EFFECT OF CHITOSAN AND DEXTROSE CROSSLINKING ON APPLE DERIVED CELLULOSE SCAFFOLD IN REGARD TO THEIR MECHANICAL PROPERTIES AND PROLIFERATION OF CELLS

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Abstract

Natural and synthetic scaffolds can be used as a substrate for the seeding and proliferation of cells. As cellulose and its derivatives have been successfully employed as biomaterials in various applications, the use of cellulose as a scaffold is viable. Easily produced, inexpensive apple derived cellulose scaffold, due to their natural origin and porosity, may prove to be a viable and even a better scaffold option compared to synthetic scaffolds. This research shows that the mechanical strength and the cell holding capacity of the apple derived cellulose scaffolds can be manipulated by crosslinking with glutaraldehyde, chitosan and dextrose. To compare the mechanical properties, swelling and solubility tests were performed along with the drug release capacity test; porosities of the scaffolds were compared and FTIR analysis was used to show the composition and crosslinking in the scaffolds. HeLa cells were used for the recellularization of the scaffolds and MTT assay was done to determine the cell proliferation in each type of scaffold. The scaffolds cross-linked with chitosan showed better swelling capacity and better cell proliferation compared to other cross-linked scaffolds. Glutaraldehyde cross-linked scaffolds had better mechanical strength, but the cell proliferation was comparatively low whereas the dextrose cross linking had no significant effect on mechanical properties but supported cell proliferation.

Keyword: cellulose, scaffold, glutaraldehyde, HeLa cells, MTT assay, chitosan, dextrose

1.INTRODUCTION

Body cells combined with scaffolds is a helpful way to regenerate damaged tissues. The development of substrate materials that can provide suitable microenvironment for cell matrix interactions by mimicking biological conditions is an important requirement for tissue regeneration by cell transplantation. Biomaterials with high porosity which is based on renewable source are required for the tissue engineering applications. Cellulose is one of the most abundant, sustainable and easily sourced biomaterials on earth which can be used for tissue engineering applications as it is organic and plant-based fiber. It is used extensively in many applications and use of cellulose as a scaffold base is one of them. Pulkkinen H et al, cellulose and cellulose/recombinant type II collagen sponges used as a scaffold and infiltrated with chondrocytes were found to be biocompatible for at least four weeks in cultivation, and gradual filling of the scaffold was also observed (1). Myocytes cultured on synthetic cellulose scaffolds contained periodic myofibrils as well as enhanced connectivity in the form of increased gap junction density and electrochemical connectivity which resulted from 3D culture in comparison to cells grown on glass (2). The cellulose scaffolds can also be produced by decellularization of tissues using detergents which leaves behind just the

ECM framework. Decellularization techniques have been applied to different plant species and tissues to generate acellular, pre-vascularized tissue engineering scaffolds. Joshua R. Gershlak et al conducted a research on decellularized Spinach leaves. Human mesenchymal stem cells and human pluripotent stem cell derived cardiomyocytes adhered to the outer surfaces of plant scaffolds. Cardiomyocytes demonstrated contractile function and calcium handling capabilities over the course of 21 days (3). Due to the presence of soft hypanthium tissue in apple and the presence of pores and abundant air pockets that facilitates nutrients transport, it is a suitable candidate for decellularization for scaffold production. In 2013, a research conducted by Modulevsky DJ et al found that three mammalian cell types, NIH3T3 Fibroblasts, mouse C2C12 Muscle myoblast and Human HeLa Epithelial Cells proliferated, migrated, and remained viable in decellularized apple cellulose scaffolds for up to 12 weeks (4). Due to the abundance and rapid growth of many plant species, this technique provides a less costly, more abundant and sustainable scaffold material. Cross-linking of these scaffolds with other agents also help alter their mechanical properties to desired level. Glutaraldehyde establishes the carbon and nitrogen double bond formation which results in increase of mechanical strength and thermal stability as well as life span of the scaffold (5). Modulevsky DJ et al found that the mechanical properties of the cellulose scaffolds could be altered by means of cross-linking with collagen and glutaraldehyde (4). Due to chitosan's biocompatibility, low toxicity, and biodegradability, it can also be used as cross-linking agent. Lie Ma et al fabricated a porous chitosan/collagen scaffold that showed enhanced biostability and biocompatibility as well as increase in their swelling capacity when they were treated with glutaraldehyde (6). Dextrose can also be used as cross-linking agent which in limited concentrations induce tissue repair hence increasing cell viability (7).

2. EXPERIMENTS

2.1. Preparation of Apple Scaffold

2.1.1. Preparation of Apple Segments

Fuji Apples were stored at 4°C in dark for maximum 2 weeks, then apple was chilled in 20°C freezer for 5 minutes. Apple were cut with a mandolin slicer to a uniform thickness of 1.20±0.14 mm as measured by Vernier caliper. Only the fleshy part of the apple (hypanthium tissue) were used, not the ovary-core. Apple slices were cut into 0.5*0.5 cm segments parallel to the direction of the apple pedicel for cell seeding whereas, for characterization apples slices were cut into 2.0*0.5 cm segments parallel to the direction of the apple pedicel for cell seeding.

2.1.2. Decellularization and Storage of Apple Tissue

Individual apple samples were placed in sterilized 2.5 mL micro centrifuge tubes and 2 mL of 0.5% sodium dodecyl sulphate (SDS) solution was added to each tube. Samples were shaken for 12 hours at 160 RPM at room temperature. The resultant cellulose scaffolds are then transferred into new sterile microcentrifuge tubes, washed and incubated for 6 hours in PBS with 1% streptomycin/penicillin. At this point, the samples were immediately used or stored in PBS at 4°C for no more than 2 weeks.

2.1.3. Post-Decellularization Treatments

The scaffolds were crosslinked with four different crosslinking agents. For crosslinking the scaffold sample with glutaraldehyde, the scaffolds were incubated in 1% EM grade glutaraldehyde for 6 hours. Then they were rinsed in PBS then incubated in 1% of sodium borohydride and kept overnight to reduce unreacted glutaraldehyde.

For crosslinking with chitosan, a 2.5 % chitosan solution was prepared by mixing chitosan powder in 5% glacial acetic acid solution in a magnetic stirrer until the mixture became homogeneous. The scaffolds each were incubated with 2 ml of chitosan solution for 24 hrs at 37˚C.

For crosslinking with dextrose, 2.5% of dextrose solution was prepared in distilled water. The scaffolds were incubated for 24 hrs in 2ml of dextrose solution at 37˚C. For crosslinking with both chitosan & glutaraldehyde, the scaffolds that were previously crosslinked with

glutaraldehyde were mixed with 2.5% of chitosan solution and incubated for 24 hrs at 37˚C. Before seeding cells, samples were incubated in cell culture medium (EMEM) for 2 hours in a CO2 incubator at 37°C and 5% of CO2.

2.2. Cell culture

HepG2 cell line was used along with medium consisting of sub confluent monolayers in DMEM along with 10% fetal bovine serum and 1% streptomycin and penicillin at 37 °C with 5% CO2. Then those frozen cells were thawed into a 175cm^2 flask containing 30 ml of medium and incubated at 37˚C, 5% CO_2 and allowed to attach and fill out the dish. The media was changed next day.

Similarly, HeLa cells were also cultured. HeLa cell line were used along with medium consisting of sub confluent monolayers but unlike HepG2 cell line it was done in EMEM along with 10% fetal bovine serum and 1% of streptomycin and penicillin at 37 °C with 5% CO2. Then those frozen cells were thawed into a 175 cm2 flask containing 30 ml of medium and incubated at 37˚C, 5% CO2 and allowed to attach and fill out the dish. The media was changed next day. Then it was trypsinized with 0.05% or 0.25% trypsin and split it as 1:8 – 1:16. The media were changed twice a week. These cells were grown either in 15cm dishes or cell stacks.

2.3. Recellularization

For recellularization of Scaffolds 24-well tissue culture plates were used for cell seeding. Scaffolds were cut into 0.5×0.5 cm pieces and placed within each well. A 40 µL droplet containing 2×104 cells were carefully formed on top of each scaffold. The samples were placed in the incubator for 30 minutes to allow the cells to adhere to the scaffolds. Then, 0.5mL of DMEM was added to each well and the samples were incubated for 48 hours. At this point, samples containing HeLa cells were then carefully transferred into new 24-well tissue culture plates. For continued cell proliferation, the culture media was exchanged every two days.

2.4. Characterization

2.4.1. Solubility Test for Scaffolds

The solubility tests for scaffolds were performed for native decellularized samples and the samples crosslinked with glutaraldehyde, chitosan, glutaraldehyde & chitosan and dextrose. First, the scaffolds were patted dry and weighed to obtain the dry weight. Then these samples were dipped in 20ml of distilled water. The scaffolds were taken out in 24 hours and gently tapped and dried with the help of filter paper and weighed. The solubility percentage were calculated by the following formula. The mean and standard deviation were also calculated.

$$\text{Soluble mass } (\%) = \frac{(m_i - m_f)100}{m_i}$$

Where, mi is the initial dry weight of the scaffolds and mf is the final dry weight of the scaffolds after 24 hours.

2.4.2. Swelling Capacity Test

The swelling capacity test was further performed for native decellularized samples along with the samples crosslinked with glutaraldehyde, chitosan, glutaraldehyde & chitosan and dextrose. First, the scaffolds were patted dry and weighed to obtain its dry weight. Then these samples were dipped in just 20ml of distilled water. The scaffolds were taken out in 1minute, 5minutes, 10minutes and 24 hours and gently tapped with filter paper and weighed. The swelling percentage was calculated by the following formula. The mean and standard deviation were calculated.

$$\text{Swelling}(\%) = \frac{\{W(s) - W(d)\}}{W(s)} \text{ x } 100$$

Where, W(d) is the weight of the dry scaffold and W(s) is the weight of the swollen scaffold.

2.4.3. Drug Release Rate

The scaffolds were further studied on the matter of their rate of drug dispersion. The samples cross-linked with dextrose, chitosan and glutaraldehyde along with the native samples were loaded with gentamicin. For this, first the calibration curve of gentamicin in phosphate buffer saline (PBS) was obtained. Gentamicin was first diluted in PBS and solutions of concentration 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml were prepared.

Then using UV spectrophotometer, the absorbance of each sample was recorded at wavelength of 256 nm. The various absorbance was used to generate a calibration curve of gentamicin in PBS.

To load the scaffolds with gentamicin, the scaffolds were taken in triplicates and immersed in gentamicin solution of concentration 16mg/ml in phosphate buffer saline (PBS). They were incubated for 48 hours to incorporate the drug inside the scaffold.

The controlled release of gentamicin was studied after immersion of scaffolds in 10 mL of a PBS without calcium and magnesium to avoid the chemical reaction of bioactivity. The release of gentamicin was studied after 10 and 40 minutes and 1, 2, 3 and 4 hours in triplicate. These notices were chosen because some studies have proved that gentamicin sulfate is released quickly. PBS solutions were analyzed at 256 nm using UV spectrophotometer to measure the concentration of gentamicin and the cumulative drug release was determined.

2.4.4. FTIR Analysis

Different types of samples were sent for FTIR analysis to determine their composition and the crosslinking in the scaffolds. The transmittance data was obtained which was first converted to absorbance and the FTIR graph was obtained.

2.4.5. Porosity

The porosities of the different scaffolds were measured using liquid displacement method where distilled water (non-solvent of hemicellulose) was used as the displacement liquid. Scaffolds samples measuring about 2x0.5x0.1 cm3 were immersed in a known volume of the liquid (V1) in a graduated cylinder for 10 mins. The porosity was obtained by:

$$Porosity = \frac{(V1 - V3)}{(V2 - V3)}$$

Where, the total volume of scaffolds in the water along with the water was recorded as (V2) and the residual water in the cylinder after removal of scaffold was recorded as (V3).

2.5. Cell Proliferation Test

2.5.1. MTT assay

The MTT reagent solution was made for 56 wells with each well receiving 20µL MTT reagent solution. So, 2ml MTT assay reagent solution was made. For this, the reagent was first weighed, and 0.014 gram was taken in a falcon tube. 2 ml of distilled water was added to the tube and the mixture was kept in vortex mixer for thorough mixing. As the solution was light sensitive, it was wrapped in aluminum foil and kept for further use.

All the different cross-linked scaffolds which were recellularized were taken out of the incubator. The samples which were seeded prior 5 days, 3 days and 1 day were transferred from the 24-well plate into new 96 well plate with proper marking to differentiate each type of scaffold. The negative control was taken as hydrophobic plastic. Then, 150µL media was added to each well where the scaffolds were kept. After that, 30µL MTT assay reagent was added to each well and the 96 well plate was completely covered by paraffin wax and placed in the incubator for 3 hours at 37°C. After that the 96 well plate was taken out of the incubator and 100µL DMSO was added to each well which was again incubated for 15 minutes. After that, the absorbance of solution present in each well was recorded in ELISA reader.

2.6. Statistical Analysis

Using Graph Pad Prism two-way ANOVA was used to analyze the data for statistical significance between the experimental groups. The results are given as mean ± standard deviation (SD) with $P < 0.05$ for significance. The degree of significant difference was denoted by the stars.

3. RESULTS

3.1. Swelling

The ability of scaffold to uptake fluids from the surrounding medium plays an important role in its use in tissue engineering. With the increase of inflow of the medium and its ability to swell, the scaffold could hold a greater number of cells within its dimensions.

The comparison between native scaffold, glutaraldehyde crosslinked scaffold, glutaraldehyde crosslinked scaffold treated with NaBH4 showed that the native scaffolds retained excess water than the ones cross linked with glutaraldehyde. The swelling capacity of the scaffolds crosslinked with glutaraldehyde and treated with NaBH4 was the least and most consistent among the three as shown in Fig. 1. These tests testified the fact that crosslinking the scaffolds with glutaraldehyde increased their rigidity and mechanical strength.

The comparison between the chitosan cross-linked scaffolds, dextrose cross-linked scaffolds and the scaffolds cross-linked with both chitosan and glutaraldehyde showed that the scaffolds which were cross-linked with chitosan showed increased swelling in comparison to other cross-linking (Fig. 1). The dextrose cross-linked scaffolds showed least swelling capacity and the scaffolds with both glutaraldehyde and chitosan crosslinking showed good swelling in comparison to dextrose and less swelling than chitosan crosslinked scaffolds (Fig 2). The scaffolds which were crosslinked with chitosan showed a considerable increase in swelling capacity with increase in time as compared to other scaffolds. The scaffolds crosslinked with dextrose showed the least variation in swelling capacity with passing time. The difference in the scaffolds swelling capacities were significant for most of the cases. The effect of crosslinking was prominent in rest of the scaffolds which were crosslinked with chitosan showed high degree of swelling with passage of time.

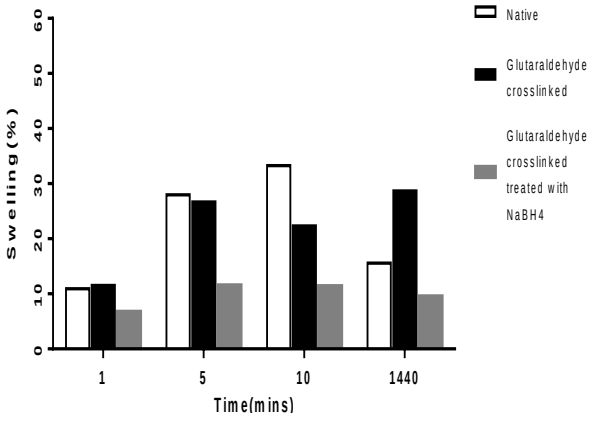

Fig. 1: Comparison between Native Scaffold, Glutaraldehyde crosslinked Scaffold, Glutaraldehyde crosslinked Scaffold treated with NaBH4

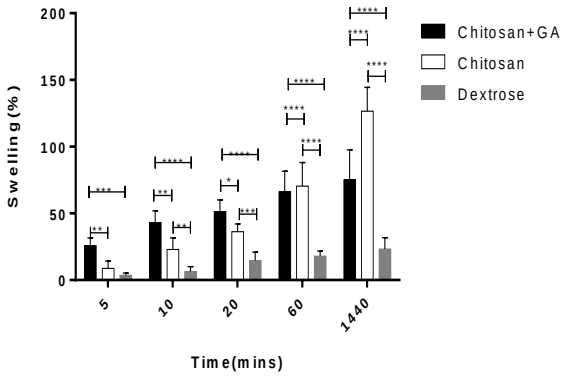

Fig. 2: Comparison of swelling capacity between different cross-linked scaffolds.

3.*2. Solubility*

The solubility test gives the amount ofdegradation that the scaffolds undergowhen placed in liquid. It is a means ofestimating the mechanical strength of thescaffolds. The results of solubility test were analyzed through (Fig. 3). Glutaraldehyde cross-linked scaffolds showed less degradation and were lesssoluble compared to the native scaffolds.This correlates to the findings of researchdone by DJ Modulevsky et al. regardingthe strength of the scaffolds (4). Thesolubility of chitosan and glutaraldehyde& chitosan cross-linked scaffolds were lessthan the native scaffolds which proved theeffectiveness of the cross-linking.

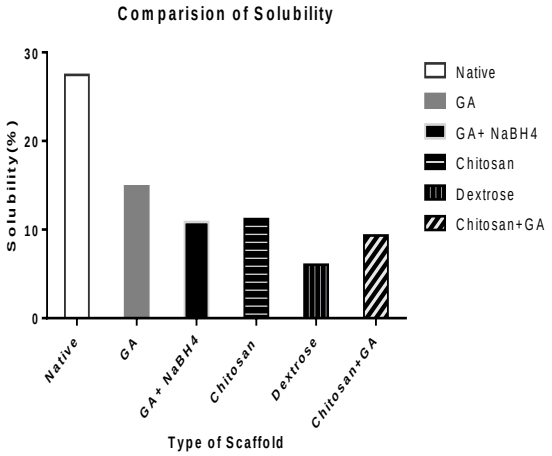

Fig. 3: Comparison of mean solubility of differenttype of scaffolds

3.3. FTIR Analysis

The results from the FTIR analysis (Fig. 4) showed that the decellularized nativescaffolds were indeed made up ofhemicellulose (a type of cellulose). The presence of the –OH stretching represents the characteristic of a cellulose structure. The results further showed the crosslinking of the hemicellulose with chitosan. The cross-linking with dextrose was notseen to be prominent but the chitosancross-linking was clearly seen. The peaksin the range 1046cm-1 denote C-C, C-Oand CH bonding in the cellulose which isnot seen in the chitosan cross-linkedscaffolds. Further the N-H peak at 1654-59cm-1 confirmed the crosslinking withchitosan.

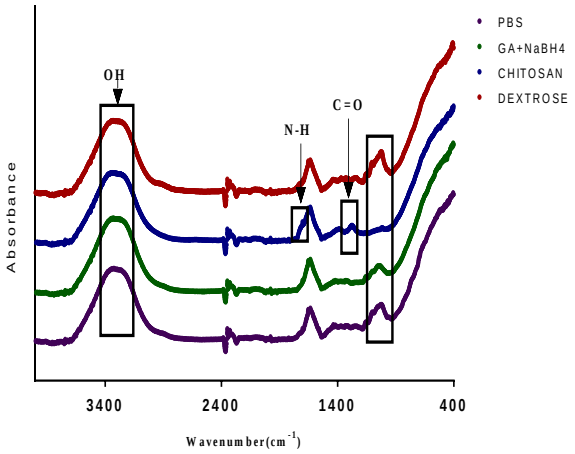

Fig. 4: Comparison of FTIR of various scaffolds

The FTIR graph obtained from the native scaffolds showed that the scaffolds were indeed made up of cellulose. The peaks seen after the cross-linking of chitosan were the most prominent and hence showed that the best cross linking was seen with chitosan. FTIR didn't show much variation in other cross-linkedscaffolds

3.4. Porosity

As the porosity of scaffolds influence thecell adherence and media intake in thescaffolds, it is an important considerationfactor. The porosity of the different type ofscaffolds were found using liquiddisplacement method. The result showed that the porosity decreased to certain extent after cross-linking though the difference was not as significant (Fig. 5). Furthermore, the scaffolds were examined under inverted microscope which showed the air pockets and the pores in differently prepared scaffolds. So, with cross linking,porosity was tried to be manipulated. The results showed that the cross-linking with chitosan and glutaraldehyde decreased the porosity though not significantly, but the dextrose cross-linking had negligible effect on porosity and hence proved the effectiveness of cross-linking in celladherence and nutrient uptake.

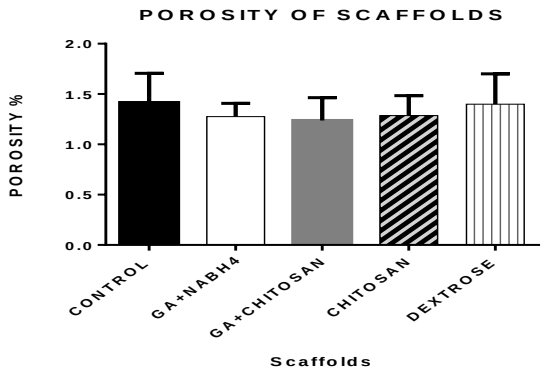

Fig. 5: Comparison of porosities of different types of scaffolds

3.5. Cumulative Drug Release

The calibration curve (Fig. 6) was plottedusing Graph Pad Prism 6 software with thehelp of the absorbance reading of differentconcentration of gentamicin solution inPBS at 256 nm.

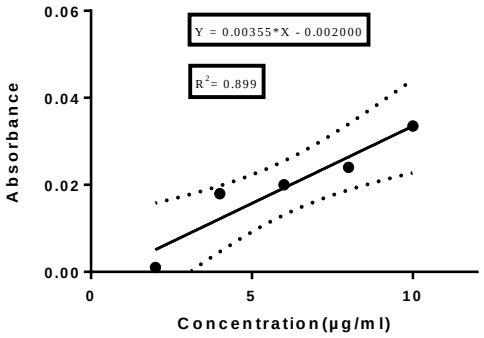

Fig. 6: Calibration Curve of Gentamicin in PBS

The cumulative drug release test was doneto evaluate the drug release rate andcapacity of different scaffolds. Thecumulative drug release test gave someidea about the use of scaffolds to deliverdrug in-vivo in addition to substrate forcell proliferation. It also gave an overviewof the mechanical structure of the scaffold.All the scaffolds showed good andsustained drug release. The scaffoldscross-linked with chitosan and bothglutaraldehyde and chitosan showed a higher rate of drug release and this correlates to the increased swelling of thescaffolds. The scaffolds cross-linked withglutaraldehyde being rigid, had noabsorbing ability and showed rapid drugrelease compared to native scaffolds. Thedrug release test was analyzed through (Fig.7).

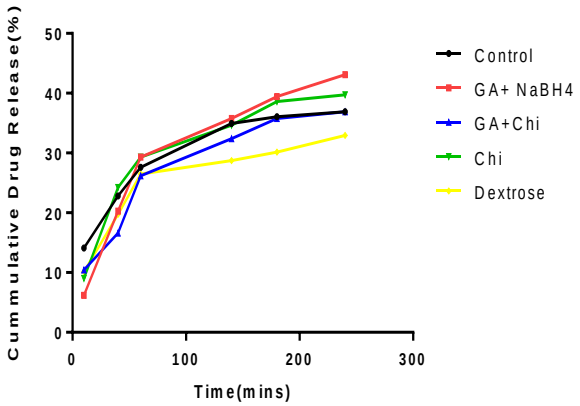

Fig. 7: Cumulative Drug Release of different scaffolds with time

3.6. Cell Proliferation

MTT assay was used to determine the cell proliferation in different cross-linked scaffolds. The different scaffolds which were seeded with cells prior 1, 3and 5 days showed increasing proliferationof cells with time. The absorbance readingshowed that the cell proliferation in everyscaffold increased with time. The difference in proliferation was not thatsignificant between 1 and 3 days in any ofthe scaffold types but the proliferation increased by a considerable margin in the fifth day as shown by the absorbance reading. There was significant difference between the absorbance reading between 1& 5 days and 3 & 5 days in the chitosancross-linked scaffold samples which was shown by the star ($p<0.05$) in Fig. 8.

When comparing absorbance in different scaffolds in different days, the results showed that the dextrose cross-linked sample had more cell proliferation compared to other scaffolds but the difference between the scaffolds was not that significant after 1 day of cell seeding.The results from the third day showed thecells proliferated more in every scaffold but still the dextrose cross-linked scaffold showed more absorbance. On Day 5, the results showed that the absorbance reading of

chitosan cross-linked scaffold was the highest followed by dextrose cross-linked scaffold and glutaraldehyde & chitosan cross-linked scaffold. Comparing theresults with negative control, all thescaffolds showed cell proliferation, but the most significant difference was shown by the scaffolds cross-linked with chitosan. After comparing the results by performing ANOVA test, the significant difference was found and denoted by a star ($p<0.05$) in the Fig. 9.

After the recellularization of different scaffolds with HeLa cells, the degree of proliferation of the cells in each type of scaffold was determined using MTT assaywhich showed that the scaffolds with chitosan cross-linking showed maximum proliferation after 5 days. The proliferation of cells in the scaffolds which were crosslinked with either glutaraldehyde or with both glutaraldehyde and chitosan was comparatively low. This might be due tothe fact that glutaraldehyde is cytotoxicand even when precautions were taken toreduce the excess glutaraldehyde, the toxicity is due to glutaraldehyde in the surface of the scaffolds (8)(9). Comparedto the negative control sample, all thecross-linked scaffolds showed increasedcell proliferation and the proliferation ofcells was found to increase with time.

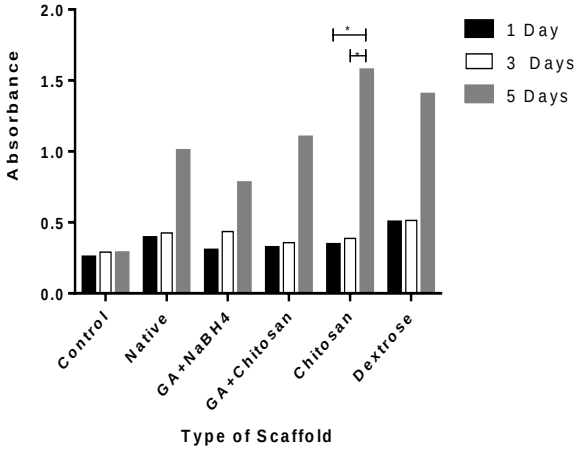

Fig. 8: Comparison of cell proliferation through absorbance reading after 1,3 and 5 days of cell seeding.

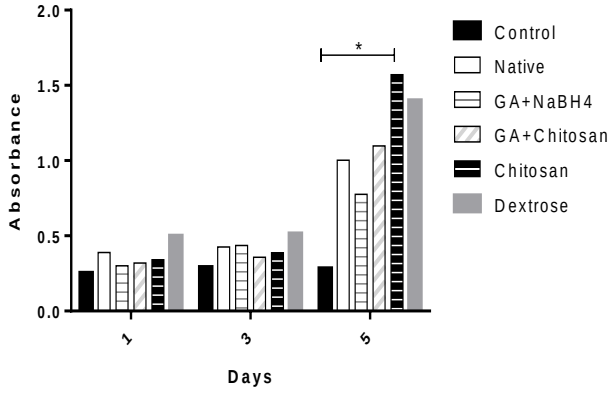

Fig. 9: Comparison of absorbance in various types of scaffolds after 1 day, 3days and 5 days of cell seeding.

4.CONCLUSION

Scaffolds work as a template for theregeneration of tissue that providesguidance for the growth of new tissue. Themajor determining factor for the suitabilityof a scaffold for use in tissue engineeringare the biocompatibility, biodegradability,consistent mechanical property and porousarchitecture. Ideally, the scaffold should beconsistent as well as it must be strongenough to allow surgical handling duringimplantation. The composition of scaffoldalso plays an important part in determiningit's use in tissue engineering.

The result presented in this study gives anoverview about the use of decellularizedapple cellulose scaffolds as a means for 3Dcell culture. It also gives an idea about theeffect of cross-linking on the cellulosescaffolds. The process of cross-linkingprovides a greater depth to the study anduse of the cellulose scaffolds in tissue engineering. The result also highlights thedegree of proliferation of cells withindifferent scaffolds and provides a correlation between their mechanicalproperties and their effect on cellproliferation.

During the decellularization of the applesamples, it was found that the species ofapple and the time the apples are left tochill in the freezer also played animportant role. Red Thai Fuji apples werethe ones that showed most degree ofdecellularization when compared to

otherFuji apples. Also, the best storage timebefore preparation of samples in thefreezer was found to be 9 days. The appleswhich were taken out of the freezer forsample preparation before that timeshowed less decellularization in the sameconditions. Also, the rate of shaking waskept at 160 rpm which was found to bebest for preparing the scaffolds.

Cell culture of HepG2 cells require specialattention and special care as these cells arevery sensitive and slow growing. Thedoubling time of HepG2 cells is 48hours (10). HepG2 cell culture was notsuccessful in the laboratory for thisproject. This might be due tocontamination in the provided seed. Also, itwas found that the cells require the samecell culture medium as they werepreviously grown in for good proliferationrate and the use of DMEM instead ofEMEM might have played a role initiallybut the failure of cell culture even inEMEM strongly suggests contamination inthe seed provided by the supplier. Due tothis reason, the HepG2 cells were replacedby HeLa cells in the project.

Overall the scaffolds which were crosslinkedwith chitosan showed better resultsregarding cell proliferation in long term and they also had significantly better mechanical properties. The presence of glutaraldehyde in the scaffolds increased their rigidity, thus decreasing their water uptake which directly affected the cell proliferation. Also, the cytotoxicity of glutaraldehyde played an important role in decreasing the cell viability in thesescaffolds. But the scaffolds which were cross-linked with both glutaraldehyde and chitosan showed less toxicity towards the cells compared to glutaraldehyde cross-linked scaffolds and showed more cell proliferation. This could be because of chitosan which enhanced the bio stability and decreased the cytotoxic effect of glutaraldehyde. The results also showed that the dextrose cross-linking had no significant effect on the swelling and solubility of the scaffolds but since the dextrose used was in limited concentration it showed good cell proliferation. So, by controlling the surface biochemistry and mechanical properties by cross linking the scaffolds, the cell proliferation can be manipulated to obtain optimum result.

All these results proved that cross linking of decellularized scaffolds with suitable agents yield better cell proliferation. So, further studies can be conducted with other cross-linking agents such as collagen to study the cell proliferation and mechanical properties in long term. Furthermore, recellularization could be enhanced with cells which have the property to proliferate in 3D microenvironment.

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