Effects of static and dynamic culture conditions

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Tissue engineering has been investigating the properties of scaffolds and cell culture conditions for better cell attachment, viability and proliferation. This study compares the two cell culture conditions: Static and spinner flask / dynamic cell conditions over a period of 7 days on polyglycolyic acid. The scaffolds were statistically seeded by mouse dermal 3T3 fibroblast in static culture method and on other hand seeded scaffolds were transferred to spinner flask at approx. 60 rpm in dynamic culture method. Significant improvement in cell viability was not observed in both the conditions after 7 days of culturing. The cells adhesion successfully took place and expressed cytoskeleton Î²-actin in both the methods but achieving maximum distribution of cells on the scaffold in dynamic method. This study reports that static culture method could produce increase in cell number approximately six times more after 7 days of culture i. e. from 1. 2 x 10â ¶ µ (±0. 16×10â ¶ µ) cells to 6. 3 x10â ¶ µ (±1×10â ¶ µ) cells. Surprisingly, instead of enhancing the growth of 3T3 fibroblast cells in dynamic condition, they seems to be probably undergoing cell death/loss as reported by alamar blue, hoechst DNA assays, toludine blue and western blot. Overall, static condition favoured the cell adhesion, proliferation and Î²-actin expression gradually with days and produced better reproducible data compared to dynamic condition. The techniques involved in dynamic culture method needs to be more carefully investigated and improved further to draw a strong conclusion.

The aim of the study is to implement the principles of fundamental techniques in tissue engineering in culture method on the three dimensional polyglycolic acid (PGA) scaffolds seeded with 3T3 fibroblast. To compare and

contrast the effects on cells in spinner flask or dynamic culture condition method with the static culture condition method by observing and analysing on factors like cell adhesion, distribution, proliferation, viability and expression of cytoskeleton after culturing in the same system for 7 days using alamar blue, hoechst 33258 DNA assays, toludine blue staining and western blot analysis.

Tissue engineering is a multidisciplinary field which aims in developing new approaches for functional substitutes applicable in restoration of damaged or injured tissues. These substitutes are complex constructs of living cells, bioactive molecules and three dimensional porous scaffolds, which supports cell attachment, proliferation and differentiation. Therefore, its main objective to achieve in therapy is to form a living tissue from small population of mammalian cells. For this, the ideal tissue engineering strategy so far has remained to develop tissue by seeding the specific population of cells on three-dimensional constructs which not only provides a structural support to cell mass but also can effectively influence cell attachment, growth and differentiation either by incorporation of adhesion molecules or controlled release on bioactive molecules from the scaffold. After seeding of cells onto the 3-D scaffolds construct, the cells starts proliferating which results in deposition of extracellular matrix components and biodegradation of scaffolds. The latter makes the porous construct of scaffold more solid 3-D.

Several other factors affect the 3D tissue growth including scaffold design, seeding method and the culture condition methods. Studies have reported that high degree of cell attachment to biocompatible and biodegradable

particles, while avoiding aggregate formation can be achieved using poly co-glycolic acid (PGA) scaffold of 50-100mm spherical size fabricated by electro-spinning technique. This method provides reliable, reproducible and well-characterized PGA scaffold. The surface chemistry of the scaffold helps to determine the particle size, shape, morphology and distribution. Depending on the experiments, surface modifications are performed like formation of poly l-lactide-coglycolide (PLGA) via ring opening polymerisation and fibronectin coating to scaffolds. However, it is not the part of standard protocol. Depending on the size, the required cell density for maximum attachment may differ to obtained optimal cell attachment.

The seeding is usually done using the cell suspension of a particular seeding density which allows for maximum dispersion of cells and well integration into the pores of the scaffolds. But for therapeutic purposes, however, this strategy is not sufficient enough to result in an overall improvement in conditions due to severe tissue damages. This can be overcome only by achieving relatively high degree of cell attachment to the micro-particle. Several factors and parameters influence the cell adhesion like the curvature of the particles, the particle material, the electrostatic charge of the particles, the surface motif of the particles, the interaction between cell and particles, the number of cells in the tissue culture and type of cell culture method implemented. It is also important to obtain homogenous cell adhesion to the scaffolds and avoiding clumping which will lead to the formation of cell-particle aggregates. This will prevent cells from appropriate uptake of nutrient from the media and hinder their subsequent growth.

The mammalian cells are usually cultured in static or bioreactors condition. Here in this study, spinner flask system is employed which is also a kind of bioreactor as it provides the 3D environment. It is a flask provided with magnetic rod which keeps rotating constantly at specified speed. The nature of growing cells requires such dynamic condition to mimic the environment similar inside the body which gives sufficient nutrient supply, waste exchange, enhances ECM and gap junction formation, and cell-cell interaction. Most importantly it also helps maintain the cells differentiated in 3D which is needed for tissue formation. This characteristic is not maintained by static culture method. Hence, many 3D culture methods have been developed such as perfusion chambers, rotary vessels and commercial perfused bioreactors with improved capacity for mass transport of nutrients and waste product. They help in formation of relatively good quality of tissue by more enhanced cell differentiation and also maintaining in that state. The static culture method used in this study, tissue culture plastic with seeded scaffolds remains untouched in the incubator. But with static culture, alternative shaking on a shaker and resting can also be employed to provide better supply of nutrient through media.

The attachment characteristic of ECM proteins such as laminin, will also depend upon the cell type used. There are particular conditions needed to be optimised with each cell type. Most of the tissue engineering experiments uses 3T3 fibroblast only to optimise the cell culture condition where there is optimum cell adhesion is obtained before using the actual stem cell of interest. This is because, 3T3 fibroblast are known to easily attach to any surfaces due to presence of the high density of integrins on their cell surface.

This will not only enhance the cell attachment but will also give maximum possible interaction with the particle. Cells that have spread on the particles exhibit a clear halo of cytoplasm surrounding their nucleus after the rearrangement of their actin skeleton. The attachment and spreading of cells to a substrate surface is often seen as a basic characteristic, but is, in fact, the initial process that subsequently influences and regulates cell growth, survival, migration and differentiation. In addition, cell-to-substrate interaction, mediated by integrins, also influence cell behaviour and signalling pathways leading to modifications in upstream and/or downstream cellular activity. Thus, a desirable substrate should allow sufficient and optimal cell attachment and spreading characteristics to occur. The 3T3 fibroblast media is used in which DMEM supplemented with 10% FCS enhances the cell attachment as the serum is highly protein rich and therefore, helps in cell in adhesion by supplying the ECM-proteins as well as nourishing them. Hence, the serum conditioning step is of critical importance in maintaining cells health and attachment in the culture.

Materials and Methods

Scaffold preparation and serum conditioning

PGA FELT Scaffolds disc of 2mm x 10mm and 45mg/cc (TE005-50-10) was provided by Smith and Nephew research group, University of Nottingham. These non-culture scaffolds were then treated in 24 well tissue culture plastics (TCP) plates with 3T3 fibroblast media containing 500ml DMEM (Sigma G7513) supplemented with 10% FCS, 2mM L-glutamine and 1% AB/AM (Sigma A5955).

All the scaffolds were statistically seeded on day 1 using non-culture treated well plates to encourage the cells of mouse dermal 3T3 fibroblast to adhere to the scaffolds at seeding density of 1x 10â ¶cells/ml. 3T3 fibroblast cell suspension was added in TCP plates for all test and no cells in the blanks. The plates are then incubated overnight at 37°C, 5%COâ,, in air. The remaining cell suspension was then again resuspended in warm media to achieve 4 x 10â ¶ cells/ml cell density and was stored at -20°C till day 7 for Hoechst analysis.

3T3 fibroblast cells were used to seed the scaffolds to observe the cell viability, cell proliferation and Î²-actin expression on day 1, when the cell culture condition was maintained static and day 7, after applying the two cell culture conditions (static & dynamic) and maintaining for 7 days.

Static culture

In static culture condition, the seeded and non-seeded (blanks) scaffolds were kept in 1ml of warm 3T3 fibroblast media per well. These five culture plates were kept in incubator and cultured for 7 days at 37°C, 5%COâ,, in air.

Spinner flask culture

Two separate spinner flask filled with 50ml warm media each was used for seeded scaffolds and non-seeded (blanks) scaffolds. These flasks were kept in incubator by loosening the side arms and setting the magnetic stirrer approximately at 60rpm and cultured for 7 days at 37°C, 5%COâ,, in air.

After following 7 days for culture conditions, the construct was then sacrificed for alamar blue, toludine blue and Hoechst analysis. Also, in

addition cytoskeleton analysis using western blot was also carried out. The assessment of two culture methods, static and dynamic was done by producing five set of readings for static condition and four set of readings for dynamic condition where the experimental analysis were conducted using three replicates for test and blanks on day1 and day7

Alamar Blue Assay

Staining was done using 10% alamar blue containing 1ml alamar blue (Serotec BUF012B) and 9ml HBSS without phenol red (Sigma H1387). The stain was kept in dark at 37°C. The scaffold was transferred from seeding and culture conditions to new 24-TCP non-cultured plate with 1ml warm alamar blue after washing three times with PBS. The plates were then incubated at 37°C, 5%COâ,, for 1hr. The aliquots of 3 x 100µl of alamar blue were transferred to 3 wells of 96 microtitre well plate including the blanks to measure fluorescence using plate reader (Ex530nm/Em590nm). The excess of alamar blue solution was aspirated and washed with 1ml sterile PBS.

Toludine Blue Staining

Scaffold for toludine blue staining was transferred to new non-culture treated 24well TCP plate and was treated with 1ml ice cold 95% (v/v) methanol in dHâ,, O for 5 mins after washing 3 times with 1ml warm PBS. Then fixative was discarded and scaffold was allowed to air dry at RT followed by treatment with 1ml aqueous 0. 1% (w/v) toludine blue (Fisher chemicals BPE107-10) for 5 mins. The scaffold was again allowed to air dry at RT.

Papain Digest and Hoechst 33258 DNA Assay

The aliquot of cell pellet (4 x 10â ¶ cells) prepared on day1 was treated with 1ml of papain solution (1. 06mg/ml, pH 6. 5) (Sigma P4762) followed by overnight incubation in waterbath at 60°C. The serial dilutions of the papain digested cell pellet using hydrolysed papain solution as diluent was prepared for 4. 0, 2. 0, 1. 0, 0. 5, 0. 25, 0. 125, 0. 0625, 0. 0312 and 0 x 10â ¶ cells. In the Hoechst 33258 DNA assay, the hydrolysed papain solution was used as blank. 5µl of each aliquots + 70 µl Hoechst dilution buffer was added in triplicates in black 96-well plate including the blank. In each well, 100 µl Hoechst 33258 working solution (Sigma S6639) was also added and fluorescence was measured using plate reader (Ex 360nm/Em 460 nm)

Western Blot

100µl cold RIPA buffer (Sigma R0278) was added to the cell pellet (4 x 10â ¶ cells) and the seeded scaffolds in eppendorf from day 1 and was kept on ice for 20 mins while vortexing every 5 mins. The cells were then snap freezed by placing it on dry ice for 1 min then 1-3 min at RT. The cells are resuspended by grating and spinning for 30 mins. The supernatant was used for western blot. 10µl of molecular weight marker and each sample were loaded onto SDS polyacrylamide gel. The electrophoresis was carried out for 90 mins at 125V. After SDS-page electrophoresis, the filter paper, nitrocellulose and sponge were soak in transfer buffer (Invitrogen NP0006) with 20% (v/v) methanol. The assembled western blot tank was run for 1 hr at 25V. The immune-detection of protein Î²-actin was performed using primary antibody anti-mouse Î²-actin (Sigma A2006) and secondary antibody anti-mouse horse radish peroxidises (HRP) (Invitrogen G21234).

Statistical analysis

All the data obtained was calculated using MS-Excel spreadsheet and statistic Independent t-test and paired t-test analysis was performed using SPSS software.

Results and Discussion

Morphology of 3T3 fibroblast cells

The cell of mouse dermal 3T3 fibroblast was obtained from T180 flask by trypsin digest method is shown in figure1. The flask was confluent enough (80%) and morphology of the cells seems to be intact and healthy. No sign of contamination was observed prior to seeding procedure. The morphology of 3T3 fibroblast cells are of flat and spindled shape. These cells form a well-characterised and established mesh like interconnected networks. This property of fibroblast cells make them ideal for cell attachment as they show anchorage property due to presence of integrins in ECM. Hence, using this cell type achieving maximum cell adhesion onto the scaffolds becomes ideal for this experiment.

Effect on Cell viability in static and dynamic conditions

The alamar blue assay was performed on the static and dynamic culture condition to observe its effect on 3T3 fibroblast cell viability is shown in figure 2a and 2b. The culture method employed aims to maintain or increase the cell viability when cultured for seven days. Under static condition (Fig 2a), only 1 group out of five showed significant increase in fluorescence whereas other two groups showed more or less no change in their fluorescence produced from day 1 to day 7. Also, on contrary two groups showed significant decrease in fluorescence on day 7 (Fig 2a). Hence,

variable of results were obtained between groups. On the other hand, under dynamic condition, the cell produced more fluorescence on day 7 compared to day1 expect for one group. Therefore on an average, when mean of the static absorbance reading was taken, it showed that there is significant decrease in fluorescence (fig 2b). But in dynamic method, the increase in fluorescence day 7 (Fig 2b) was not significant enough. The 3-D construct of PGA scaffold provides with an environment to the cells where they remain viable in culture for several weeks. Moreover, they should successfully increase the cell viability after some days. However, our study reported that the cell viability decreased tremendously in cell seeded PGA scaffolds in static cell culture condition whereas the dynamic cell culture method was able to increase the cell viability over 7 days of culture. So, when comparing the two culture methods statistically showed difference in their overall effect on the viability of 3T3 fibroblast cells where dynamic condition is more but not effective enough. So, static condition did not improve the cell viability more than dynamic culture method.

Effect on Cell distribution in PGA scaffolds

The three-dimensional PGA scaffolds constructs enables the fibroblastic cells to adhere and to evenly distribute throughout the porous structure. To assess the uniform 3T3 fibroblast cell distribution in two different culture conditions, toludine blue staining was carried out on day 1 and day 7 on both conditions is shown in fig 3. Toludine blue stains cell dark blue within the 3-D construct. As observed in static condition, on day 1 the cells were successfully seeded onto the scaffold but compared to day 7 the cells are not evenly distributed throughout the scaffold. Also, the scaffolds were efficiently

seeded on day1 under dynamic condition as the figure 3c shows cells stained with toludine blue. Surprisingly, on day 7 (Fig 3d), the scaffolds shows no cells at all. This means, that the 3T3 fibroblast cells under dynamic condition was eventually lost or died. The spinner flask culture system might have loose the cells by day 7 due to poor adhesion or vigorous rotation. The cell seeded on day 1 was too low or error in carryout the technique. But this was observed with all the spinner flask condition system, where the success was 2 out of 4 groups (Supplementary data 3). However, this observation is more of debate because no other factors expect the condition itself could affect cell distribution as uniform distribution was achieved in all the five static condition (supplementary data 3) which used the same scaffolds and cell type.

Effect on Cell proliferation in static and dynamic conditions

3T3 fibroblast was culture over 7 days in both conditions to also observe its effect on the cell proliferation are shown in figure 4 (a, b, c & d). The standard curve obtained with known cell density for both static and dynamic of all the groups (fig 4a & 4b) showed increase in cell density with increase in the fluorescence. The unknown cell density of the cells from these two culture methods on day 1 and day 7 was calculated and found that 2 out 4 groups from dynamic conditions had no cells in the culture on day 7. Therefore, only other two groups were considered to evaluate the cell number on day 1 and day 7. There was significant difference in cell density over 7 days of culture in static method (n= 5)(fig 4c) and on contrast there was no significant difference in cell density in dynamic method (n= 2)(fig 4c). Almost all the groups showed cell density on day 1 around 1 x10â µ

cells/ml which was the actual cell density seeded on day 1 (supplementary data 4). This shows that seeding performed on scaffold achieved effective adhesion of all the cells present.

The mean cell number from 1. 2 x 10â ‡ µ (± 0. 16 x 10â ‡ µ) cells on day 1 increased to 6. 3 x 10â ‡ µ (± 1 x 10â ‡ µ) cells on day 7 under static culture method (fig 4d). On the other hand, dynamic culture methods showed hardly any change in cell number over 7 days of culture i. e. 2. 0 x 10â ‡ µ (± 0. 92 x 10â ‡ µ) cells on day 1 to 2. 5 x 10â ‡ µ (± 1. 96 x 10â ‡ µ) cells on day 7 (fig 4d). Previous studies have reported using other cell types that they start proliferating within 24 hrs after seeding cells on scaffolds employing dynamic culture methods. Contradicting this, our results have shown that dynamic had really poor effect on cell proliferation. Moreover, 3T3 fibroblast cells were undergoing death during seven days of culture. Whereas, static culture method shows drastic increment in the cell number and thus supporting 3T3 fibroblast cell proliferation efficiently. The scaffolds used for alamar blue assay on day 1 were used for Hoechst DNA assay with same after washing step (same for day 7 scaffolds). The washing might have been too vigorous which resulted in cell loss. It could also be possible that cells are being aspirated off from the culture which gave poor or no cell proliferation. It should be also taken into account that the success rate with dynamic culture method on cell proliferation was null out of 4 demonstrations.

Expression of Cytoskeleton

For the analysis of expression of cytoskeleton Î²-actin on 3T3 fibroblast in two different conditions was done by western blot as shown in figure 5. The cell pellet of density 4 x10â ‡ ¶ cells/ml was loaded against the cells obtained on

day1 and day 7 from static and spinner flask culture method. The density of Î²-actin obtained from the cell pellet was maximum. The amount of Î²-actin detected on day 1 was lower than day 7 in static culture condition. It was the opposite scenario with spinner flak method where day 7 had minimum amount of Î²-actin compared to day1. In some cases of spinner flask method Î²-actin was not even detected on day 7 (supplementary data 5). Hence, comparatively the expression of Î²-actin was higher in static culture method. Perhaps, it could be because the cell could not proliferate much as expected. Also, the culture didn’t have enough cells left to express Î²-actin on western blot. The formation of ECM cytoskeleton was not shown to be supported by spinner flask method.

Conclusion and future work

The tissue engineering scaffolds constructs have been shown more effective on cells containing serum in spinner flask/dynamic culture method rather than in static culture method. But from our data, it shows that dynamic condition only favoured cell adhesion and distribution. It was also able to produce a small increment in cell viability unlike static culture method. Contradicting the other data, cells were virtually not detected on day 7 and so is the expression of Î²-actin. Not only this, all the 4 demonstration failed to show that cell growth can be effectively supported in dynamic culture method. Three seeded scaffolds were kept in spinner flask together, where there is increased chance for it to come in contact with each other. Cells may get detached from the scaffolds as it might be loosely adhere to the scaffold. The continuous rotation of magnetic rod in the flask circulates the media to provide nutrients to cells more effectively then static. Despite of this fact,

the cells were either undergoing cell death or dislodged from the scaffolds or may be aspirated off from the culture.

The static culture method have been effective in 3T3 fibroblast adhesion on the construct after seeding and eventually could improve tremendous cell growth by showing increase in cell proliferation over a period of seven days in culture. However, better distribution and increased Î²-actin expression could only be achieved by the static culture method after 7 days as the cells proliferated more. Moreover, the success rate for this method was more compared to dynamic and produced more reliable and reproducible data. Hence, it can be concluded that static culture method supported cell growth better then the dynamic culture method.

It would be interesting to investigate the technique involved in dynamic culture method more carefully to produce reliable data where it could be compared with the static method to give better understanding of the environment cells require to grow in artificial ECM-like structure and culture media. Since, within the body the cells are continuously under the force by blood flow in 3D environment, it would be useful to derive cell culture growth better in dynamic condition with enhanced technique. It is strongly recommended to carry out further research in this area to conclude spinner flask method’s effect on 3T3-fibroblast cells with more reliable data.

Evaluation

The practical session assessed my learning in the techniques and concepts involved in tissue engineering. The demonstration on different techniques to prepare scaffolds assessed my understanding better and was helpful to

apply same in this practical session by evaluating the different parameters that can be influenced by the scaffold design alone. As earlier discussed troubleshoot, implementing the technique given in protocol helped to produce the good replicates and contamination free-blanks and controls. While working in the hood with the partner, things were discussed prior to carrying out the experiment and working space was kept ready which helped in managing the use of same equipments, solution and incubation time effectively to avoid any source of contamination. Also, the exchange of results and data between several groups also led to the exchange of ideas and different cause for their results. However, the exact reason for spinner flask method to not work out is still not clear as all the groups got same reading where cells were present onto the scaffolds during alamar blue assay on day1 and day 7 but eventually lost when subsequent assays were done for same day. Overall, the difference between the effects of two culture method was evaluated.

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Figures

Figure1. 3T3 fibroblast cells in T180 flask (10X). The image shows morphology of 3T3 fibroblast prior the trypsin digest followed by static seeding. The image was taken using Nikon (Scale bar: 80µm)

Figure 2a. Alamar blue assay for all static (n= 5) and for dynamic (n= 4) culture methods on day1 and day 7. The graph shows fluorescence detected ± SD for both the culture condition. The absorbance value of non-seeded scaffold (control, Ac) was subtracted from the absorbance value obtained for seeded scaffold (As) to optimise the calculated fluorescence i. e. As-Ac. This was done for all the static and dynamic culture methods data. The statistical analysis paired t-test was at 95% significance level was done using SPSS. The calculated data is provided in the supplementary data.

Figure 2b. Alamar blue assay of static and dynamic condition on day 1 and day 7. The mean of all the values on day 1 and day 7 for static (n= 5) as well as dynamic (n= 4) was done. The graphs shows the mean of absorbance (O. D) ± SD. The statistical analysis was performed using paired t-test and independent t-test at 95% significance level.

DAY 1 DAY 7

Figure 3. Toludine blue assay. The toludine blue staining was performed on static culture condition on day 1 (a) and on day 7 (b). Similarly for dynamic culture condition on day 1 (c) and day 7 (d) was carried out. In (a) and (b) there is darker background staining but (c) shows proper stained 3T3 fibroblast cells. No cells staining can be detected in (d) (Scale bar: 100µm).

Figure 4a. Standard curve for all static condition using Hoechst 33258 DNA assay. The standard curve was plotted using the known concentration 4. 0, 2. 0, 1. 0, 0. 5, 0. 25, 0. 125, 0. 0625, 0. 0312 and 0 x 10â ¶ (blank) of 3T3 fibroblast cells against the absorbance obtained. The blank was subtracted from the test reading to standardise the graph. The graph was produced using MS-Excel, to obtain the linear regression and linear equation for each group to calculate the cell density in static culture condition.

Figure 4b. Standard curve for only two dynamic condition using Hoechst 33258 DNA assay. The standard curve was plotted using the known concentration of 4. 0, 2. 0, 1. 0, 0. 5, 0. 25, 0. 125, 0. 0625, 0. 0312 and 0 x 10â ¶ (blank) of 3T3 fibroblast cells against the absorbance obtained. The blank was subtracted from the test reading to standardise the graph. The graph was produced using MS-Excel, to obtain the linear regression and linear equation to calculate the cell density in dynamic culture condition on day 1 and day 7.

Figure 4 c. Hoechst 33258 DNA assay was carried out on all static (n= 5) and dynamic (n= 2) culture condition on day 1 and day 7. The cell density was calculated using the standard curve for its own respective group. The graph shows cell density (x 10â µ cells/ml) ± SD for static and dynamic condition. The calculation was performed on excel-sheet and statistical analysis of paired t-test was done using SPSS.

Figure 4 d. Hoechst 33258 DNA assay. The unknown cell density calculated from standard curve was averaged (mean) for static (n= 5) and dynamic (n= 2) culture methods. The graph shows cell density (x 10â µ cells/ml) ± SD

for static and dynamic condition. The calculation was performed on excel-sheet and statistical analysis of paired t-test and independent t-test was done were appropriate using SPSS.

Figure 5. Western blot analysis of 3T3 fibroblast cell from static and dynamic on day 1 and day 7. The expression of Î²-actin in both culture methods are analysed using the rainbow marker and compared with the actual pellet of 3T3 fibroblast to cells extracted from two different culture methods on different days.