 Alcian blue staining

Alginate discs containing cells were stained using Alcian blue dye [20, 21]. The encapsulated cells were fixed with 10% formalin for 20 min then washed with PBS. The discs were then stained with Alcian blue dye for 48h [20]. The alginate/cells were then washed sequentially with 3% acetic acid, 3% acetic acid and 25% ethanol, 3% acetic acid and 50% ethanol and 70% ethanol. The encapsulated cell were observed using a light microscope (Axiolab, Zeiss, Oberkochen, Germany) [20, 21].

2.2.8 Sample preparation for SEM

CP5 encapsulated in alginate disc were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 1 h. After that the samples were dehydrated in alcohol (50%, 70%, 90%,
100%) twice for 15 min for each respective alcohol change. Then the samples were dried using critical point CO$_2$ and the dried samples were mounted on a stub and coated with platinum. The samples were then viewed using scanning electron microscopy (Philips XL30 ESEM FEG, Netherlands).

**2.2.9 Statistical analyses**

The Shapiro-Wilk test [22] for the normality test was done for all of the data presented here. The data were analyzed using the Independent t-test for normal data and Mann-Whitney test for non-normal data [22]. Differences were considered to be significant if the probability $p < 0.05$.

**3.0 Results**

The cell viability and proliferation of chondrocytes in the 3D alginate culture for both groups (control and PLIUS) shows a fluctuating pattern with the total cell number in the range 17 - 39 x10$^5$ cells/ml for the control group and 27 - 47 x10$^5$ cells/ml for PLIUS group (Figure 1). There is no significant different between treated and control groups.

The hydroxyproline assay (Figure 2) shows that PLIUS may have a tendency to enhance collagen production in the treated group and appears to be significant at day 4. However, there is no evidence for PLIUS consistently enhancing collagen production for encapsulated bovine chondrocytes.

GaG content was quantified using the DMB assay. With this assay, PLIUS treatment for 10 days in culture did not represent any significant effect in increasing GaG production (Figure 3). The graph itself shows a decreasing amount of GaGs with time.

Alcian blue staining was done to confirm GaG accumulation around the encapsulated chondrocytes. After 8 days, as shown in Figure 4, the stained sample showed halos of Alcian blue around the cells indicating GaG production. Alcian blue staining revealed the formation of GaG for the whole experimental period. There is no significant difference in GaG production for control and treated groups.

Figure 1 shows that PLIUS did not have any effect on chondrocyte viability and proliferation. In SEM micrographs, the cells showed no differences compared to the control group. However, the chondrocytes encapsulated in alginate had a rounded chondrocyte-like appearance (Figure 5). This was supported by previous findings that also show that chondrocytes maintain their rounded morphology in 3D culture [23-25] but have a spread, fibroblast-like, shape in 2D culture (i.e., in a monolayer) [23-25]. It appears that chondrocytes maintain a rounded morphology when encapsulated in alginate while fibroblasts do not maintain their fibroblast-like shape when encapsulated in alginate gels[9, 10].

**4.0 Discussion**

The objective of this study was to determine whether PLIUS can increase cell proliferation, enhance collagen production
and stimulate GaG production as in our previous study [10]. PLIUS have been proven to promote cell proliferation [2, 3] and matrix deposition by chondrocytes in 3D culture systems [2, 3, 8].

However it is shown in this study that the PLIUS did not promote chondrocyte proliferation in culture. The total cell numbers appear to fluctuate during the 10 days of the study. Previous studies showed no proliferation of chondrocytes when cultured in an alginate matrix [26] and Parvizi et al. (1999) reported no proliferation of chondrocytes when treated with PLIUS in a 2D culture system. There is no previous study of chondrocytes cultured in alginate that have been exposed to PLIUS that used the same dose as in this study. From the results presented here, it can be concluded that PLIUS exposure has no effect in stimulating chondrocyte proliferation when cultured in alginate matrix.

A possible explanation, for this result, is that the alginate matrix is limiting the mass transfer of essential nutrients through the gel [24]. As a result, the supply of nutrients becomes low and the cell population stabilizes (i.e., maintenance of viability without proliferation). However, a previous study reported that chondrocyte cell numbers increase when they are seeded in alginate with a low seeding cell density (1x10^4 cells/ml) [27]. This might be another reason why the chondrocytes in the present study did not show any proliferation in culture. Furthermore, Zhang et al. (2003) stated that PLIUS influenced chondrocyte proliferation in an alginate matrix in an intensity-dependent manner, which means there is a possibility that the PLIUS intensity used in present study was not suitable to promote chondrocyte proliferation. A previous study also reported that the buffer system for culturing chondrocytes in an alginate matrix also plays an important role [28]. According to their observations, supplemented DMEM with HEPES and bicarbonate buffer improves chondrocytes growth and matrix production in 3D alginate beads. In the present study, DMEM was supplemented only with HEPES buffer and this may be another reason for low cell proliferation and low collagen and GaG production.

Collagen production only showed a significant difference at 4 days of PLIUS treatment. Since that it only appear once for the whole experimental period, it can be considered PLIUS dose in this study might not has effect in stimulating collagen production.

GaG detection also shows no difference between control and PLIUS-treated groups. As discussed before [9, 10], there was a decrease in the amount of GaG produced, with time in culture, for the other cell types. Alcian blue staining showed.
qualitatively that GaGs accumulated around the cells [26] in alginate discs.

SEM micrographs show a spherical morphology [3, 29] for chondrocytes when cultured in alginate discs and no damage to the cells after exposure to PLIUS when compared to the control group. The cells did not proliferate in alginate when treated with PLIUS and there was no defect or injury to the cells seen in the SEM micrographs.

TGF-β1 was reported to be able to stimulate collagen and GaG biosynthesis by chondrocytes in 2D culture [30]. However, in this study there was only PLIUS exposure (physical stimulation) and no growth factor or biochemical stimulation involved. Another study showed that TGF-β1 did not stimulate collagen and GaG synthesis in 3D culture in agarose [31].

Chondrocytes are reported to require a 3D scaffold in order to maintain their morphology [25] as in alginate hydrogel, with the capability of increasing in number and viability as well as showing increased GaG synthesis [25, 26]. In this study, there was no significant chondrocyte proliferation (Figure 1), collagen production (Figure 2) and GaG production (Figure 3) in alginate when exposed to PLIUS. A possible reason for this behaviour is that the kind of alginate [32] used might be different in different studies but the exact reasons for the conflicting findings is still unclear [33].

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References


