Effect of Transforming Growth Factor-β3 on mono and multilayer chondrocytes

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Abstract

Articular cartilage is an avascular and flexible connective tissue found in joints. It produces a cushioning effect at the joints and provides low friction to protect the ends of the bones from wear and tear/damage. It has poor repair capacity and any injury can result pain and loss of mobility. Transforming growth factor-beta (TGF-β), a cytokine superfamily, regulates cell function, including differentiation and proliferation. Although the function of the TGF-βs in various cell types has been investigated, their function in cartilage repair is as yet not fully understood. The effect of TGF-β3 in biological regulation of primary chondrocyte was investigated in this work. TGF-β3 provide fibroblastic morphology to chondrocytes and therefore overall reduction in cell proliferation was observed. The length of the cells supplemented with TGF-β3 were larger than the cells without TGF-β3 treatment. This was caused by the fibroblast like cells (dedifferentiated chondrocytes) which occupied larger areas compared to cells without TGF-β3 addition. The healing process of the model wound closure assay of chondrocyte multilayer was slowed down by TGF-β3, and this cytokine negatively affected the strength of chondrocyte adhesion to the cell culture surface.

Keywords: Primary chondrocyte multilayer; TGF-β3; Cell length; Cell proliferation; Model wound closure assay

1. Introduction

Cytokines are extremely potent biomolecules produced almost by all type of cells. They regulate cellular functions and play multiple roles in initiation and inhibition of disease. Transforming growth factor-beta (TGF-β) superfamily is a group of pleiotropic cytokines with high molecular weight of 25kD homodimeric peptide (Lawrence, 2001, Sefat, 2010, Sefat 2015, Li, 2015). These highly specialised molecules are actively involved in control of cellular proliferation, apoptosis, cell migration and adhesion (Sefat, 2014a). Different kinds of TGF-βs are involved in signal transduction between the extracellular environment and the nucleus. TGF-β 1, 2 and 3 are potent regulators in cellular development (Raftery and Sutherland, 2002, Sefat, 2014b).

Shah et al (1999) investigated the effect of manipulation of TGF-β on the wound healing process and found that the effects of TGF-β3 is inhibited by the high levels of TGF-β1and 2 from the inflammatory cells in adult tissue. Thus increasing the levels of TGF-β3 caused scar-free wound healing (Shah et al., 1999). Related work was also carried out by Ferguson and O'Kane (2004) who identified some TGF-β1 related therapeutic targets. They found that the growth factor profiles differ significantly in embryonic and adult tissue. For example, levels of TGF-β1 and TGF-β2 in embryonic wounds are very low, however the levels of TGF-β3 is much higher in comparison to adult wounds (Ferguson and O'Kane, 2004). Similarly, Gorvy et al (2005) found that TGF-β1 and TGF-β2 have inhibiting effects whilst simultaneously increasing TGF-β3 concentrations in adult wounds, near embryonic repair could be achieved in adults (Gorvy et al., 2005). One year later Davidson et al (2006) investigated TGF-β induced cartilage repair. They found that the cartilage was maintained by TGF-β but fibrosis of cartilage was blocked in the presence of Smad7 which is an inhibitor of TGF-β superfamily signalling (Davidson et al., 2006, Benchabane, 2003).

The TGF-β superfamily consists of various types of polypeptides effecting up and down-regulation of membrane proteins (Spagnoli et al., 2007) and consequently inducing cell adhesion, proliferation, differentiation, activation, migration and apoptosis (Krauss, 2006). This may indicate how cartilage repair is initiated and how this could be accelerated by TGF- β1 (Bos et al., 2007). It has also been reported that seeding chondrocytes at high cell density induced chondrocytic morphology (Bashey et al., 2006) and also fibroblastic morphology was obtained by culturing in low density after isolation (Martin et al., 2001). The ability of chondrocyte to produce a correct matrix is inherent (Schuurman et al., 2009), but is also affected by other influences such as mechanical stress (Byers et al., 2008), hormones and specific proteins and growth factors (Maitre et al., 2007).

The effect of some cytokines, such as interleukin (IL), transforming growth factor-beta (TGF-β) and bone morphogenic protein (BMP), in the regulation of some other cartilage-related proteins have been studied previously (Joosten et al., 2004). However, the effect of these factors in the biological regulation of chondrocyte, proliferation, cell size, cell adhesion and wound repair are still not clearly reported or understood.

The limitation of the repair capacity of cartilage is directly related to chondrocyte cell and the adhesion, proliferation and migration of chondrocyte into the wound site. There are only few evaluations of the effect of TGF- βs and their manipulated form on chondrocyte cell proliferation and cartilage wound repair. In this work, therefore, the characteristics of the primary chondrocyte cell in terms of cell phenotype, alignment, adhesion, ECM production and wound healing were investigated in relation to exposure to TGF- β3 in multilayer culture system.

2. Materials and Methods

The following method was designed to investigate the effect of TGF- β3 on the biological regulation of chondrocyte.

Primary chondrocyte cells from the fourth passage were cultured in multilayers, expanded and, therefore, prepared for this study. The cells were cultured for a period of 132 hours to measure cell length and evaluate the proliferation rate. Wound-closure assessment was performed by cultivation of chondrocyte on a multilayer culture system and performing a scratch assay.

Microstructural analysis was performed regularly on daily basis using light microscopy, Image J software and statistical analysis using Excel and SPSS. The experiments were performed three times identically to ensure repeatability and reliability of the results.

2.1. Culture of Chondrocyte Cells

Primary chondrocyte cells were isolated from the articular joint of six five-day old neonate Sprague-Dawley rats and purified according to the protocol described previously (Khaghani et al., 2009, Khaghani, 2012a, Khaghani 2012b). Epiphyseal plates were carefully separated from the end of both tibias and femurs derived from five days old SpragueDawley rats. Tissue was then immersed in 4ml of 0.25% trypsin and 2g per litre EDTA (Sigma) and stirred for 15 minutes at 37℃. The supernatant was aspirated after 15 minutes and transferred into a 15ml centrifuge tube. Trypsin was deactivated by adding 4 ml of 10% FCS contained Dulbecco's Modified Eagle Medium (DMEM). The process of digestion by trypsin was repeated three times. The aspirated supernatants and epiphyseal plates were centrifuged at 2000 rpm and the obtained pellet was immersed in 4ml of 0.1% collagenase type-I solution (Sigma) for 90 minutes. Following treatment with collagenase type -1 solution the supernatant was mixed with 4ml of FCS containing cell culture media and re- centrifuged at 2000 rpm. The supernatant was discarded and the obtained pellet was resuspended with 5 ml chondrocyte culture media, seeded in a 25 cm² tissue culture flask and incubated at 37℃. After 24 hours the epiphyseal plates and non-attached cells were discarded, fresh media was added to the cell culture and incubated at 37℃ until 70-80% confluency. To obtain the chondrocytes with chondrocytic morphology the cells were resuspended at cell density of 250,000 cells/ml in 10ml high glucose DMEM with 10% FCS supplementation and 0.1mg/ml hyaluronic acid (Sigma). Five millilitres of cell suspension were transferred into a 25cm² TG grade falcon cell culture flask. To acquire chondrocytes with a fibroblast like phenotype 1ml of cell suspension was added to 4ml chondrocyte culture media to achieve a cell density of 50,000 cells/ml. Both cultures were incubated at 37℃ until 70-80% confluency. Isolated chondrocytes were cultured in high glucose DMEM (Sigma Aldrich, UK), supplemented with 10% fetal calf serum (Promocell, UK) and 0.1% w/v hyaluronic acid (Sigma Aldrich, UK) in multilayer culture system after expansion, and utilised for the present experiments.

2.2. Solvent

TGF-β3 is a type of protein produced by almost all types of cells (Schwartz et al., 1993) ant it is soluble in an acidic environment as well as water resulting in a sticky jelly (Wallace and Rosenblatt, 2003) and, thus, it is not appropriate to dissolve it in water and instead aliquot in an micro-litre tube with the final solution less than 1ml.

Hydrochloric acid (HCl) is an appropriate solvent for all types of TGF- βs and provides a TGF-β solution allowing for proper aliquot. The solvent also contained 1mg/ml bovine serum albumin (BSA) (Sigma Aldrich, UK) as a protein carrier.

2.2.1. Solution preparation

Human recombined TGF- β (Sigma Aldrich, UK) was utilised for this experiment. It was diluted, according to supplier's recommendation, with HCl and BSA. The dilution equation was used to prepare 4mM HCl from an available 2.5M HCl. According to the supplier's instructions, 10mg bovine serum albumin was dissolved in 10ml of 4mM hydrochloric acid to obtain 1mg/ml HCl/BSA. Following this, the solution was sterilised, using a 0.22μm filter.

Various TGF-β with different concentrations of 5, 10 and 50ng/ml were used previously in our research while measuring the percentage of wound closure for bone, cartilage and skin monolayers (Sefat, 2009a, Sefat 2009b, Sefat 2010). Among these TGF-β isomers TGF-β3 represented the optimum result for bone and chondrocytes. Therefore, for this research work 2μg of TGF-β3 was dissolved in 0.4ml of 1mg/ml HCl/BSA, aliquoted into forty 10μl vials and stored at -20ºC.

2.3. Cell length Analysis

3.5 x 10^5 chondrocyte cells were resuspended in 5ml high glucose DMEM (4500 mg/L) (Sigma Aldrich, UK), supplemented with FCS 10% v/v (Promocell, UK), 2.5 mM U/ml L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 1μg Amphotericin B (Fungizone) (Sigma Aldrich, UK), and assigned as control. The cell suspension was seeded in a 25cm² cell culture flask and incubated at 37ºC until 70-80% confluency.

50µl of prepared TGF-β3 was added to another chondrocyte cell suspension with the same volume and cell density as control to make a concentration of 10ng/ml TGF- β3. After 12 hours the non-attached cells were removed, fresh media were added to both control and TGF-β3 contained culture flasks. Every 24 hours the process of confluency was microstructurally analysed and imaged by light microscope. The cell length of 270 randomly selected cells were measured for analysis, and culture flasks were incubated again at 37°C until full confluency.

2.4. Cell Proliferation Analysis

To evaluate the effect of TGF-β3 on chondrocyte cell proliferation, primary chondrocytes were cultured in multilayer with and without TGF-β3 supplementation. 10ml cell suspension with a cell density of $7x10^4$cells/ml was subjected to this experiment. 50µl of human, recombined TGF-β3 (Sigma Aldrich, UK), with a dilution ratio of 10µg/ml, was added to 4,950µl cell suspension to obtain a final concentration of 10ng/ml and seeded in a 25cm² tissue culture flask. The remaining 5ml cell suspension was seeded in a similar culture flask without addition of TGF-β3. Both cell cultures were incubated at 37ºC for 132 hours. The media were replaced with fresh media every 48 hours.

After 132 hours both cell cultures were 100% confluent. The media were removed and the culture flasks washed three times with Hank's balanced salt solution (HBSS). After aspiration of third HBSS, 2ml of 0.25% Trypsin-EDTA was added to the culture flask to detach chondrocyte cells from the surface of the culture flask. Detached cells were counted using a haemocytometer and compared with the initial cells. The amount of initial cells at the beginning and at the end of the experiment were standardised and a graph was produced to show how TGF-β3 affected proliferation of chondrocyte in a multilayer cell culture system.

2.5. Wound Healing Assay

The primary chondrocyte cells were cultured in 25 cm² tissue culture flasks without and with 10 ng/ml TGF-β3 supplementation. The cultures were incubated at 37ºC until full confluency. The media were changed every 48 hours.

To assess the effect of TGF-β3 on the chondrocyte repair capacity, a wound model was created via scratching the confluent chondrocyte multilayer cultured in both culture flasks. The scratch was performed by the tip of a sterilised polyethylene plastic pipette with a tip size of 1mm in diameter. The average of initial wound size was ~261.3µm.

The model wounds were imaged using ImageJ (NIH) software every two hours until the first wound gap was closed, which was the sign of a complete wound closure. The earliest wound repair was observed after 48 hours for control. All images were recorded and saved for further analysis. For precise statistical analysis, the experiment was repeated three times and the mean wound size (± SE) was calculated.

2.6. Cell Adhesion Analysis

The chondrocyte cells were cultured in 25 cm² TC grade culture flasks without and with 10ng/ml TGF- β3 and incubated at 37°C until full confluency. To analyse the strength of chondrocyte attachment on the solid surface, the confluent multilayer chondrocyte culture was trypsinized by 0.25% Trypsin-EDTA solution (Sigma Aldrich, UK). Before trypsinization, the media were removed and the cell culture was washed three times with PBS or alternatively with HBSS (Sigma Aldrich, UK). HBSS was aspirated and 4ml of trypsin was added to the cell culture. Following the trypsinization process, the chondrocyte culture was imaged by light microscope for further microstructural and statistical analysis.

The chondrocyte detachment time in both control and TGF-β3 treated cultures was measured and compared. The trypsinization assay was performed at room temperature, the data was used to evaluate cell adhesion and a graph was drawn to demonstrate different cell detachment time.

2.7. Statistical Analysis

Statistical analysis was performed to determine the normality and accuracy of the data using One-way ANOVA, with graphical results and comparison. The means of all data are given with ± standard error (±SE).

3. Results and Discussion

3.1. Cell length Analysis

Addition of TGF- β3 to the cell culture media did not influence the length of the primary chondrocytes. The initial length of the chondrocyte cells in both TGF-β3 contained media and the control culture were 15.68μm ±0.7SE. However, the length of the cells in the control culture after 12, 36, 60, 84, 108, and 132 hours were 29.29 ± 0.46, 39.14 ± 0.54, 45.34 ± 1.43, 44.99 ± 2.91, 40.45 ± 2.45, 44.05 ± 0.33μm, respectively. These length for chondrocytes in the TGF-β3-contained environment were 33.11± 0.54, 39.83 ± 0.46, 40.36 ± 0.28, 44.04 ± 0.64, 48.55 ± 1.92, 49.76 ± 1.58μm, respectively (Figures 1-3). One-way ANOVA test was used to compare the length of the cells in both cultures. Statistical analyses of variation of cell length versus time indicated that the assumption of null hypothesis was accepted (Table 1). Null hypothesis was an assumption of equal mean cell length of chondrocytes cultured in control, and TGF-β3 supplemented media. The results of ANOVA test ($F = 0.086$, $F_{crit} = 4.747$, $P = 0.774$) showed that $F < F$ crit, and $P > 0.05$, which is an acceptance of the null hypothesis.

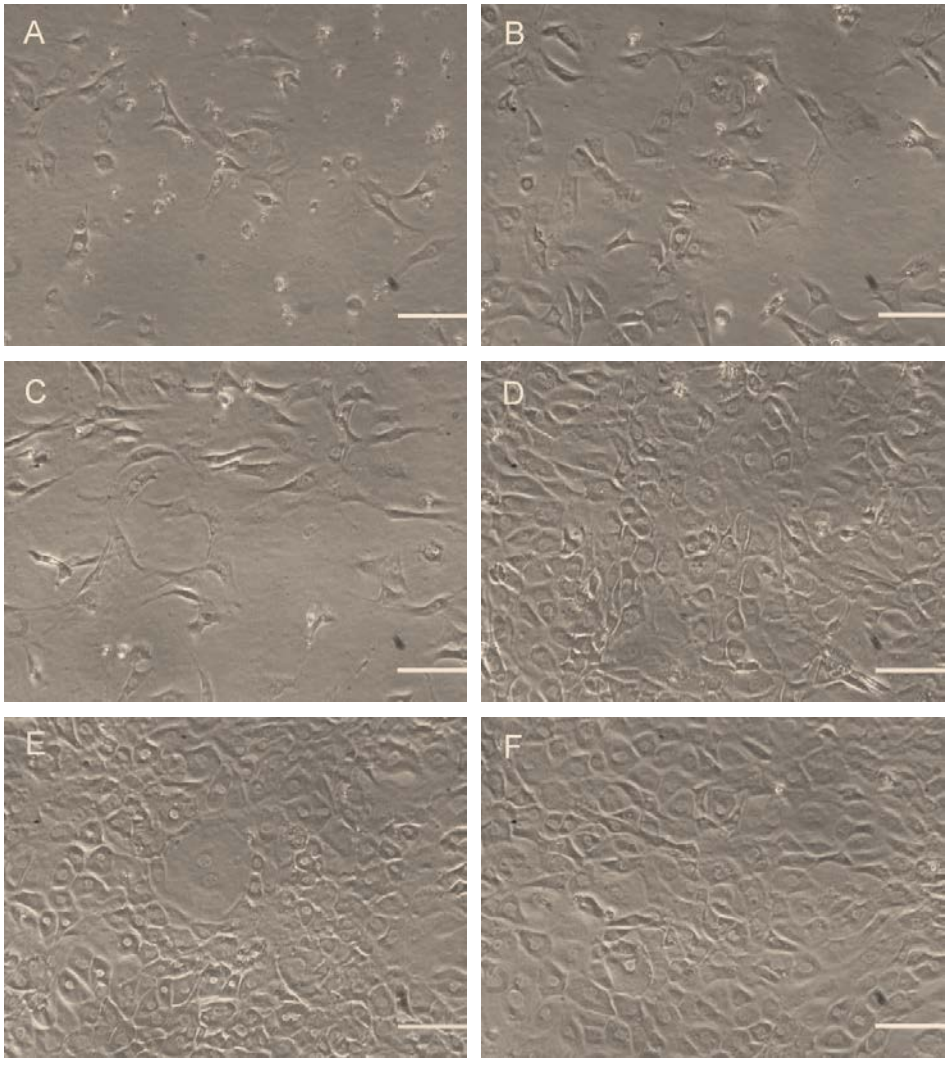


Figure 1. Culture of primary chondrocyte without TGF-β3 addition after: A) 12, B) 36, C) 60, D) 84, E) 108 and F) 132 hours; (Scale bar = 100μm).

Primary chondrocyte culture without addition of TGF-β3 exhibited a rounded morphology, which was a sign of chondrocyte at differentiation stage (Kim et al., 2009).

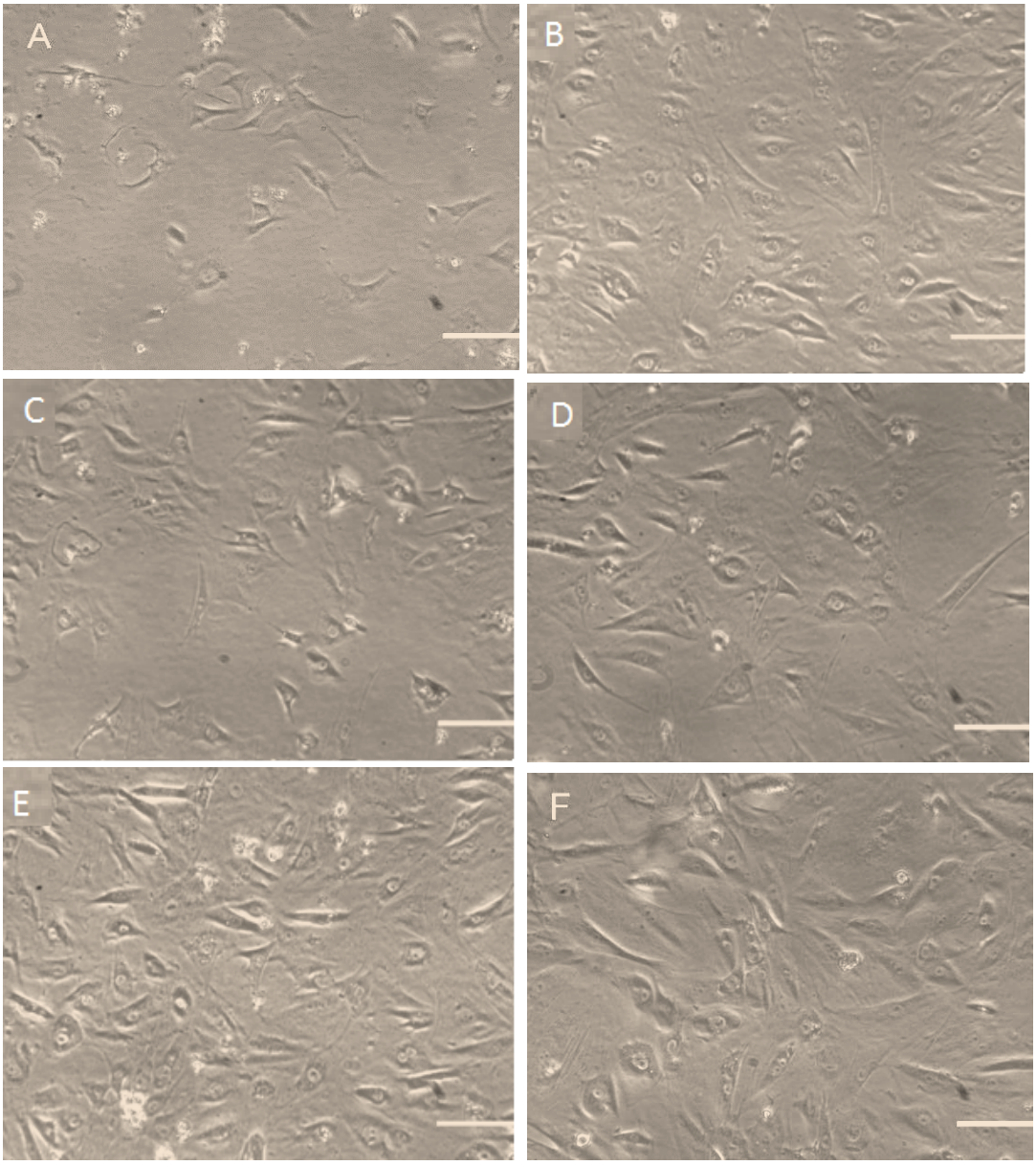


Figure 2. Chondrocyte cell length, supplemented with TGF-β3 after: A) 12, B) 36, C) 60, D) 84, E) 108 and F) 132 hours; (Scale bar = 100µm).

Control culture of chondrocytes over 132 hours also showed an increase in cell length, (See Table 1). However, after 60 hours the cell length decreased from 45.34µm to 40.45µm. This reduction may have happened at the stage of chondrocyte mitosis. In contrast, the cells cultured in media with TGF-β3 supplementation revealed an increasing cell length up to 132 hours (Table 1).

Hours	0	12	36	60	84	108	132
Control (Without)	15.68µm ± 0.63	29.29µm ± 0.46	39.14µm ± 0.54	45.34µm ± 1.43	44.99µm ± 2.91	40.45µm ± 2.45	44.05µm ± 0.33
TGF-β3 (With)	15.68µm ± 0.63	33.11µm ± 0.54	39.83µm ± 0.46	40.36µm ± 0.28	44.04µm ± 0.64	48.55µm ± 1.92	49.76µm ± 1.58
P-value	P = 1.000	P < 0.05	P = 1.000	P < 0.05	P = 1.000	P < 0.05	P < 0.05

Table 1. Alteration of cell length of chondrocytes cultured with and without TGF-β3 treatment during 132 hours (Mean ± SE).

The statistical data was used to plot a graph to compare the change in cell length with and without addition of TGF-β3 as shown in Figure 3. Results from the ONE-WAY ANOVA test (F = 268.16, P = 0.0001, F crit = 5.31) confirmed a significant difference between the proliferation rate of the control culture and the TGF-β3 contained culture, as F > F crit and P < 0.05.

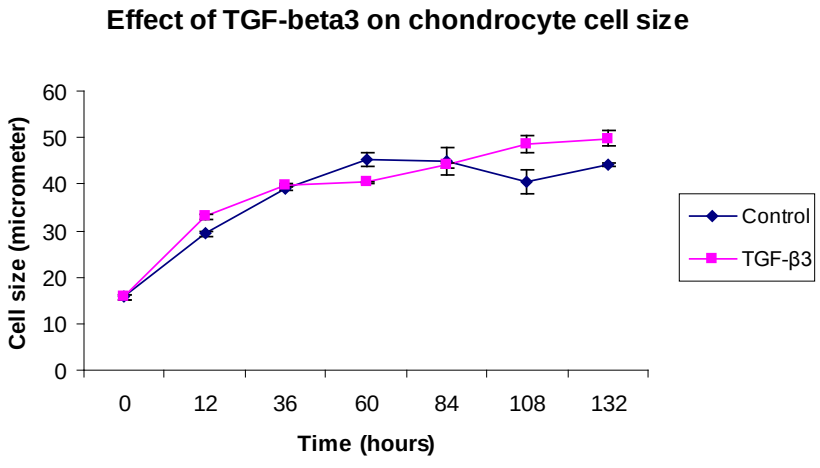


Figure 3. Graph showing change in chondrocyte cell length cultured for 132 hours with and without TGF-β3.

3.2 **Cell Proliferation Analysis**

The result of the cultivation of primary chondrocyte culture with and without TGF-β3 treatment showed that TGF- β3 reduces chondrocyte cell proliferation by increasing cell death (Figure 4).

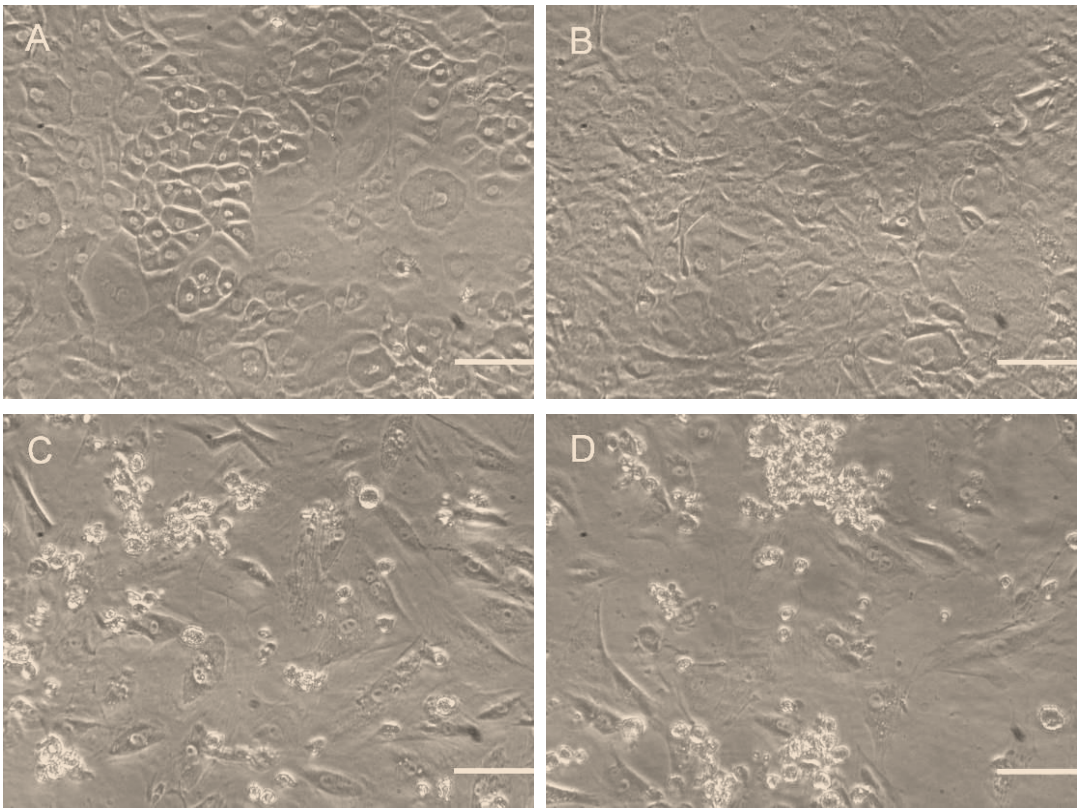


Figure 4. Proliferation and cell death of chondrocyte cells: A) and B) without, C) and D) with TGF-β3 supplementation; (Scale bar = 100μm).

Microstructural analysis of chondrocyte cells cultured in TGF- β3 contained medium showed partially rounded and shiny cells, which are the sign of detached, dead cells as can be seen in Figure 4.

After 132 hours of chondrocyte cell culture, the number of initial seeded cells, 7 x 10^4 cells/ml ± 4000 SE, was increased to 27 x 10^4 cells/ml ± 13.416 SE. In contrast, the number of initial cultured chondrocytes, 7 x 10^4

cells/ml ± 4000 SE, was decreased to 4.6 x 10^4cells/ml ± 2666 SE for TGF-β3 contained culture flask (See Table 2).

Hours	0	132
Control	7 x 10^4 ± 4000	2.7 x 10^5 ± 13,416
TGF-β3	7 x 10^4 ± 4000	4.6 x 10^4± 2,666

Table 2. Comparison of chondrocyte cell proliferation between control and TGF-β3 treated media.

The statistical data was used to plot a graph (Figure 5) showing the number of cells for initial, control and TGF-β3 contained culture media after 132 hours.

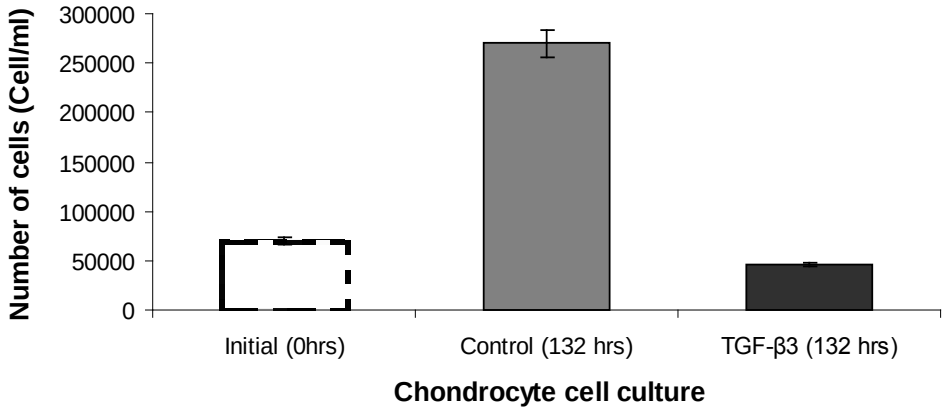
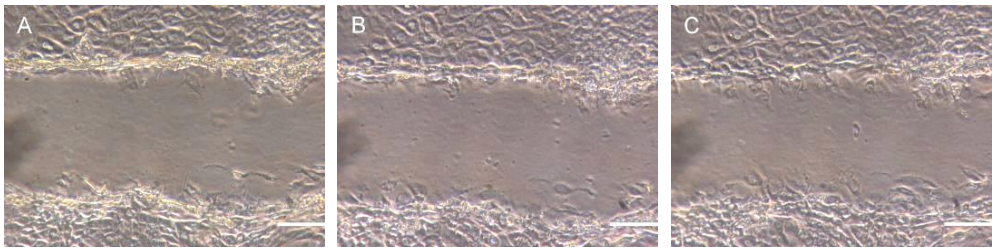


Figure 5. Graph showing the initial number of chondrocyte cells and after 132 hours in medium with and without TGF-β3 supplementation.

3.3. Wound-Healing Assay

Wound-closure assay of primary chondrocyte cells cultured on multilayer culture system showed that the modified wound model on control culture was repaired after 48 hours. The created wound width was initially 238.07μm ± 5.37μm and after 48 hours this wound was closed completely (Figure 6). There was ca. 4-6 hours' delay in the wound-closure process and in the migration of cells into the wound area after creation of the model wound. The cells started to migrate into the gap after partial removal of the dead cells from the wound edge. This delay was a demonstration of stress-responsive cells.



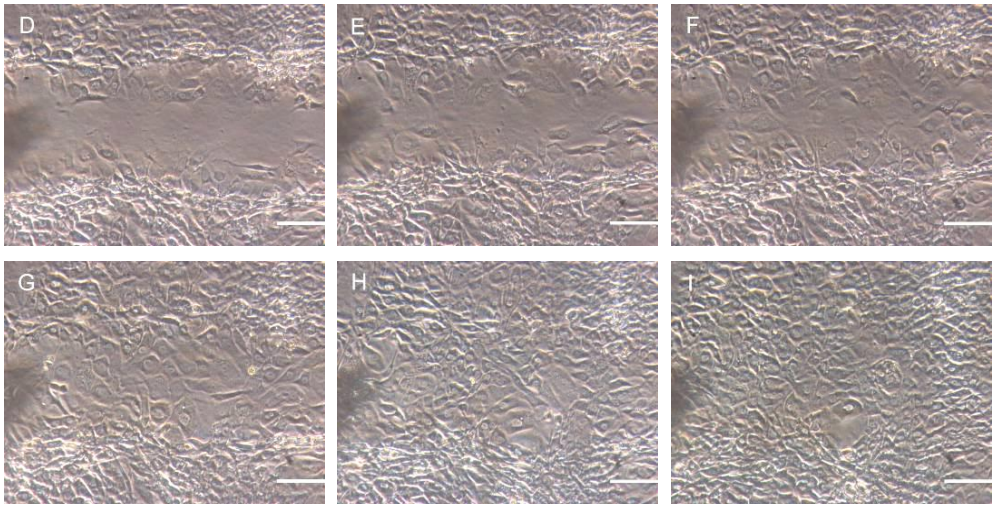


Figure 6. Images of wound closure of multilayer chondrocytes for the control culture of:
A) 0, B) 2, C) 4, D) 6, E) 8, F) 10, G) 18, H) 24 and
I) 48 hours, respectively; (Scale bar = 100μm).

In contrast, the generated model wound on chondrocytes multilayer with TGF-β3 supplementation did not show no complete repair after 48 hours. The initial wound width was 283.95μm ± 12.19μm and after 48 hours reduced to only 130.11μm ± 22.05μm (Figure 7).

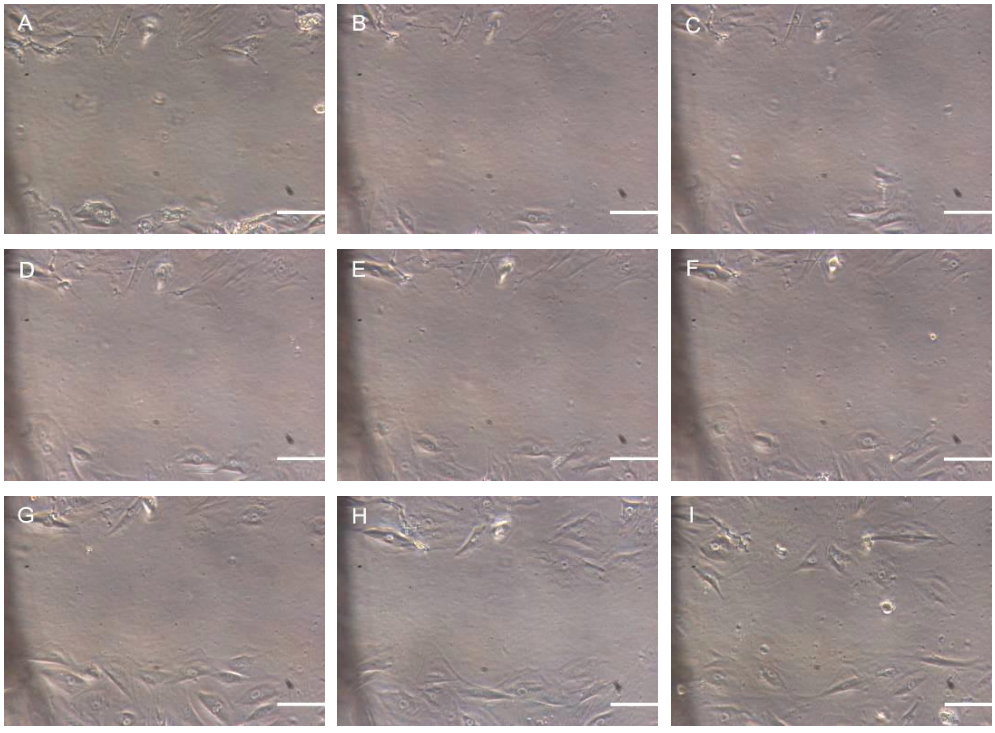


Figure 7. Images of wound closure assay of chondrocyte multilayer culture
with 10 ng/ml TGF-β3 supplementation: A) 0, B) 2, C) 4, D) 6, E) 8, F) 10,
G) 18, H) 24 and I) 48 hours; (Scale bar = 100μm).

Figure 8 also shows and compares the progression of wound healing of chondrocyte in a multilayer culture system without and with TGF-β3 supplementation. As can be seen from the graph in Figure 8, the wound width

created on control culture is zero after 48 hours, as compared to the wound width of the TGF-β3 culture showing only 60% closure and no sign of complete wound closure, even after 48 hours. Slowing down the chondrocytes proliferation by TGF-β3 (See Table 2 for detail) might be a reason for incomplete wound closure (Takahashi, 2000, Im, 2002). High levels of nitric oxide in wound edge produced by damaged cells could be another cause of increase in cell death and consequently slowing down of the wound repair process.

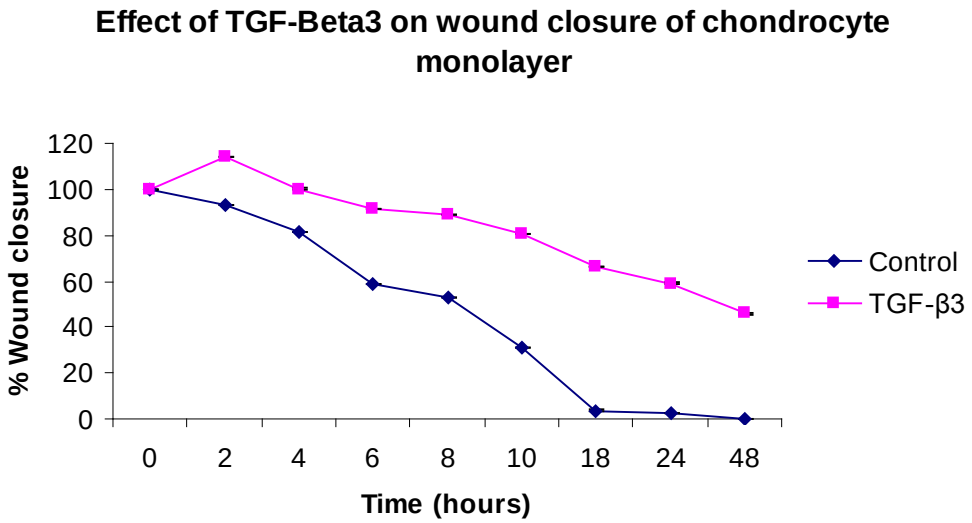
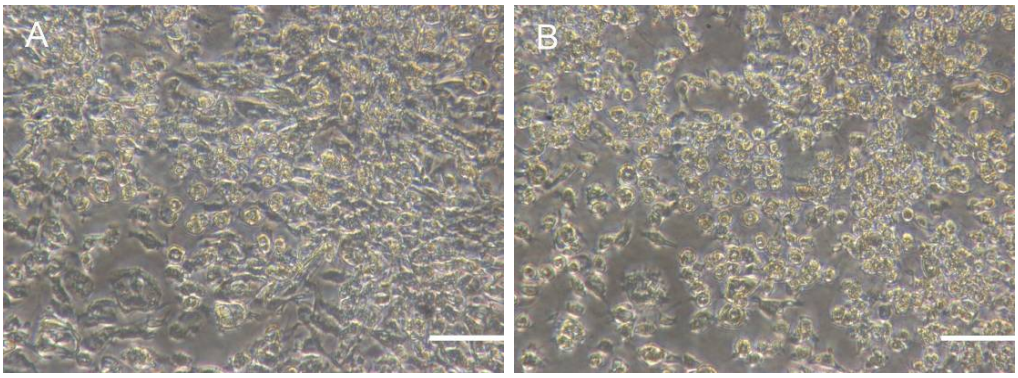


Figure 8. Graph of %wound closure vs time for the chondrocyte multilayers with and without TGF-β3 treatment.

A ONE-WAY ANOVA test was used to examine the wound width difference for the both wound closure assays. The results of statistical data analysis showed that the F (5.63) is greater than F crit (4.49) and P (0.03) < 0.05. According to these results, the null hypothesis, the assumption of equal wound closure process, was rejected.

3.4. Cell-adhesion assay

Results from trypsinization assay showed that the chondrocyte cells of the control culture detached completely after 240 seconds. In contrast, the entire cells from the surface of culture flask with TGF-β3 supplementation detached in 90 seconds (Figures 9 and 10).



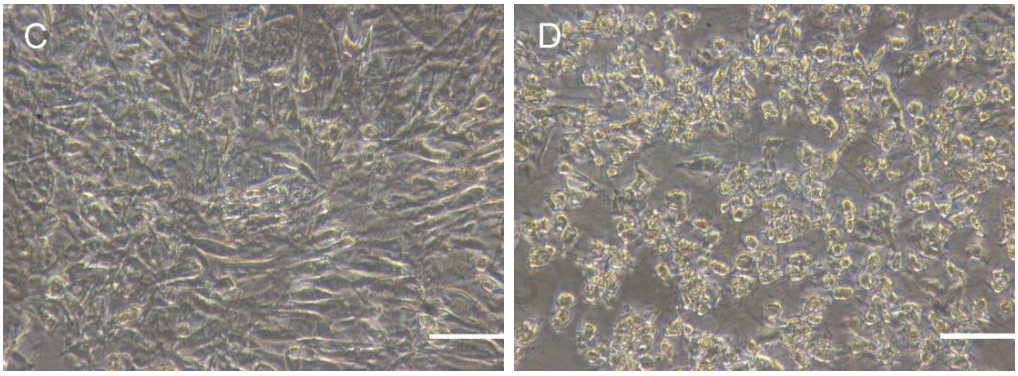


Figure 9. Images of chondrocyte cells' detachment: A) control at 0 seconds, B) control after 240 seconds, C) TGF-β3 contained culture at 0 seconds, and D) TGF-β3 after 90 seconds; (Scale bar = 100 µm).

After about 90 seconds, chondrocyte cells were detached from the surface of the tissue culture flask, which was supplemented with 10ng/ml TGF-β3. Unlike the chondrocyte culture with TGF-β3 addition, the cells of the control culture required 240 seconds to detach from the surface of the culture flask (Figure 10).

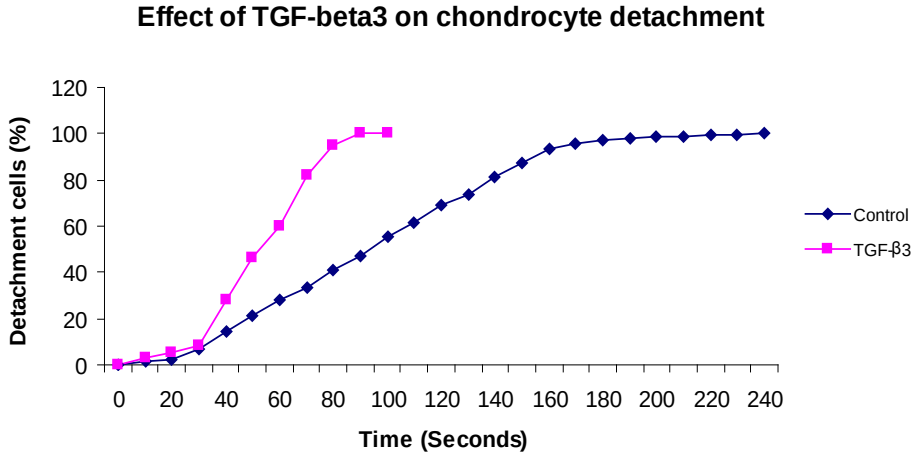


Figure 10. Graph of trypsinization assay for chondrocyte cell cultured with and without TGF-β3 treatment.

The difference between chondrocytes detached from the control culture and the TGF-β3 contained media was ~150 seconds. This time difference revealed that the TGF-β3 facilitated the detachment of chondrocyte cells from a solid surface, by almost 1.5 times.

4. Discussion and Conclusion

This work dealt with the effect of TGF-β3 isomer in chondrocyte behaviour in multilayer culture system. The aim has been to develop appropriate condition and supplementations for chondrocyte to maintain its differentiation state and promote proliferation, and to identify the effect of TGF-β3 isomer in biological regulation of primary chondrocyte. Quite a large number of experiments were developed, and several conclusive results have been found during this research.

It was found that TGF-β3 slow down cell proliferation and caused the chondrocytes undergo dedifferentiation with fibroblast like morphology. Fibroblastic phenotype of the chondrocytes caused the cells to occupy larger areas resulting in increased contact inhibition which led to decrease in cell proliferation.

TGF-β3 had no significant effect on chondrocyte cell length. However, this cytokine caused regulation of chondrocyte cell death and therefore facilitated the process of cells occupying larger area, spreading around and

getting a flatter shape. This occurrence caused the cells to develop a fibroblast-like morphology. Although the chondrocytes with fibroblastic shape occupied a larger area, the mean cell length of the chondrocytes in the TGF-β3 contained culture was only 5μm larger than the mean cell length in the control culture.

The process of wound repair for the chondrocytes monolayer culture with TGF-β3 treated was much slower than wound repair progression for the control culture. The effect of TGF-β3 on slowing down the chondrocyte proliferation may be the reason for the incomplete wound closure. Although the model wounds in both control and TGF-β3 contained media were created by the same pipette tip, the wound width on the TGF-β3 supplemented chondrocytes monolayer was 283.95μm ± 12.19μm, whereas the wound width for control culture was 238.07μm ± 5.37μm, and thus a normalized wound width was necessary.

TGF-β3 had a negative effect on cell-adhesion strength. This is due to the ability of adherent cells to sense and adapt to the mechanical stress created at the focal adhesions (FAs) which is directly dependent on integrin-ECM and ECM-cytoskeleton interaction (Bordeleau et al., 2008). This might also be the explanation for the increase in wound width and delay in the wound repair process suggesting that TGF-β3 slow down the expression of some types of integrins such as α3β1 (Saito, 2004, Srichai, 2010).

Chondrocytes isolated from articular cartilage undergo a rapid change in phenotype and gene expression in line with dedifferentiation. Consequently, they lose their round morphology in monolayer culture and shift their gene expression from type-II collagen to type-I, which is caused by the increase of the transcription levels of both SOX9 and type II collagen. This is because SOX9 activates a number of genes expressed in chondrocytes, including typical cartilage ECM genes Col2a1, Col9a1, Col11a2, Aggrecan, CD-rap (Chun-do Oh et al., 2014), and others, thus further studies are needed to investigate all the molecular changes leading to affinity or alignment of primary chondrocytes on ECM proteins for optimising a suitable bioscaffolding for cartilage regeneration.

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