A combination of biomolecules enhances expression of E-cadherin and peroxisome proliferator-activated receptor gene leading to increased cell proliferation in primary human meniscal cells: an in vitro study

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Abstract The present study investigates the impact of biomolecules (biotin, glucose, chondroitin sulphate, proline) as supplement, (individual and in combination) on primary human meniscus cell proliferation. Primary human meniscus cells isolated from patients undergoing meniscectomy were maintained in Dulbecco's Modified Eagle's Medium (DMEM). The isolated cells were treated with above mentioned biomolecules as individual (0–100 µg/ml) and in combinations, as a supplement to DMEM. Based on the individual biomolecule study, a unique combination of biomolecules (UCM) was

finalized using one way ANOVA analysis. With the addition of UCM as supplement to DMEM, meniscal cells reached 100 % confluency within 4 days in 60 mm culture plate; whereas the cells in medium devoid of UCM, required 36 days for reaching confluency. The impact of UCM on cell viability, doubling time, histology, gene expression, biomarkers expression, extra cellular matrix synthesis, meniscus cell proliferation with respect to passages and donor's age were investigated. The gene expression studies for E-cadherin and peroxisome proliferator-activated receptor (PPAR) using RT-qPCR and immunohistochemical analysis for Ki67, CD34 and Vimentin confirmed that UCM has significant impact on cell proliferation. The extracellular collagen and glycosaminoglycan secretion in cells supplemented with UCM were found to increase by 31 and 37 fold respectively, when compared to control on the 4th day. The cell doubling time was reduced significantly when supplemented with UCM. The addition of UCM showed positive influence on different passages and age groups. Hence, this optimized UCM can be used as an effective supplement for meniscal tissue engineering.

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Introduction

Human meniscus is a cartilage present in between femur and tibia which tends to get damaged or torn due to

excessive physical or sports activities, trauma, etc. Healing and repairing such damaged meniscus is always limited due to its avascularity in the inner region. Surgical intervention like meniscectomy or suture fixation gives only a short term relief and does not initiate repair or regeneration. Such surgical interventions lead to osteoarthritis in most cases and hence, a tissue engineering approach with long term benefits is essential to overcome this problem. The major hurdle faced during meniscal tissue engineering is the poor in vitro growth rate of primary meniscal cells (Baker et al. 2009). Since the cellular phenotype and cell density seeded onto the scaffold play important role in determining the biochemical and biomechanical characteristics of the scaffolds used for tissue engineering (Van Der Bracht et al. 2007), there is a need to significantly increase the growth and proliferation rate of the primary meniscal cells. Scientists have tried various growth media like F12 (Heidari et al. 2011), DMEM (Nakata et al. 2001), RPMI (Kreuz et al. 2013) etc. to study their impact on meniscal cell growth. Among them, DMEM was reported to be efficient for meniscal tissue engineering (Nakata et al. 2001) and is widely used. The number of days required to achieve higher confluency of meniscal fibrochondrocytes was considerably high using DMEM (10 days to reach 70–80 % confluency in 35 mm culture dish; Zhang et al. 2015). Freymann et al. (2012) observed that in a 3-D bioresorbable PGA–hyaluronan scaffold, it took 21 days for cell growth using DMEM. Hence, in order to enhance the cell proliferation rate, addition of growth factors like TGF- β (Demoor et al. 2014), BMP (Perrier-Groult et al. 2013), FGF (Matsiko et al. 2013) etc. were investigated. As an alternative to these growth factors, various inducers/stimulants like amino acids (Fruchtl et al. 2015) and vitamins (Mason 2013) have been used to obtain increased cellular proliferation. Biotin, a water-soluble vitamin, acts as a prosthetic group of carboxylases, regulates gene expression at both transcriptional and translational level and has an effect on glucose metabolism (Aswani et al. 2013). Glucose, a central source of energy, has been reported to promote human mesangial cell proliferation and fibronectin expression in vitro at high concentration (Yano et al. 2009). Chondroitin sulfate (CS) is a proteoglycan used for mimicking the meniscus extracellular matrix (ECM) for cartilage regeneration (Lee et al. 2014). CS also significantly induces proteoglycan production in differentiated human articular chondrocytes (Kubo et al.

2009). L-Proline, a cyclic amino acid, is involved in numerous physiologic processes, including gluconeogenesis, lipogenesis, neurotransmission and cell growth. In addition, L-proline is also an essential precursor for the synthesis of many structural proteins. Studies on embryonic stem cells proved its role in regulation of cell differentiation (Washington et al. 2010). These studies clearly indicate the positive influence of biomolecules (biotin, glucose, CS and proline) on cell growth, proliferation and differentiation.

In this study, the impact of biomolecules like biotin, glucose, chondroitin sulfate and proline on the proliferation rate of primary human meniscal cells has been studied in vitro, separately and in combination. Based on the study, a unique combination of biomolecule (UCM) supplement that can be added along with DMEM has been formulated for enhancing the proliferation rate of primary human meniscal cells.

Materials and methods

Sample collection

Human menisci excised from patients undergoing meniscectomy during arthroscopic knee surgeries at Ortho One hospital and PSG Institute of Medical Sciences and Research, Coimbatore, were used in this study after having received informed consent from the patients. The study was approved by Institutional Human Ethical Committee (No. 12/193, January 2013) at the PSG Institute of Medical Sciences and Research, Coimbatore, India.

Cell isolation and expansion

Excised human meniscus from 10 patients aged between 20 and 60 years were used for cell isolation. Meniscal cells were released through sequential enzymatic digestion using 0.2 % (w/v) trypsin (Sigma-Aldrich, St Louis, MO, USA) and 0.2 % (w/v) collagenase type II (Sigma-Aldrich, St Louis, MO, USA). The harvested meniscal cells were suspended in DMEM medium (HiMedia laboratories, Mumbai, India) supplemented with 10 % fetal bovine serum (FBS) (HiMedia laboratories, Mumbai, India) and 0.1 % penicillin, streptomycin and amphotericin B (HiMedia laboratories, Mumbai, India). After reaching 100 % confluency, meniscal cells were

subsequently detached using 0.25 % trypsin/1 mM ethylene diamine tetra acetic acid (EDTA) (HiMedia laboratories, Mumbai, India) and subcultured as first passage cells (P1) until they reached confluency. All plates were maintained throughout the study at 37 °C, 5 % CO₂, inside an incubator (Eppendorf 170S, Hamburg, Germany) under humid conditions.

Impact of biomolecules on meniscal cell proliferation

Meniscal cells isolated from 43 year old male donor were supplemented with different concentrations (0–100 µg/ml) of individual biomolecules like biotin, glucose, chondroitin sulphate and proline along with DMEM medium and the best concentration yielding higher cell proliferation was selected for the treatment in combination. Cells at approximately 70 % confluence in 60 mm dishes (P1) were harvested and prepared as suspensions ($1-4 \times 10^5$ cells/ml). Viable cells were counted using trypan blue dye exclusion method in a hemocytometer (Shapiro 1988). Cells were transferred to 60 mm plates to achieve an initial plating densities of ~7000 cells and allowed to attach. The doubling time (DT) of cell during the exponential growth phase was calculated for three consecutive passages using the following formula (Ruohola et al. 2001):

$$DT = (t_2 - t_1) / (3.32 \times (\log N_2 - \log N_1)) \quad (1)$$

where, N_1 and N_2 are the cell numbers at time t_1 and t_2 respectively. The cells were monitored using inverted phase contrast epifluorescence microscopy (Nikon Ti-S Eclipse, Tokyo, Japan). The impact of UCM addition on meniscal cell proliferation was studied at four different age groups viz., Group A: 20–30 years; Group B: 30–40 years; Group C: 40–50 years and Group D: 50–60 years. All experiments were carried out in triplicate. Cells were harvested from the four groups as mentioned earlier from a defined sample weight of 0.4 g. The cells were exposed to control and UCM supplemented medium and cell density was quantified on the 4th day.

Nuclear staining

Hoechst-33258 stain (Sigma-Aldrich, St Louis, MO, USA) was used for cell nuclei visualization by staining the chromatin (Latt and Stetten 1976). Cells were prewashed with PBS buffer and incubated for 10 min in room temperature after adding Carnoy's fixative

(1:3 acetic acid (Loba Chemie, Mumbai, India): methanol (HiMedia laboratories, Mumbai, India)). The fixed cells were treated with Hoechst-33258 stain and incubated for 30 min. Cells were monitored for its fluorescence using inverted phase contrast epifluorescence microscope (Nikon Ti-S Eclipse, Japan) at excitation and emission wavelength of 350 and 450 nm, respectively.

Viability assay

The viability of the cells was estimated using the standard 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) tetrazolium bromide (MTT) (HiMedia laboratories, Mumbai, India) assay before and after supplementation of UCM. MTT assay is based on the ability of viable cells to convert the tetrazolium salt to purple formazan. The amount of purple formazan formed is directly proportional to the number of viable cells present (Ghasemi-Mobarakeh et al. 2008). Optical density of the formazan compound formed was read at 570 nm using 96 well MultiskanTM GO Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Estimation of total glycosaminoglycan (GAG) and collagen

The total ECM content can be correlated with GAG and collagen content in the medium. Total GAG and collagen secreted by the cells into their medium (with and without biomolecule supplementation) were estimated after 4 days of biomolecule treatment as individual and in combination. The medium was separated from the cells by centrifugation and a known quantity of supernatant was used for estimation of GAG and collagen. 1,9-dimethylmethylene blue (DMMB) (Sigma-Aldrich, St Louis, MO, USA) assay was used to estimate the GAG content in the sample, spectrophotometrically (Whitley et al. 1989). The aliquots of supernatant from different biomolecule treated samples were mixed with DMMB dye and its absorbance was measured at 525 nm in MultiskanTM GO Microplate Spectrophotometer. Chondroitin sulphate A sodium salt (Sigma-Aldrich, St Louis, MO, USA) was used as standard for GAG estimation. Similarly, the collagen secreted into the medium was estimated using modified sirius red dye method (Reinert and Jundt 1999). A known concentration of

sirius red dye (Sigma-Aldrich, St Louis, MO, USA) prepared in 0.5 M acetic acid solution was added to a known quantity of medium from the cell culture plates and mixed well for 5 s. The content was incubated undisturbed for 30 min, centrifuged at 1500 rpm for 10 min and the pellet was washed with 0.01 N hydrochloric acid (HCl) to remove unbound dye. The pellet was re-suspended in 0.1 N potassium hydroxide (KOH) (Merck, Mumbai, India) and absorbance was measured at 540 nm using a microplate reader. Calf collagen (Sigma-Aldrich, St Louis, MO, USA) was used as standard for collagen estimation.

Immunohistochemistry

The expression of three different markers viz., Ki67 (marker for cell proliferation), CD34 (marker of stem/progenitor cells) and vimentin (marker for cytoskeleton of meniscal cells) were studied for understanding the impact of UCM on meniscal cells. Cells treated with or without UCM were trypsinised after 4 days and pelleted. Sections of cells were prepared in paraffin blocks and 4 µm sections were cut using microtome, incubated at 37 °C for one day and further incubated at 58 °C overnight. Sections were deparaffinized and rehydrated by the addition of graded alcohols to water. Antigen retrieval was carried out by baking samples at 60 °C for 30 min in citrate buffer (Merck, Mumbai, India) (pH 6.0). After reaching room temperature, slides were transferred to tris buffer saline. After this, procedures were followed as per manufactures instruction (Dako Real EnVision Detection System, Glostrup, Denmark). Finally, slides were counterstained with hematoxylin and visualized using a Nikon Eclipse Ci S microscope.

Real time-quantitative PCR (RT-qPCR)

Total RNA was isolated from primary human meniscus cells (with and without UCM supplement) using TRIZOL reagent, as per the manufacturer's

instructions (Merck Ltd, Mumbai, India). Total mRNA was quantified spectrophotometrically (Nanodrop, ThermoFisher, USA) at 260 nm and purity was confirmed based on 260/280 and 260/230 ratio. 2 µg of total RNA was used for cDNA synthesis by M-MULV Reverse Transcriptase (Merck Ltd., India), as per the manufacturer's instructions. The resultant cDNA along with SYBR Green (Clontech, Mountain View, CA, USA) was used for RT-qPCR (StepOne, Applied Biosystems, Foster City, CA, USA). The conditions of the PCR amplification were as follows: incubation of samples for 4 min at 94 °C, followed by 40 cycles of denaturation for 10 s at 94 °C, annealing for 1 min at the suitable temperature of primers, and extension for 1 min at 72 °C. The details of primers and amplicon sequences used are given in Table 1. The primers were designed using Primer Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) ensuring that at least one primer in a set spanned an exon–exon junction to minimize the chances of amplification from genomic DNA contamination. Relative quantity (RQ) to the control for gene expression was calculated by normalizing with housekeeping gene (GAPDH). Fold change was calculated as $2^{-\Delta\Delta CT}$ (Mendenhall et al. 2013).

Statistical analyses

All experiments were carried out in triplicates, and the results are presented as mean $\pm$ standard deviation. The experimental data were analyzed using Minitab® software. One way ANOVA analysis with 95 % confidence level ($p \leq 0.05$) was carried out to test the statistical significance for cell proliferation. Optimization of their concentration was first carried out individually with the null hypothesis “there is no significant difference between the concentrations of X”, where X is individual biomolecules. With that guideline, different combinations of biomolecules were selected and response surface contour was plotted to select the best combination of biomolecules at different composition for meniscal cell proliferation.

Table 1 Selected primer sequences

S. No.	Primer	Forward sequence (5'–3')	Reverse sequence (3'–5')
1	GAPDH	TGCCTCCTGCACCACCAA CT	GCC TGC TTC ACCACCTTC
2	PPARA	CGTGATACTCACACAGTGCC	TTCCCATCAGCCTTGAAGCA
3	E CAD	GCTGCTCTTGCTGTT TCTTCG	CCGCCTCCTTCTTCATCATAG

Fig. 1 **a** Effect of biotin on meniscal cell counts at different concentrations and time intervals (mean $\pm$ SD). Phase contrast images of human meniscus cells on the 24th day **b** control and **c** cells with biotin at 20 $\mu\text{g/ml}$ concentration (* $p < 0.05$)

Results

Effect of biomolecules: individual study

Effect of biotin

Supplementation of biotin was found to induce meniscus cell proliferation (Fig. 1). Cell proliferation in biotin supplemented medium (20–100 $\mu\text{g/ml}$) showed faster growth rate when compared to control. Cell count was taken at an interval of 12, 24 and 36 days to study the cell density (Fig. 1a). After 12 days, the difference observed between concentrations of biotin was not statistically significant ($p > 0.05$). On increasing the incubation time to 24 days, significant variations in the cell count between the concentrations were observed. The highest proliferation rate was found to be with 20 $\mu\text{g/ml}$ of biotin which was 2.20 times greater than that of control. Further increase in biotin concentration did not show any significant effect even after extending the incubation period. Hence, 20 $\mu\text{g/ml}$ of biotin concentration was selected for combination studies. On the 36th day, all concentrations including control showed good proliferation. The meniscus cells in this medium with 20 $\mu\text{g/ml}$ of biotin appeared densely packed (Fig. 1c) when compared to control on the 24th day (Fig. 1b).

Effect of glucose

As the concentration of glucose in the medium was increased from 0 to 100 $\mu\text{g/ml}$, there was a gradual

increase in the cell density (Fig. 2). After 12 and 24 days, the cells without glucose supplementation showed significantly less cell growth when compared to medium supplemented with glucose. However, no significant variation was observed within different concentration of glucose used till the 24th day (Fig. 2a). The trend was same as on the 36th day. However, the highest growth rate was observed with 60 $\mu\text{g/ml}$ glucose which is 46 % higher than the control. Beyond that (80 and 100 $\mu\text{g/ml}$), the cell growth rate showed a statistically significant decrease. Hence, the glucose concentration was selected as 60 $\mu\text{g/ml}$ for combination studies. The cells observed under phase contrast microscope showed similar cellular extensions in the case of both control (Fig. 2b) and glucose supplemented culture (Fig. 2c).

Effect of chondroitin sulphate (CS)

Meniscus primary cells were highly responsive to CS concentrations (Fig. 3). However, there were no significant differences observed between all the concentrations after 12 days. After the 24th day, CS concentrations of 40, 60, 80 and 100 $\mu\text{g/ml}$ showed similar cell count ranging from 2.91×10^6 to 3.4×10^6 cells/ml. However, samples having 0 and 20 $\mu\text{g/ml}$ of CS showed significantly less cell count than the samples exposed to higher concentrations of CS. On the 36th day, all CS concentrations significantly stimulated the cell growth when compared with control. CS 60 $\mu\text{g/ml}$ showed highest mean

Fig. 2 **a** Meniscal cell count at different days with varying concentrations of glucose (mean $\pm$ SD). Phase contrast images of human meniscus cells on the 24th day **b** control and **c** cells with glucose at 60 μ g/ml concentration (* $p < 0.05$)

Fig. 3 **a** Effect of CS on meniscal cell counts at different concentrations and time intervals (mean $\pm$ SD). Phase contrast images of human meniscus cells on the 24th day **b** control and **c** cells with CS at 60 μ g/ml concentration (* $p < 0.05$)

value among all other concentrations, although there was no significant difference between the samples having 60, 80 and 100 μ g/ml of CS (Fig. 3a). Since the standard deviation of sample data having 60 μ g/ml was high, the optimum concentration of CS could be finalized at this point. Meniscus cells in this medium with and without CS appeared similar in morphology (Fig. 3b, c).

Effect of proline

Proline concentrations were found to be effective in enhancing meniscus cell division with increasing number of days (Fig. 4). Although the initial

response of cells towards proline was not good on the 12th day, on further incubation, it promoted cell proliferation (Fig. 4a). After 24 days of culture, samples having 20 μ g/ml proline showed highest cell count. However, the 36th day result indicated significant proliferation of meniscus cells with addition of proline, except for 20 μ g/ml, when compared to control (i.e., $p < 0.05$). Step wise elimination of sample data revealed that variation of proline concentration from 40 to 100 μ g/ml has no significant difference. The cell count was found to be highest (1.5-folds as compared to control) at a concentration of 80 μ g/ml of proline on the 36th day (Fig. 4a). From these analyses, no conclusion can

Fig. 4 **a** Meniscal cell count at different days with varying concentrations of proline (mean $\pm$ SD). Phase contrast images of human meniscus cells on the 24th day **b** control and **c** cells with at 20 μ g/ml concentration (* $p < 0.05$)

be drawn on the optimal concentration of proline, since the variation of its concentration have varying influence on meniscal cell proliferation. Hence, similar to CS, proline concentration was also selected as a variable for combination studies. In proline supplemented medium, cells appeared stellate shape with fibroblast-like morphology and had cytoplasmic extensions in both control and treated samples (Fig. 4b, c).

Effect of biomolecules: in combination

Based on individual biomolecule study, the optimal concentrations required for attaining maximum cell growth with respect to biotin and glucose was fixed as

20 and 60 μ g/ml, respectively. CS and proline concentrations were kept as variable for combination studies. From this, eight different combinations were selected to study their effect on meniscal cell growth and proliferation (Table 2). The cell density was in the following order: V > VII > IV > VI > III > VIII > II > C>I. A contour was plotted using all combination data (except control) to find out the optimum concentration of CS and proline (Fig. 5a). I and II combinations were found to have same stimulatory impact on proliferation as compared to control. Combination III, IV, VI and VIII showed similar cell proliferation ($2.0\text{--}2.5 \times 10^6$ cells/ml). Combination VII induced higher cell proliferation of $2.5\text{--}3.0 \times 10^6$ cells/ml. A small region surrounding

Table 2 Impact of combination of biomolecules on cell density

Combinations	Biotin (μ g/ml)	Glucose (μ g/ml)	Chondroitin sulphate (μ g/ml)	Proline (μ g/ml)	4th day cell count ($\times 10^6$ cells/ml)
C	0	0	0	0	1.26 ± 0.12
I	20	60	0	0	1.20 ± 0.12
II	20	60	20	60	1.35 ± 0.18
III	20	60	30	40	2.28 ± 0.04
IV	20	60	40	20	2.40 ± 0.13
V	20	60	60	20	4.06 ± 0.04
VI	20	60	60	0	2.30 ± 0.09
VII	20	60	80	0	2.63 ± 0.09
VIII	20	60	0	80	2.23 ± 0.19

C control

Fig. 5 **a** Contour plot showing effect of different combinations on meniscal cell proliferation on the 4th day. Hoechst stained images of **b** control and **c** combination V

the combination V was found to have maximum cell proliferation and growth when compared to all other combinations studied. The cell count was found to be threefold (4.06×10^6 cells/ml) higher than the control (1.26×10^6 cells/ml). Hence, any concentration within the small region surrounding combination V can be selected for obtaining higher cell proliferation. Based on the contour diagram, the UCM having biotin at 20 µg/ml, glucose at 60 µg/ml, CS at 60 µg/ml and proline at 20 µg/ml, was selected for further studies. Figure 5b, c shows fluorescence stained images of the control and combination V treated cells, respectively. The entire optimization process was re-confirmed with meniscal cells isolated from another sample (38 years, male donor) and the results obtained were highly reproducible (data not shown). Doubling time and cell proliferation rate was calculated using medium supplemented with combination V in three subsequent passages. The doubling time was calculated to be 8.86 ± 0.11 , 8.34 ± 0.11 and 8.34 ± 0.01 h for control in first, second and third passages, respectively. However, after supplementation with UCM, the doubling time was found to be reduced significantly (7.56 ± 0.04 , 7.22 ± 0.03 and 7.29 ± 0.04 h for passage 1, 2 and 3, respectively; Fig. 6). Hence, DMEM supplemented with combination V decreased the doubling time with all passages when compared to control. Although the doubling time was reduced, there was no morphological changes observed with

Fig. 6 Doubling time (mean $\pm$ SD) of cells grown in control and UCM supplemented medium. P1-C: passage 1 control; P2-C: passage 2 control. P1-CM: passage 1 with UCM supplementation; P2-CM: passage 2 with UCM supplementation

cells in control and UCM treated medium (Fig. 7). This study clearly indicates that the combination V of biomolecules (UCM) has significant impact on doubling time in all passages.

Immunohistochemistry

Immunohistochemistry was performed using antibody markers Ki67, CD34 and vimentin (Fig. 8) after

Fig. 7 Phase contrast images of meniscus cell proliferation with passages on the 4th day. Passage 1: **a** control and **b** UCM supplemented; Passage 2: **c** control and **d** UCM supplemented

4 days of treatment and compared with control. Ki-67 used cell proliferation marker. The Ki67 proliferative index was found to be <1 % in control. However, after UCM supplementation in medium, the Ki67 marker proliferation index raised to 2–3 % (Fig. 8a, b). The increase in proliferation index of Ki67 marker in UCM supplemented cells when compared to control cells is also given as intensity plot (respective inset of Fig. 8a, b). CD34, a stem cell/progenitor marker was also used to analyze the impact of UCM in in vitro meniscus cell differentiation which was found to be negative in both control (Fig. 8c) and UCM treated cells (Fig. 8d). UCM treated cells were found to be strongly positive for Vimentin (Fig. 8e) than control meniscus cells (Fig. 8f).

Biochemical quantitative analysis

The cell viability (MTT assay) after exposure to individual biomolecules and UCM is given in Fig. 9a. Medium supplemented with individual biomolecules and UCM showed 2.7-folds increased cell viability when compared to control. The viability of cells were in the following order; UCM > CS-60 > G-60 > B-20 > P-20 > C. Figure 9b shows relative quantity of

gene expression to control for PPAR Δ and E-cadherin. Gene expression of PPAR Δ and E-cadherin in UCM supplemented cells were higher than that of control (3.79 ± 1.31 and 2.25 ± 0.18 , respectively). ECM secretion (collagen and GAG) into the medium in response to the supplementation of UCM was studied and compared with individual biomolecules and control. After 4 days of incubation, all samples (except control) showed very high collagen and GAG secretion. Collagen and GAG synthesis in UCM supplemented samples was significantly higher than individual concentrations and control (Fig. 9c). Among the individual biomolecules, proline (20 $\mu\text{g}/\text{ml}$) showed higher collagen synthesis and CS (60 $\mu\text{g}/\text{ml}$) showed increased GAG secretion. Hence, it was found that UCM supplementation has profound impact on viability, PPAR Δ and E-Cadherin gene expression and on ECM synthesis.

Effect of donor's age on cell proliferation

The quantity of meniscal cells isolated from the donors of different age groups varied significantly. Cell proliferation in the control samples (with DMEM

Fig. 8 Photomicrographs of immunohistochemical staining. Ki67 biomarker staining of control **a** UCM treated cells, **b** (stained nuclei indicated by *arrow*) and intensity plot of control and UCM treated cells (**a**, **b** insert respectively). CD34

marker staining of control **c** and UCM treated cells **d**. Vimentin staining of control **e** and UCM treated meniscus cells **f**. All images were taken after 4 days of treatment

alone) was significantly less in age Groups C and D (between 40 and 60 years) when compared to Group A (between 20 and 30 years) and B (between 20 and 40 years; Fig. 10). On supplementation of UCM, the cell proliferation increased significantly for all age groups.

Discussion

The results clearly indicate that human meniscal cell growth rate can be significantly increased by the addition of selected biomolecules in medium individually and in combination. In this study, the addition of

Fig. 9 a MTT assay with medium supplemented with individual biomolecules and UCM at the 4th day.

b Relative quantity to control for E-cadherin and PPAR Δ genes. **c** Collagen and GAG secreted into medium supplemented with individual biomolecules and UCM at the 4th day (where, B-20: biotin 20 μ g/ml; G-60: glucose 60 μ g/ml; P-20: Proline 20 μ g/ml; CS-60: chondroitin sulphate 60 μ g/ml; UCM: unique combination medium; C: control)

Fig. 10 Impact of age group on cell viability after supplementation with UCM on the 6th day (age groups in years, A: 20–30; B: 30–40; C: 40–50; D: 50–60)

20 μ g/ml biotin as a supplement in DMEM showed significant increase in cell density. Gopinathan et al. (2015) reported that biotin impregnated polycaprolactone nanofibrous scaffold can enhance human knee meniscus cell attachment and proliferation. Biotin

plays an important role in the modification of histones which leads to replication of DNA and transcription. As cell proliferation requires an increased rate of both replication and transcription, uptake of biotin by cells results in enhanced cell proliferation (Zempleni and Mock 2001). Biotin acts as a cofactor for biotin dependent carboxylases that is involved in cellular biosynthetic pathways and cell proliferation (Takechi et al. 2008). Biotin also increases cell proliferation through biotinylation of histones (Stanley et al. 2001). The increased concentration of glucose has been reported to stimulate TGF- β 1 synthesis (Fraser et al. 2003) which in turn enhances cell division and proteoglycan synthesis in human knee meniscus cells (McNulty and Guilak 2008). Hence, supplementation of excess glucose along with the glucose available in the DMEM is expected to increase the growth and cell proliferation in meniscal cells. In our study, we also observed that the addition of 60 μ g/ml glucose supplementation enhanced maximum growth rate. In case of CS and proline, all concentrations promoted cell proliferation rate significantly. Supplementation of CS into the culture medium has been reported to influence cell proliferation in 3D cultures of

chondrocytes (Jerosch 2011) and stimulate anabolic processes leading to neocartilage formation (Levett et al. 2014). Muzzarelli et al. (2012) reported that CS plays an important role in cartilage regeneration by creating a chondrocyte inducing environment which paves way for cell growth and ECM deposition. CS can bind with core proteins to produce aggrecan that acts as a shock absorber inside cartilage (Little et al. 2014). The ability of amino acids to function as regulators in cell communication, differentiation and cell growth has been well documented (Chalisova et al. 2013). Moreover, proline acts as a precursor for hydroxyproline for collagen formation and tissue repair as reported by Barbul (2008). Hence, for combination studies, CS and proline concentrations were altered between 0 and 80 µg/ml keeping biotin and glucose concentrations as constant (20 and 60 µg/ml, respectively). When the above mentioned biomolecules were used in combination, we observed some synergistic and stimulatory effect on human meniscal cell growth and proliferation. Among the combinations, combination V (UCM) was found to be the most effective one. The proliferation of meniscus cells by addition of UCM was validated by investigating the expression of key markers like Ki67, CD34 and vimentin. Ki67 marker is widely used for the analysis of cell proliferation by counting the positive stained nuclei. This marker is expressed in all stages of cell cycle except G0 stage (Brown et al. 2014). The increased expression of Ki67 marker in UCM treated cells compared to control, clearly indicates that supplementation of UCM to DMEM significantly influence meniscal cell proliferation. Similar increase in Ki67 positive expression was reported in meniscus cells grown in collagen type I scaffold along with infrapatellar fat pad (Oda et al. 2015). CD34 marker expression was found to be absent in both the control and UCM supplemented meniscal cells indicating that the UCM does not induce any differentiation during the proliferation. CD34 has been reported to be expressed as surface antigen in lympho hematopoietic stem cells and progenitor cells (Verdonk et al. 2005). The fibrochondrocyte cells present in the inner surface of the meniscus do not show any CD34 markers on surface. Our studies were in correlation with this report. Both control and UCM treated were devoid of CD34 antigens indicating that there was no differentiation of meniscus cells. Vimentin present in chondrocyte cytoskeleton have an important role in

maintaining cellular mechanical integrity along with actin and tubulin and is a determinant of cell stiffness (Haudenschild et al. 2011). In the present study, vimentin expression was found to be higher in UCM supplemented cells than the untreated control cells (Fig. 8e, f). Increased expression of vimentin marker indicates that addition of UCM increases the structural integrity of meniscus cells. All these studies clearly indicate that UCM supplementation not only increases proliferation but also increases the cell membrane integrity without inducing any differentiation in meniscal cells. Further effect of UCM in cell proliferation was quantitatively analyzed using qPCR with specific primers for E-cadherin and PPAR Δ . Cadherin superfamily consists of transmembrane proteins which mediate Ca²⁺ dependent cell–cell adhesion and cell signaling (Maitre and Heisenberg 2013). E-cadherin functions as a regulator of cell adhesion and self-renewal (Chen et al. 2013). PPAR Δ is a regulator of lipid and glucose energy metabolism in adipose and muscular tissues, and stimulates Wnt signaling pathway in mesenchymal stem cells (Niehrs 2012; Scholtysek et al. 2013). Wnt proteins have multiple functions such as cell differentiation, proliferation etc. (Niehrs 2012). When human meniscus primary cells were treated with UCM, expression level of E-cadherin and PPAR Δ increased significantly resulting in higher cell proliferation rate.

Hence, the results achieved in this study may be due to increased cell–cell adhesion, lipid and glucose energy metabolism, or through stimulation of Wnt signaling pathway after addition of UCM. Further study is required to understand the exact mechanism of increased cell proliferation due to UCM addition. ECM estimation results indicate that the addition of UCM and individual biomolecules into the meniscal cell culture upregulates collagen and GAG secretion along with increased cell proliferation. The effect of biomolecules such as biotin (Gopinathan et al. 2015), glucose (Ayo et al. 1990), CS (Kubo et al. 2009) and proline (Barbul 2008) was reported to enhance ECM like collagen and GAG. However, the combination of such biomolecules and ECM synthesis has not been reported so far to our knowledge. The impact of UCM on meniscal cell growth for donors with different age groups was quite significant. The cell proliferation rate was found to be less in Group D (50–60 years) donors. This may be due to fewer numbers of viable cells and lack of cell–cell communication in higher age groups

which ultimately leads to lower rate of cell adhesion and proliferation (Tran-Khanh et al. 2005; Stalling and Nicoll 2008; Brink et al. 2009) and increased production of reactive oxygen species (ROS). Previous studies reported that cells isolated from tissues such as anterior cruciate ligament (Stalling and Nicoll 2008), chondrocytes (Brink et al. 2009) and tendon (Tran-Khanh et al. 2005) lose the ability to proliferate with increasing age. The combination of biomolecules (UCM) reported in our study is promising and a cost effective supplement for human knee meniscus tissue engineering.

Conclusion

The major problem faced in human meniscal tissue engineering is to achieve a higher cell proliferation rate from isolated primary culture. Many researchers have used various expensive growth factors to achieve this in a short time period. In this study, we have optimized a UCM (biotin 20 µg/ml, glucose 60 µg/ml, CS 60 µg/ml and proline 20 µg/ml) which enhanced meniscal cell proliferation rate and reduced the doubling time significantly. Using this combination, time to reach full confluency has been reduced by a large extent (from 36 to 4 days) without any significant change in morphology. The proliferation effect of UCM was confirmed by analyzing the expression of E-cadherin and PPAR Δ which was significantly higher in UCM supplemented cells when compared to control. Immunohistochemistry results were also in line with qPCR results. The cells supplemented with UCM showed increased expression for Ki67 proliferative marker and vimentin. Supplementation of UCM also enhanced the ECM secretion (collagen and GAG). Increased cell proliferation was achieved with cells of different passages and age groups after UCM supplementation. These results clearly indicate that addition of UCM increases the proliferation of human meniscal cells by inducing expression of E-cadherin and PPAR Δ , enhancing GAG and collagen secretion and by increasing the expression of proliferative and cytoskeleton markers. Since, the biomolecules used in the study are cost effective and affordable, UCM can be used as an effective alternative to other expensive growth factors for tissue engineering studies.

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Compliance with ethical standards

Conflict of interest None of the authors have a conflict of interests including direct or indirect financial relations with any of the trademarks and companies mentioned in this paper.

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