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Biodiversity

Biomechanical Assessment of the Aggregate Encapsulated Agarose Gels for Production of Efficient Bone and Cartilage Mini Tissues

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Abstract | **Original Research Article**

The potential of the scaffolds to make the cells to differentiate into a higher level of maturation is an essential factor. The high-density culture methods such as cell aggregation are found to improve the maturation of the osteochondral bioengineered tissues. In this research, we utilized the cell aggregation of rat mesenchymal stem cells in soft and stiff agarose concentration for assessment of the osteogenic and chondrogenic differentiation in the static and dynamic environment. The displacement of aggregates was tracked via Optical Coherence Elastography in the presence of hydrostatic force while the overall young's modulus of the tissues was assessed via conventional mechanical testing. It was found that the usage of 4% of agarose and application of dynamic shear force to the cells could improve the stiffness and the glycosaminoglycan content of the bioengineered tissues. This study showed that the stiffness of the aggregate can be dependent on the size of the aggregate particles. The increase in mechanical properties of the aggregates, higher expression of glycosaminoglycan and the stiffness of the engineered tissues can be regarded as signs of maturity.

Keywords: biomechanical analysis, optical coherence elastography, bone, cartilage, tissue engineering, aggregates.

INTRODUCTION

Between the musculoskeletal conditions, Joint diseases such as osteoarthritis (OA) are known as the most prevalent type of musculoskeletal disease [1]. While the autografts are regarded as gold standard for tissue engineering based treatment approaches for musculoskeletal conditions, there are several complications associated with usage of autografts. A high number of cells for treatment and the need for the extra surgery [2, 3] are the examples of these complications. As a result, usage of allografts for graft-based bone and cartilage treatment is desirable. However, one of the most important problems associated with the usage of allografts is the compromised potential of these type of grafts for osteogenic and chondrogenic maturation [4]. This effect is more obvious in osteogenic treatment where the osteoinduction of the graft is reduced when allografts are used [5]. The maturation problem should be addressed by engineering better tissue-like structures.

Mesenchymal Stem Cells (MSCs) are found a good source of osteo/chondrogenic differentiation. MSCs show better differentiation potential compared to chondrocytes or osteoblast [6]. MSCs are multipotent stem cells and are less subjective to ethical issues [7]. Unlike embryonic stem cells (ESCs), MSCs are not subjective to tumorigenesis [7]. The anti-inflammatory response of MSCs promotes their biocompatibility and their usage in bone and cartilage grafts [8]. However, the problem of tissue maturation is still seen in both osteogenic and chondrogenic treatment of the MSCs.

High-density culture system is one of the methods that have been used for increasing the rate of cell differentiation and improving the level of maturation in osteocytes and chondrocytes [9-11]. Aggregate culture is one of the methods of high-density culture that is found to have a great impact on the differentiation of osteocytes [11, 12] and chondrocytes [13]. A study, by Deegan et al. (2014) showed aggregation of osteoblasts pushed the cells toward higher levels of differentiation and mineralization. A similar study in Fibrin- poly (lactide-co-caprolactone) scaffold and using BMSCs caused the cells to differentiate into better cartilage-like tissues [14], however, the size of aggregates in this study was not controlled. Yet, there have been no studies about the effect of high-density aggregate culture and the size of aggregates on osteogenic and chondrogenic differentiation of the MSCs. We expect that the

application of MSCs aggregates in a scaffold can push the cells toward further maturation. Additionally, we expect that the size of aggregates, the application of the loading regime and the stiffness of the scaffolds can impact the quality of the tissues and the level of their differentiation. The differentiation of the chondrocytes in the bone and tissue cartilage increases the mechanical properties of the tissue by their maturation and start producing matrix in their substrate [11], tracking the mechanical properties of the cell aggregate, as well as the mechanical properties of engineered tissue, can be a good indication of the tissue maturity.

For measuring the stiffness of hard materials such as bone, usage of conventional ultra-sound technics is popular. This method can measure the stiffness and density of the targeted biomaterial [15-17]. Ultrasound is accurate when it is used for dense and stiff materials, however, when it gets to the looser materials the accuracy reduces. Application of mechanical compression for studying the properties of bone tissues such as cancellous [18] or cortical bone [19] has been reported. This method can measure the mechanical properties by manual or preprogrammed stress cycles [20] and can get a good picture of the overall mechanical properties of the biomaterial. Measurement of the mechanical properties via compression reaches its highest accuracy when the biomaterial is absolutely homogeneous in terms of mechanical properties [21]. The inhomogeneity can even arise from the difference in the shape of the biomaterial. Consequently, studies try to use the similar shapes of the materials for this approach which in case of measuring the tissues taken from the in-vivo model it is challenging. However, for measurement of stiffness in inhomogeneous materials (such as cancellous bone) where the mechanical properties of the material vary from one place to another, the compression analysis is not efficient. As a result, non-invasive elastography technics are used for precise measurement of displacements. These technics utilize an external stimulus with an accurate system of detection to graph the mechanical properties of the targeted object. The usage of Magnetic Resonance Elastography (MRE) has been reported for the measurement of displacements in a tissue based on phase contrast magnetic imaging [22]. The measurements are done in the presence of acoustic waves in tissue [22]. However, the contrast is a challenge for this method which is dependent on environmental conditions and properties of the material. Ultrasound elastography is suggested for clinical usage and measurement of tissues such as cancerous tissues [23, 24]. An important problem associated with the usage of MRE and UE is their limited spatial resolving of these technics which keeps these approaches for the usage in macroscopical level [25]. As a result, Atomic Force Microscopy Elastography (AFME) has been used as a method that encompasses a high resolution [26]. This method is precise but the limitation is the field of view which makes this technic unable to measure the materials more than 10^{-5} mm and limits the usage of AFME mostly for the cell culture approaches [25]. As a result, there is a gap between the AFME and MRE/UE method for the measurement of objects with the scales between the ones supported by these approaches. The Optical Coherent Tomography (OCT) technic can address this gap.

OCT is a precise technic with microscale spatial resolution which is used clinically in ophthalmology and cardiology [27]. Usage of this technic alongside with an external stimulus is called Optical Coherent Elastography (OCE). This method was first introduced by Schmitt to assess the biomechanical deformation by localized measurement of displacement inside the samples. The early measurements of the displacements in this technic were done with inaccurate methods such as pixel-wise tracking of the displacement over the time to track the measurement of the object [29, 30]. However, the advances in mathematical concepts bring this ability to utilize the informetric phase-resolved signals to measure the displacements of the objects with nanoscale sensitivity [31]. This revolution made the OCE to be listed among one of the most precise technics for the measurement of mechanical properties of objects in-vivo as well as in-vitro.

For tracking the mechanical properties of the engineered tissues, we first designed a novel hydrostatic bioreactor platform that has this ability to be joined with an OCT platform to measure the mechanical properties of MSC seeded aggregates in chondrogenic and osteogenic treatment in both static and dynamic culture conditions and tracked the mechanical properties of the aggregates after a 28-day period of culture to find out the efficacy of the high density aggregate culture in both osteogenic and chondrogenic treatment.

MATERIALS AND METHODS

Experimental Design

We intended to study the osteogenic and chondrogenic aggregate seeded tissues in the soft and stiff substrate in the presence of mechanical force (shear stress) or static culture over a 28-day culture period. For this purpose, Rat MSCs aggregates were seeded in 2 and 4% agarose in Osteogenic (OM) and Chondrogenic Media (CM) at 37 degrees and 5% CO2 for 28 days. The 4% Isolated cell seeded (SCs), as well as 2 and 4% acellularized (Acell) gel batch, were considered as experimental controls. All the samples were cultured for five days in differentiation media, after 5 days of culture the experimental plan has been performed to the structures. The shear stress was only applied to aggregate seeded structures from day 10 until day 28. The samples were subjected to compression mechanical analysis, OCT imaging, biochemical analysis and image analysis for finding out the size and properties of the aggregates every 15 days. The image analysis of the

tissues was done in the day after the formation of tissues as well as day 0 and day 28 of the experiment.

Cell preparation and maintenance

Rat MSCs have been isolated from a 30-day old male mouse femur and cultured in Corning™ Dulbecco's Modified Eagle's Medium (DMEM) with L-Glutamine with 4.5g/L Glucose and Sodium Pyruvate (Corning; Type MT10013CV) that was supplemented with 10% of FBS, 1% Penicillin/Streptomycin (P/S) and 1% of Non-Essential Amino Acid (NEAA). This formulation was considered as proliferation media (PM). The cells were kept at 37 degrees and 5% of CO2 and the media change were done regularly. The cells where passaged whenever the confluency reached 70 – 80 percent.

Aggregate formation

Flasks of Rat MSCs were utilized at 80 - 90% confluency. The cells were digested using 1X trypsin. The amount of 0.25 million cells per well from the resulting cell solution was distributed inside the wells of a Corning® Costar® Ultra-Low Attachment 24 Well Plate in a sterile environment. The aggregate formation pattern and the morphology of the aggregates were tracked every 4 hours with the Olympus U-CMAD3 series microscope and images captured from the surface of Ultra-Low attachment plate using Micropublisher 5.0 RTV camera (Q-Imaging).

Agarose encapsulation

4 and 8% (w/v) of low gelling agarose (Sigma; Type A9045-5G) dissolved in a sterile PBS. Gel solution was kept at 80 degrees oven for 12 hours beforehand to let all the agarose particles dissolve in PBS and sterilized via UV.

The cells in Ultra-low attachment plates that were incubated for 24 hours have been used for agarose seeding (Figure 2-1). All the aggregates were caught from the surface of ultra-low attachment plate (0.25 million cells) and mixed homogeneously in 1:1 sterile agarose to aggregate solution ratio to make a final concentration of 2% and 4% cell/agarose solution. The cell/agarose solution was added to the mold and let the solution solidify inside the syringe for 30 mins prior to cutting (Figure 2.1, D). The structures were cut precisely with a diameter of 4.05 mm and a depth of 0.1 inches and 250,000 calls per structure.

Apart from aggregates batch the isolated cells have been encapsulated in agarose by direct digestion of cells in T75 flask without incubation in ultra-low attachment plate and mixing the cell solution with 8% agarose gel homogeneously in 1:1 cell to agarose to make structures with a final concentration of 4%, a diameter of 4.05 mm, depth of 0.1 inches and 250,000 calls per structure. The acellularized batch was made by 1:1 ratio addition of PM to sterile agarose solution. Macroscopic and microscopic images were taken from the structure for confirmation of homogeneous cells distribution in agarose gels and assessment of the aggregate size via image analysis.

Osteogenic and Chondrogenic culture

2 and 4% cell aggregate seeded structures were transferred to 24 well plates containing Osteogenic Media (OM) composed of DMEM (ThermoFisher Scientific) supplemented with 1% P.S., 1%NEAA, 10% FBS, L-glutamine, 4.5g/L glucose 100 nM Dexamethasone, 50 µM of 2-Phospho-L-ascorbic acid and 10mM of β-glycerophosphate and Chondrogenic Media (CM) composed of 1% ITS (25µg/ml transferrin, 25µg/ml insulin and 25µg/ml sodium selenite), 1% Sodium Pyruvate, 100 nM of Dexamethasone, 50 µg/ml ascorbic acid, 40ug/ml L-proline and 10 ng/ml of human TGF-β3 (Prospec-Tany Technogene Ltd; Type CYT-368). The cells were kept at 37 degrees and 5% CO2.

Shear Stress stimulation

The agarose structures have been mechanically stimulated using a shaking Racker instrument with the amount of 30 cycles of shear stress stimulation per minute from the 10^{th} day of the culture in differentiation media for one hour, in a daily basis at 37 degrees and 5% CO2 inside the environment of the incubator. The unloaded batch was kept in the same environment while the shear loaded batch being stimulated.

Analysis of the number and the size of aggregates

The number of aggregate colonies and the size of the aggregates were analyzed using ImageJ software (NCBI). A test of significance with an alpha value of significance = 0.05 was performed between 2 and 4% groups using Mann-Whitney test with IBM SPSS Version 24 Software.

Optical Coherence Elastography

A 50Kpa pressure was applied to the structures prior to OCT imaging. The samples were placed inside the culture well plates and placed inside the chamber. The 50Kpa pressure was applied to the agarose gels inside the chamber. The force generated a gas-liquid interface with the media topped up the agarose gels, creating a force to the gels which cause a displacement inside in agarose gel based on mechanical properties of the components.

The 1024 x 1024 pixels pictures were taken from the surface of agarose gels with optical coherence tomography imaging system TELESTO-II (Thorlabs) with the center of the wavelength of 1310nm and LMSO4 platform focus scan lenses (Thorlabs), while the hydrostatic pressure bioreactor was working. All the images were taken from doppler mode. A total number of 200 images were taken from the samples with 48

successive B-scans and over 2 successive averaging A-scans with a scanning rate of 24 kHz.

Each period of pressure change was taken as one pressure alteration cycle. The OCT images taken with the designed system were evaluated in terms of the number of pixels correlated with the aggregates in four frames in a pressure alteration cycle using ImageJ software platform and the mod value of the time points were used as size change in one pressure alteration cycle. The calculation of displacement was done in four low-pressure cycles and four high-pressure cycles.

The differences of the pixels between low and the high-pressure cycle were measured as a function of the aggregates modulus and plotted against their size.

Mechanical Compression Testing

Samples were immersed in phosphate buffer saline for 1 hour to let the previous media clear out from the gels. General mechanical properties of the gels were determined by conventional mechanical testing using ElectroForce® 3200 Series (Bose Cooperation) mechanical compressor testing system. The amount of stress resulted from compression was tracked by Wintest® Digital controlling system (Bose Cooperation) until 20% of strain with a 2 – 20 KPa load cell with pre-loading of 0.01N. The Young's modulus was calculated by considering the linear part of the stress VS strain plot of the specimens.

Cell Digestion

The wet weight of the samples that were allocated for biochemical testing was evaluated prior to analysis. The gels were kept overnight at -6 mBar and -4 degrees overnight in freeze dryer environment to let all the water content to evaporate. The samples were treated with 0.3ml of 0.5mM papain enzyme solution (Papaya latex; Type P4762) diluted in phosphate buffer containing 1mM EDTA for 5 minutes. The samples were crushed until gaining a homogeneous solution of agarose and papain. The solution was placed inside the 60 degrees oven and was vortexed once 20 a minute for the first 5 hours. The solution was placed at 60 degrees continuously for the next 13 hours for letting the Papain enzyme dilute the enzyme solution. The specimens were centrifuged, and the supernatant was used as a cell extract for biochemical analysis.

DNA and GAG analysis

Samples for biochemical testing were evaluated for the amount of sulfated GAG using DMMB protocol [32] as well as evaluation of Double-Strand DNA content in samples using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen; Type P7589). The samples absorption was read by BioTeK Synergy™ 2 Series Plate Reader (BioTek Instruments Inc). The amount of µg/g gel Wet Weight which was normalized based on DNA content was calculated for evaluation of GAG content of gels.

STATISTICAL ANALYSIS

The values gained from the experiments were statistically analyzed using IBM SPSS Statistics Version 24. A one-way analysis of significance between the samples was performed with the alpha value of significance = 0.05 using Tuckey's test. The variations between the samples have been reported as ± Standard Error (±SE).

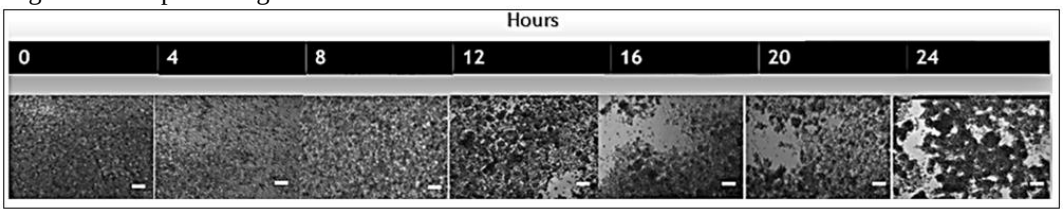

Fig-1: The morphological comparison between the Rat MSCs seeded in Ultra Low Attachment plate (ULAP) in a 24-hour period. each scale bar indicates 150µm

RESULTS

The condensation of the aggregates over a 24-hour culture treatment

Images from 24 hours of tracking the cells in ULAP showed that the aggregates on the surface of ultra-low attachment plate became concentrated by time (figure 1). Additionally, the cells from time 0 started joining together and turning from amorphous structure to circular and condense structures by the time. The number of single cells present on the surface of ULAP decreased over time and the size of aggregates reached about 150µm by 24 hours.

Generally, a mean value of 177.8± 5.7 (n=33) for the OM treatment and 152.15± 5.5 (n=24) resulted from the average number of aggregates seeded in structures (±SEM) The average diameter of the MSCs used for the ULAP was 32.085 ±0.71 (±SEM, n = 178).

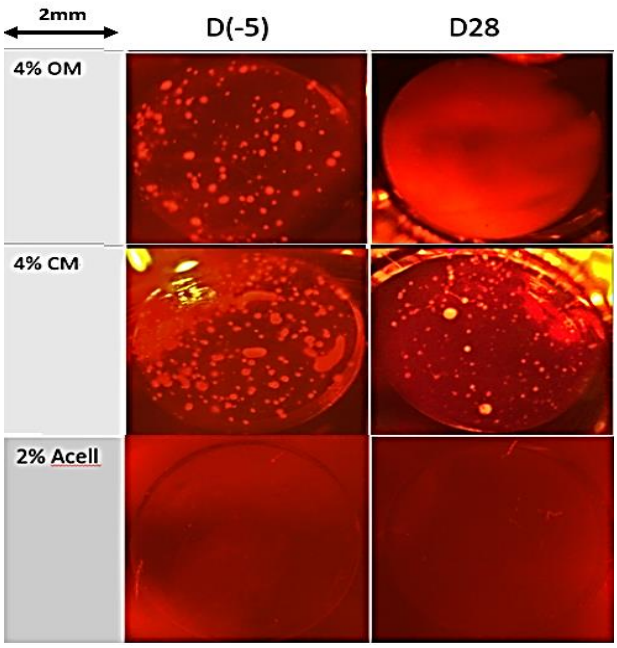

Fig-2: The macroscopic images of the whole mini-tissue structure. The OM treatment showed extensive sedimentation in all the samples. This effect was not seen in either CM or acellular batch. D(-5) is an indication of the time of gel encapsulation. The concentration and mechanical stimulation during culture period showed similar results in two batches. Consequently, only 4% loaded group was chosen as representation

The morphology of the aggregates

Extensive sedimentation occurred in the OM treatment (Figure 2). This sedimentation appeared in day 15 of culture and it became intensified over the time and distributed inside the OM gels, making the macroscopic camera unable to take detailed macroscopic images from the aggregates (Figure 2). As a result, the pictures from day 28 of OM treatment were taken with changed light settings (Figure 2). The single cell seeded batch showed extensive sedimentation in OM treatment after the 10^{th} day of culture. However, CM treatment didn't change the single cell batch macroscopically. The acellular batch didn't show any changes after 14 days of culture (Figure 2).

A significant reduction in the size of aggregates was seen in both OM and CM treatment

The microscopic inspection of cells over time showed a smaller aggregate size in OM treatment after 28 days (Figure 3). The image analysis based on microscopic images showed a significant decrease in the size of the aggregates for OM (p=0.000) and CM treatment (p=0.020) using Tuckey's analysis of significance.

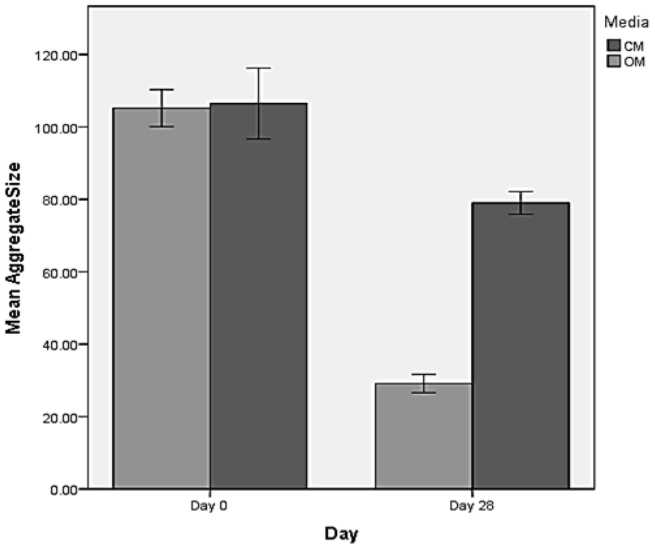

Fig-3: The Image analysis of aggregates shows a significant change in the size of the aggregates in OM (p=0.000) and CM (p=0.020)

Detection of a GAP in OM treatment between aggregates and the Gel

The OM treated aggregate seeded structures showed smaller aggregates compared to the CM-treated samples. Additionally, a gap was detected between the aggregates and agarose gels after 28 days of treatment which the same was not detected in CM (Figure 4). The effect of the gels shading in OM treatment was observed in OCT images of the OM group as well.

The Aggregates showed the highest mechanical properties in a specific size range

The plotting of modulus against the size of the aggregates shows two different patterns (Figure-1). The chondrogenic treatment shows the highest mechanical properties when the aggregates are between the range of 10 to 20 pixels in diameter (0.6 to 0.15 mm). While this pattern is different in OM as the peak in mechanical properties was seen in the range between 0.03 to 0.07 mm in diameter. Ultimate increase in the size of both OM and CM treatment causes a drastic decrease in mechanical properties. Cculture under shear stress ell didn't affect the mechanical properties of the aggregates significantly, however, the OM treatment caused the average of the modulus to increase significantly in the aggregates.

Alterations in mechanical properties of mini-estissu

The Young's modulus of the all aggregate seeded gels on day 28 except 2% OM increased compared to D0 of the experimental plan. However, no significance was found between the groups (Figure 6). The groups treated under shear stress showed higher mechanical properties compared to those that were cultured under the static regime. In 2% OM treatment, the mechanical properties of the gels were compromised after 28 days. Isolated cells encapsulation didn't affect the mechanical properties of the gels significantly, but a significant reduction ($p=0.001$) was seen in the mechanical properties compared to acellular gels as soon as the aggregates were seeded in the gels (Figure 6 A, D).

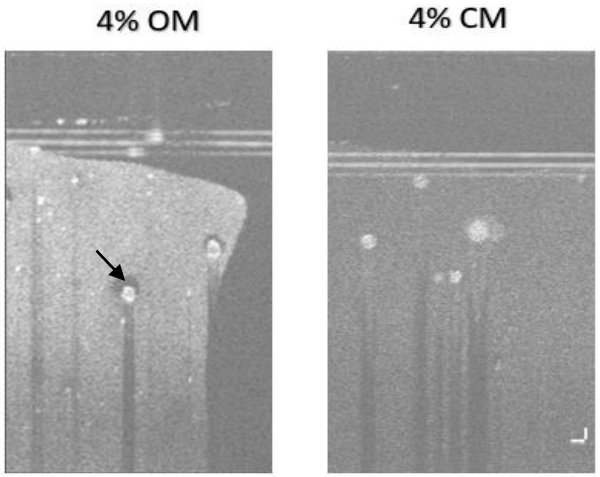

Fig-4: The comparison of OCT images taken from day 28 samples. Only 4% loaded group was chosen as representation. it was found that aggregates in OM treatment are found in small holes (indicated with an arrow) which the same was not seen in the CM group

Tracking the mechanical properties of the batches showed that there was an increase in stiffness of the gels after a reduction in the 15th day of the cell culture (Figure 6 E-G). It was found that although the increasing trend in mechanical properties of materials in both OM and CM treatment was found, the overall mechanical properties of OM was significantly lower than the mechanical properties of CM treatment (Figure 6, E). The gels treated with a high concentration of agarose showed higher speed in increasing the mechanical properties. (Figure 6, F). Also, the shear loaded group showed faster growth in Young's modulus over the static group (Figure 6, G).

A Significant Glycosaminoglycan secretion after the culture treatment

The results from considering the amount of normalized glycosaminoglycan (GAG) content from different dimensions showed a significant amount of GAG secretion by the day 28 in the samples that were under treatment of 4% agarose concentration ($p = 0.003$), static culture ($p = 0.004$) and CM treatment ($p = 0.021$; Figure 7). Performance of shear stress caused an increase in the amount of normalized GAG content only in the first five days of culture, and results from consideration of shear stress in day 28 of culture showed static regime with a higher level of GAG secretion especially in CM treatment. The separate comparison of the experimental groups showed 4% CM expressed the highest amount of GAG in day 28 and 2% OM comprised the lowest secretion of GAG over time.

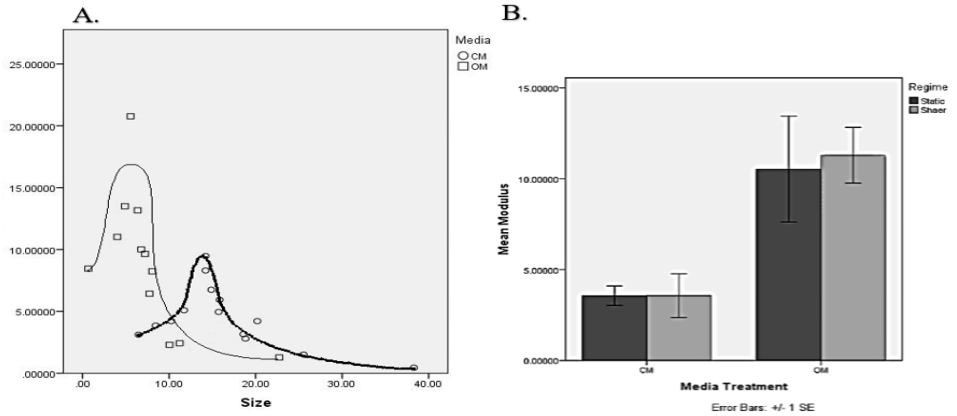

Fig-5 A: The mechanical properties of the aggregates follow a normal distribution which has different patterns in osteogenic and chondrogenic media. The aggregates in 4% OM treatment have a high level of mechanical properties in the range between 0.03 to 0.07 mm in diameter. The 4% CM treatment aggregates within the range between 0.05 to 0.15 mm diameters show the highest mechanical properties. This data is further confirmed in 2% CM gels where the aggregates show the highest mechanical properties between the range of 0.05 to 0.15 mm in diameter. B. shear stress in CM didn't affect the mechanical properties of aggregates significantly, however, the OM treatment considerably increased the mechanical properties of aggregates.

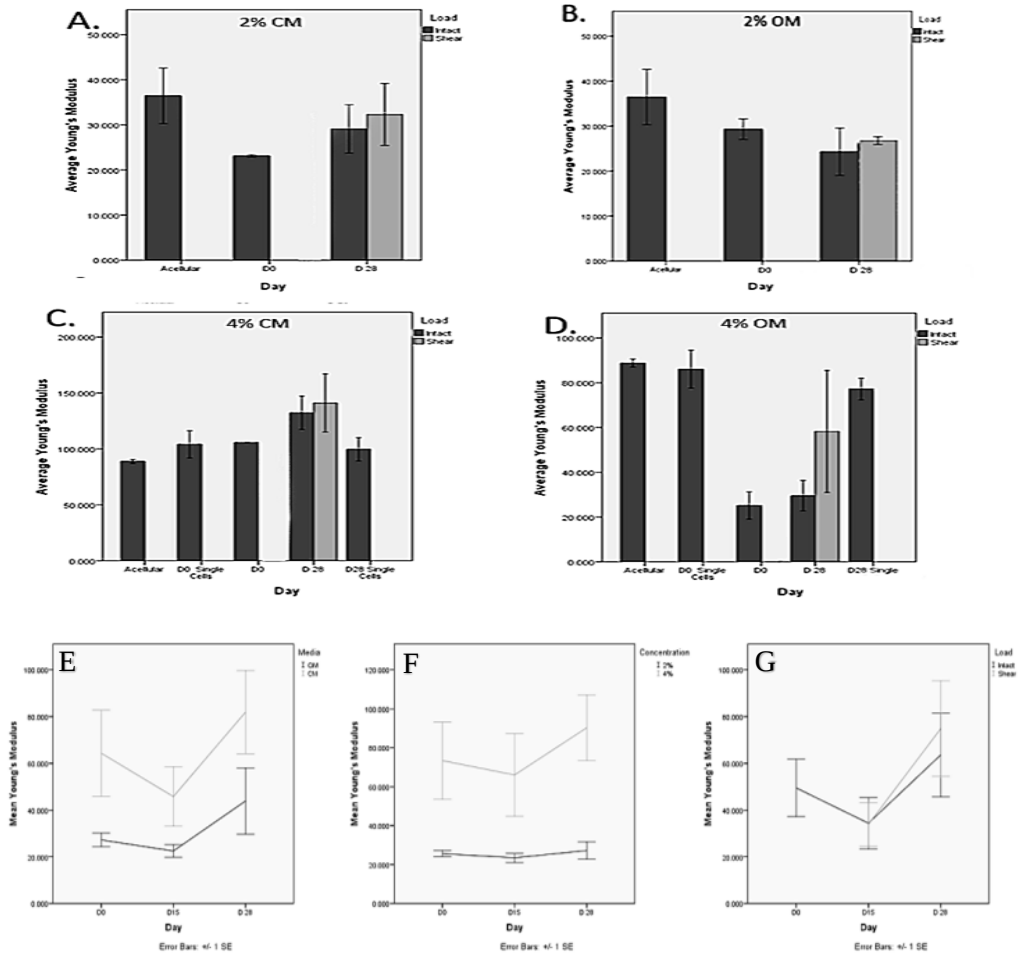

Fig-6: The mechanical properties comparison between different experimental groups, A. 2% CM showed a significant decrease (p=0.010) in mechanical properties in day 0 of the experiment. However, the increasing trend of mechanical properties was seen over time. B. 2% OM which showed reduction after cell seeding in day 0 of culture and the mechanical properties decreased over time. C. 4% CM showed an increase in mechanical properties over time and it is the only group that didn't show any reduction after encapsulation. D. 4% OM which showed a significant reduction in mechanical properties after gel encapsulation however, the mechanical properties showed an increasing trend over time. E the effect of media to mechanical properties, F. The effect of concentration on mechanical properties of gels, G. the effect of loading to mechanical properties of gels

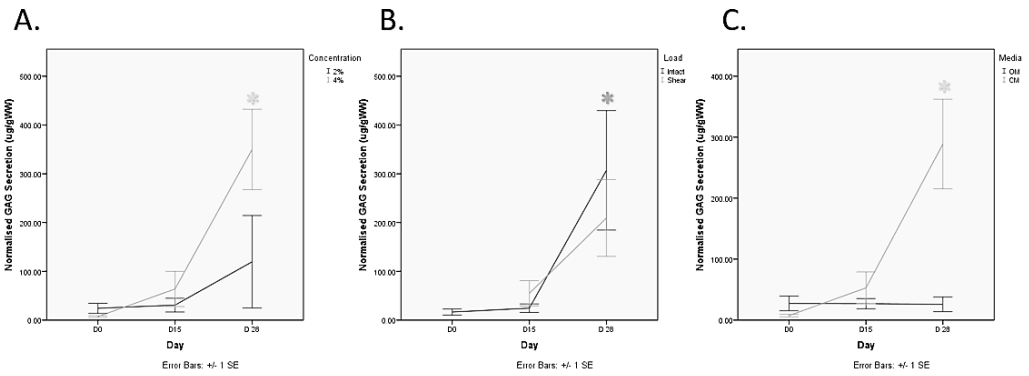

Fig-7: Alterations in normalized GAG expression of experimental groups from different dimensions, A. 4% concentration showed a significant rise in GAG expression of the gels (p = 0.003). B. Static regime showed higher GAG expression and a significant peak on day 28 (p=0.004), C. CM culture increased the GAG expression significantly (p=0.021)

DISCUSSION

We considered 2% and 4% of agarose for making gels. The 2% agarose was chosen as the most common concentration of agarose in other studies [6, 33, 34]. Since a healthy articular cartilage tissue comprises about 40KPa of Young's modulus 4% was chosen as a concentration that resembles the healthy cartilage [20]. The gels in the first 10 days of the experimental plan were cultured in a static environment, this regime made the MSCs acquire some levels of chondrogenic and osteogenic differentiation and express bone or cartilage phenotype. Extensive response to the stress is possibly one of these phenotypes [35]. Additionally, the 10-day gap can provide a period for the cells to generate their own matrix and integrate with their scaffold and as a result, make them more responsive to mechanical stress. Thorpe *et al*. [36, 37] recommended using 21 days of static culture prior to performing the stress regimes however, we hypothesized that the aggregation methods can enhance the rate of differentiation, as a result, a 10-day period was considered prior to the start of the mechanical load.

The sedimentation in OM gels can be the result of the cell secretions in the matrix

We hypothesized that the aggregation of MSCs enhances osteochondrogenic differentiation. The increase in the mechanical properties of the graft can be regarded as one of the successful signs of matrix deposition and maturation [38, 39]. The results showed intensive signs of sedimentation in OM treatment over time in macroscopic and OCT images. This deposition can be caused by Stimulated Body Fluid (SBF) effect which is reported to have the potential to cause mineralization deposition in scaffolds such as collagen and hyaluronan [40, 41]. For this reason, we tried to assess this possibility with the result of two experiments. First, the acellular group with OM treatment and same conditions didn't show any signs of mineralization after 28 days of culture (Figure 2). Secondly, an agarose gel with isolated cells was made inhomogeneously so in some part the tissue there was no MSCs present and in the other part, the cells were present. It was found that after 28 days of culture the location of sedimentation was around the cells and the area without cells didn't show any signs of deposition (Figure 8). This shows that this shading effect is probable to be caused by the cells inside of the tissue. However, the macroscopic and microscopic images taken from the structures still cannot confirm the matrix deposition of the cells. As a result, the biochemical testing and biomechanical analysis were used for further confirmation of this deposition. The mechanical data analysis could be a good indication of matrix secretion as we expected the mechanical properties of the gel should have increased over time.

Gels show a reduction in mechanical properties in the first 15 days which was followed by an increase in next 15 days of culture

Comparing the mechanical properties of real chondrocyte shows that 4% of agarose had similar mechanical properties as the real cartilage extracellular matrix (ECM) in-vivo. Blanco *et al*. [42] Have shown that an OA cartilage comprises about 25 Kpa young's modulus in the pericellular matrix. The experimental results showed that the application of 2% will cause Young's modulus about 30 Kpa in agarose gel at the first stages of differentiation which resembles OA cartilage mechanical properties.

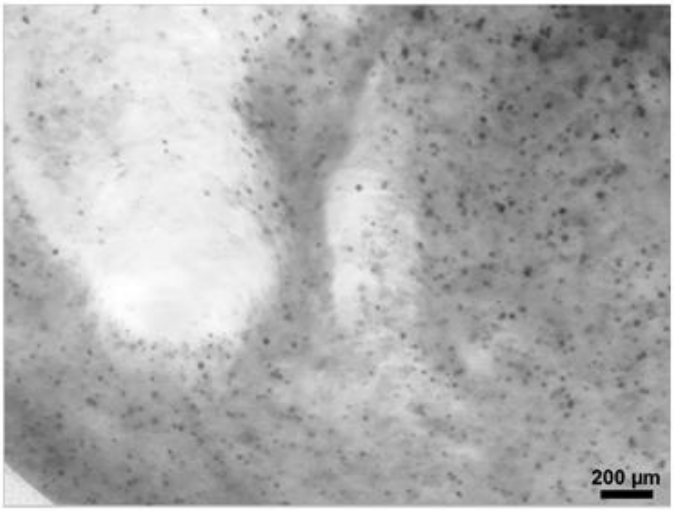

Fig-8: Inhomogeneous agarose cell seeding caused the inhomogeneous deposition of the matrix

The application of shear stress in CM treatment was able to affect the GAG secretion. Usage of shear stress can modulate the further activation of the TGF-β signaling pathway. This pathway can lead to progression either Smad1/5/8 (hypertrophy) or Smad2/3 (differentiation stopping) pathways and affect the quality of chondrogenesis [43, 44]. Maturated chondrocytes respond to the higher level of differentiation by increasing the GAG content and its secretion into the substrate. The agarose seeded tissues showed higher GAG content by application of shear stress in day 15 of the experiment. However, in 4% sample, after a peak in day 15, the shear loaded structures showed themselves lower in terms of normalized GAG content in day 28. Two explanations can be proposed for the reason for this happening. The 4% loaded group is shown to have more signs of differentiation and possibly higher condensation over other groups. It is possible that this condensation causes a limitation in the diffusion of media, making the inner cells unable to be exposed to further TGF- β3 molecules. The other possibility is based on the experiments by Thorpe [39] where he found in his study that application of loading in the presence of TGF- β may inhibit the chondrogenesis and activate the Smad2/3 molecules. Thorpe suggests either further optimization for understanding the appropriate amount of TGF- β or simply exclusion of the TGF- β from the culture before reaching to plateau phase of differentiation (in this case of study after 15 days).

The size of the aggregates affects the degree of maturity

The displacement analysis showed that the mechanical properties of the aggregates are dependent on the size of aggregates. Both CM and OM culture showed the highest amount of mechanical properties in a specific range. Additionally, OM treatment caused a significant increase in aggregates mechanical properties. This may imply that the utilization of the aggregate cell culture was successful in mineralization of aggregates with a certain size. In a study by Deegan *et al*. [11], it was shown that the bigger aggregate could show higher mechanical properties in CM treatment. This study confirms the results of the Deegan et al. and further adds this point increasing the size of aggregates after a certain level causes a significant decrease in mechanical properties in both OM and CM treatment. The aggregates in OM treatment should be in a smaller size to have the optimal mechanical properties compared to CM treatment.

Alterations in the mechanical properties of the aggregates cause defects in biomaterials

The data from mechanical properties after 5 days of agarose encapsulation (Day 0) in OM treatment showed a significant reduction, although the mechanical properties of the gels rose over time. The lower mechanical properties of OM treatment can be explained by the holes that were generated and detected in both wax embedded samples and OCT images. The disturbing effect of the holes which generate defects inside the biomaterial may explain the significant reduction in the mechanical properties of gels, especially after cell seeding. The defects can be as the result of the changes in biomechanical properties of the cell aggregates which get stiffer and consequently, dissociate from the substrate. As the cells don't produce any connection with agarose, they will remain in chambers surrounded by the substrate. The disturbing effect of defects can cause high variations of samples for mechanical testing and it can be the cause of lower mechanical properties of the initial phase of the differentiation. While in the first stages of the differentiation the generated defects have affected the agarose and compromised the stiffness of the biomaterial, in the later stages, further differentiation of the cells and production of the matrix increased the stiffness of the mini tissues. The defects in the OM treatment was bigger as there was more reduction in the size of the aggregates in OM treated aggregates compared to CM treated samples (Figure 3). This

excessive defect in OM batches can be the result of lower Young's modulus of OM gels compared to CM gels in terms of stiffness.

Inhomogeneity of the aggregates mechanical properties will cause the appearance of aggregates with different level of maturation

Deegan *et al*. [11] have proved that the level of differentiation in osteoblasts in monolayer culture is dependent on the size of aggregation. They postulated that in monolayer culture the cells will remain between the proliferation and early matrix maturation stage while the bigger aggregates will contain a higher level of maturation and they can push the cells toward mineralization. This study showed that aggregates within certain size showed the highest level of mechanical properties. With this in mind, we can consider different stages for the aggregates with various sizes in the same substrate. As the size of aggregates within the agarose gel was inhomogeneous, it is possible that aggregates that could show the highest are those that are in the level of matrix maturation, while the aggregates that comprise less maturity, as well as isolated cells, are sought to be in lower level of differentiation such as matrix maturation stage where they start production of matrix and sedimentation. However, further experimentation is needed to confirm the mineralization of the aggregates.

Future Considerations

This research intended to characterize the biomechanical properties of the aggregate seeded gels in a 28-day period of culture. Although this study was able to find the impact of the factors such as shear stress and the stiffness of substrate on the trend of biomechanical and biochemical properties, it is highly beneficial to characterize the genomic and transcriptomics analysis of the aggregates to further prove the higher level of the cell maturation after treatment. Usage of uCT for confirming the mineralization would be another method that could be considered to show the mineral content of the matrix.

CONCLUSION

This research was an attempt to find the impact of stimulating factors such as mechanical stress and substrate stiffness on a 3D aggregate encapsulated agarose tissue in osteogenic and chondrogenic treatment. We found that the usage of aggregates can improve the mechanical properties of the aggregates and agarose gel. Additionally, we found that the mechanical properties of aggregates are dependent on the size of aggregates. Aggregate cell culture was successful in increasing the stiffness of both OM and CM-treated gels. However, the OM treated tissues showed a lower value of Young's modulus compared to CM treated batches which could be arisen by the generation of the defects by the maturation of aggregates in OM culture. The increase in the level of glycosaminoglycan level, the stiffness of the agarose gels and the mechanical properties of the seeded aggregates after 28 days of treatment are the signs of the maturity of the samples. Finally, the presence of the aggregates with various size can produce the cells with different levels of maturation. Yet, more studies are needed for confirmation of the maturity of aggregates after 28 days of culture and assess the biomolecular properties of the aggregate culture.

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