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Use of cyclic strain bioreactor for the upregulation of key tenocyte gene expression on Poly(glycerol-sebacate) (PGS) sheets

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ABSTRACT

The inadequate donor source and the difficulty of using natural grafts in tendon repair and regeneration has led researchers to develop biodegradable and biocompatible synthetic based tissue equivalents. Poly(glycerol sebacate) (PGS) is a surface-erodible bioelastomer and has been increasingly investigated in a variety of biomedical applications. In this study, PGS elastomeric sheets were prepared by using a facile microwave method and used as elastomeric platform for the first time under mechanical stimulation to induct the tenocyte gene expression. It is revealed that elastomeric PGS sheets promote progenitor tendon cell structure by increasing proliferation and gene expression with regard to tendon extracellular matrix components. Human tenocytes were seeded onto poly(glycerol-sebacate) sheets and were cultured two days prior to transfer to dynamic culture in a bioreactor system. Cell culture studies were carried out for 12 days under 0%, 3% and 6% strain at 0.33 Hz. The PGS-cell constructs were examined by using Scanning Electron Microscopy (SEM), cell viability via live/dead staining using confocal microscopy, and GAG/DNA analysis. In addition, gene expression was examined using real-time polymerase chain reaction (RT-PCR). Tenocytes cultured upon PGS scaffolds under 6% cyclic strain exhibited tendon-like gene expression profile compared to 3% and 0% strain groups. The results of this study show that PGS is a suitable material in promoting tendon tissue formation under dynamic conditions.

1. Introduction

The tendon is a type of elastic connective tissue that connects the muscle and bone formations together to transmit load and force. It has a hierarchical structure that consists of collagen molecules, fibrils, fibre bundles, fascicules and tendon units which are arranged longitudinally and parallel to each other in the structure [1]. Cell content in the tendon tissue is very low. The tissue has two basic types of cells which are of mesenchymal origin, tenocytes and tenoblasts. Tenoblasts are the predominant cell type in the tendon, they mature into tenocytes by lowering their cytoplasm-to-nucleus ratio and their metabolic activity. Tenoblasts have a fusiform shape and their numerous organelles can be observed in their cytoplasm that reflects high metabolic activities. Tenocytes on the other hand have the function of reshaping and maintaining the ECM via the synthesis of components such as collagen and proteoglycans in response to mechanical stimuli [2]. Tenocytes are located on the surface of collagen fibers and arranged in parallel to the fibres. Tenocyte differentiation can be observed according to different locations in the tendon. For example, interfascicular tenocytes are more productive and have a rounded morphology compared to intra fascicular ones [3,4]. The main component of the tendon is water, which constitutes 55–70% of the wet weight of the tendon. The main molecular component of the extracellular matrix of tendon is collagen, which accounts for 60–85% of the dry weight of the tendon [5]. The ability of tendons to resist various mechanical stress types is directly based on the structural organization of the extracellular matrix. The dynamic and mechanical forces they are exposed to *in vivo* environments cause the fiber patterns and viscoelastic properties of the tendons to change and improve. These properties of collagen molecules which are exposed to the forces mainly arise from the binding patterns and the relationship of these molecules with other extracellular matrix elements. Different biomechanical forces can cause different organization and compositions on the same tendon. In sections where tension is applied in a single direction, the fibres show a uniform and unidirectional structure.

Tendons typically appear white due to their low-level

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vascularization, but they contain small, thin-walled blood vessels. It is known that some tendons, for example tendons around bone protrusions, have completely avascular zones [6]. Considering the high proportion of ECM in the tissue, combined with a relatively small number of cells with low proliferative potential and limited blood supply, tendons cannot heal effectively when damaged [7].

Damages to human tendon tissues and degenerative diseases cause loss of function and severe pain. The key to the development of successful tendon tissue construct *in vitro* is to generate the culture medium that mimics the dynamics of the environment *in vivo*. Successful healing of tendon injuries depends on factors such as anatomical localization, vasculature, skeletal maturity and amount of tissue loss. Spontaneous healing may occur, but this healing usually results in the formation of a scar tissue, unlike morphological, biochemical and biomechanically healthy one. If the resulting tissue is different, it adversely affects the tendon's functionality. Tendon tissue engineering aims to initiate spontaneous regeneration of tendon tissue *in vivo* or to produce a functional tissue *in vitro* that can then be implanted into the body [8]. For this reason, bioreactors have been used to create favourable conditions in tendon tissue engineering. The designed bioreactors aim to create a mimicking environment in the light of mechanobiological cues of tendon regeneration. The inductive effect of mechanical stimulation regarding to synthesis of specific ECM proteins in connective tissues have been approved [9,10]. However, the optimal stimulation pattern is still unclear and need to be developed for each specific biomaterial designed for tendon/ligament reconstruction. Until now, acellular human and rabbit flexor tendons [11,12], fibrous scaffolds [13,14], dense collagen substrats [15] and hydrogels [16] have been investigated under mechanical stimulation. For instance, human tenocytes in collagen gel subjected to cyclic strain under 5% cyclic uniaxial strain at 1 Hz for 48 h have been shown regulated multiple protease and matrix genes [16]. Elastomeric polyurethane meshes with different fibre diameters seeded with mesenchymal stem cells subjected to 4% cyclic loading at a rate of 0.25 Hz for 30 min have also been showed to alter cell morphology and longer culture periods may be required to display the differences in cell phenotype.

The difficulty of using natural grafts in tendon tissue engineering has directed researchers to develop biodegradable and biocompatible synthetic-based tissue scaffolds. Poly (glycerol-sebacate) (PGS) is a new generation elastomer that exhibits surface degradation and thermoset elastomer properties and has been increasingly used in biomedical applications [17]. PGS was first reported in 2002 as a biodegradable polyester synthesized for soft tissue engineering [18]. With its flexible and elastomeric structure, it has been developed mainly to be used in cardiac muscle, nerve, cartilage and retina tissues. The medical use of PGS elastomer has been approved by the FDA [19]. Yet, the effects of biaxial loads on elastomeric PGS sheets have not been examined. The objective of this study is to investigate the effects of different mechanical stimulation regimes under static and cyclic tensile loads upon upregulation of key tenocyte gene expression onto elastomeric poly (glycerol-sebacate) (PGS) sheets by increasing the cell density without any growth factor contribution.

2. Materials and methods

2.1. Synthesis of PGS sheets

Glycerol (Merck, Germany) and sebacic acid (Sigma-Aldrich, Germany) were used to synthesize the PGS elastomer. The polymerisation of the elastomer consists of two steps: prepolymerisation and curing [20]. In the prepolymerisation step, glycerol and sebacic acid monomers were weighed in the appropriate petri dish in equal molar (1:1) ratio, then the petri dish was taken into a microwave oven (Samsung, Korea) and run at 650 W and High/Medium settings. Equimolar sebacic acid and glycerol mixture was exposed to electromagnetic waves 5 times for 1 min each with 10 s intervals. Following the prepolymerisation process, in order to crosslink the viscous prepolymer and prepare elastomeric matrices, the prepolymer was exposed to dehydrothermal crosslinking in a vacuum oven (Thermo Scientific, USA) under 50 mbar at 150 °C for 12 h in a Petri dish. The resulting cross-linked poly(glycerol-sebacate) elastomeric sheets with 1.8 mm thickness were cooled overnight at room temperature and cut into 1 × 2.5 cm sheets before use.

2.2. Characterization of PGS sheets

In order to characterize the elastomeric PGS sheets used in the study, Hydrogen-Nuclear Magnetic Resonance (^{1}H-NMR), Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), tensile and degradation tests were performed. A Bruker Spectrospin Avance Ultrashield spectrometer (2′ 300, 400 MHz) was used to determine the ^{1}H-NMR spectrum of the synthesized polymer. The analysis was carried out at ambient temperature and a $CHCl_3$ solution at 99.8% concentration was used as solvent and tetramethylsilane (TMS) was used for internal standard. FTIR analysis was carried out using a spectrophotometer (Shimadzu, Japan) between the wavelengths of 4000 and 400 cm^{-1}. DSC (Perkin Elmer, USA) was used to investigate the thermal transitions and the thermograms were determined between −80 °C to 100 °C at a heating rate of 10 °C/min. To determine the mechanical properties of the PGS sheets (n = 3), tensile testing was performed under 0.2 N load with the speed of 5 mm/min until rupture (Universal Tester, Zwick/Roell Z250, Germany) [21]. Tensile strength (σ, MPa), elongation at break (ε, %), and Young's modulus (E, MPa) values were calculated via software. For the degradation test, discs with same weight were cut with the aid of a biopsy punch (5 mm in diameter and 1.5 mm in width). Samples were then incubated in Ringer's solution at 37 °C and solution was renewed once a week. On the day 7, 14, 21, and 28, samples were removed from the solution, placed in a vacuum oven at 90 °C, dried for 24 h and the final weights were recorded [22,23].

2.3. Scaffold sterilization and cell culture

PGS sheets were kept in 70% (v/v) ethanol for 20 min prior to cell culture in order to remove unreacted monomers. The samples were sterilized in a PBS solution containing 3% antibiotic-antimycotic (AA) for 45 min with two changes. PGS sheets were then placed in a 6-well culture dish and washed with sterile PBS to remove excessive AA solution. In order to increase cell adhesion, sterilized PGS sheets were immersed in Type-I bovine collagen solution (Devro, UK) for 1 h and dried under laminar cabin. The sheets were seeded with human tenocytes (ZenBio, USA) at passage 3 with 2 × 10^5 cells/cm^2 concentration. The incubation of the samples was carried out at 37 °C in 5% CO_2 incubator (Memmert, Germany). For the culture medium, DMEM/HAM-F12 (Merck, Germany) supplemented with 1% L-Glutamine (Capricorn, Germany), 1% AA (Capricorn, Germany) 1% ascorbic acid (Sigma, USA) and 10% FBS (Capricorn, Germany) [24,25] was used. Throughout the cell culture study, the media were replaced with fresh medium every two days.

2.4. Bioreactor working conditions

Ebers TC-3 (Spain) bioreactor was used to apply dynamic stimulation to individually clamped PGS sheets. Bioreactor operating software was set to control the three-parallel compartments. PGS sheets (1 × 2.5 cm) were attached to the clamp in each compartment where the bioreactor provides optimal conditions during biaxial loading (Fig. 1A). A total of 12 PGS sheets were used in a single test, 6 of which were in the static culture group and 6 of which were in the dynamic culture. The separable bioreactor parts were sterilized with the autoclave instrument (Nüve, Turkey) at 120 °C for 20 min prior to use. PGS sheets were carefully placed into the bioreactor after two days of static

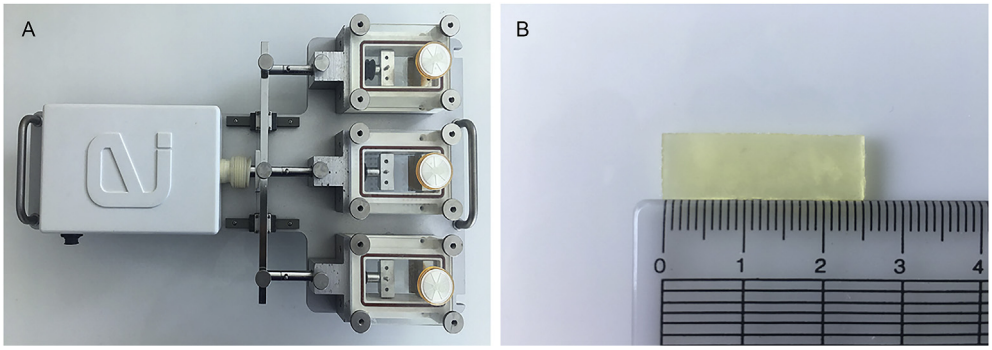

Fig. 1. A) Cyclic tensile bioreactor and its three parallel compartments, B) a representative PGS sheet used in the study.

culture to ensure complete attachment of the cells.

The study parameters were determined as cyclic tensile strain to be 3% and 6% at 0.33 Hz [26]. The samples were subjected to cyclic strain for 30 min in the first 2 days (7,200 cycles), then 60 min for the next 8 days (14,400 cycles). During the culture period, samples were removed from the compartment for both groups on the days 4, 7, and 12.

2.5. Live/dead staining assay

A Live/Dead staining kit (Sigma Live/Dead Cell Double Staining, USA) was used to evaluate the cell viability. This kit contains two fluorescent molecules, namely propidium iodide (PI) (535 nm/617 nm - excitation/emission) which allows the dead cells to luminesce while the second being calcein (490 nm/520 nm - excitation/emission) for the live cells. Propidium iodide stain is nuclear dye and it cannot pass through the cell membrane of the living cells. It passes through the irregular regions of the dead cell membrane, reaches the nucleus and then stains the double strand of the DNA to emit red fluorescent light. Acetoxymethyl ester of calcein is lipophilic and therefore has a very high cellular permeability. Although calcein is not a fluorescent molecule, when it interacts with the esterase enzyme present in living cells it creates a strong, green-coloured fluorescent light. Since both calcein and propidium iodide can be stimulated at 490 nm, living and dead cells can be monitored at the same time with a fluorescence microscope. After application of the kit procedure, imaging was performed with a confocal microscope (Zeiss-LSM510, Germany) at the days 4, 7, and 12.

2.6. Scanning Electron Microscopy (SEM) examination

Samples were taken from culture on the days 4, 7, and 12 in order to determine the cell morphologies and adhesion/spreading in PGS sheets following mechanical stimulation. The samples were carefully washed with PBS to avoid any residue from culture medium. Cultured samples were subjected to 2.5% (v/v) glutaraldehyde (Sigma, USA) for 30 min for the fixation. At the end of the fixation, the samples were washed with PBS. Prior to SEM analysis, PGS sheets were subjected to a series of alcohol treatments to remove the water in the samples. Accordingly, the scaffolds were kept in 30%, 50%, 70%, 90% and 100% (v/v) alcohol solutions for 2 min in each step. Following, samples were immersed in hexamethyldisilazane (HMDS) for 5 min, completely dried at room temperature, coated with gold (Au) -palladium (Pd) and were then imaged with SEM (Supra 50VP, Germany).

2.7. In vitro cytotoxicity assay

MTT (3- [4,5-dimethylthiazol-2-il]-diphenyltetrazolium bromide) (Invitrogen, USA) analysis was performed as an *in vitro* cytotoxicity test to determine any cytotoxic effects of PGS polymer on human tenocyte cells. According to established standards, 100 mg of PGS sheets (5 mg/mL) was weighed and then sterilized with 70% (v/v) ethanol for 20 min. It was then washed 2 times in PBS solution containing 3% AA for 45 min and washed again with PBS. Subsequently PGS sheets were exposed to UV light for 1 h and transferred into a total of 20 mL serum-free, low-glucose DMEM (Capricorn, USA) medium and kept at 37 °C in CO_2 incubator for 24 h to obtain elution. After 24 h of incubation, the medium was filtered with 0.2 μm pore sized syringe filter (Corning, Germany) and then before the cell culture, 1% L-Glutamine, 1% AA and 10% FBS was added into this sterile elution.

Tenocytes (P3) were seeded into the 24 well-plate at a density of 0.02×10^6 cells/well and cultured for 24 h before the experiment. After 24 h, the media were removed from the wells, replaced with defined elution concentrations and MTT test was performed for the following 3 days. For each day, 600 μL of serum-free medium and 60 μL of MTT solution (2.5 mg/mL of PBS) were added to each well and incubated for 3 h at 37 °C. At the end of the process, the media in the wells were removed and 400 μL of acidic isopropanol was added to each well. The isopropanol solution in the wells was pipetted to dissolve the formazan crystals. 200 μL of the obtained solution was analysed spectrophotometrically using plate reader (Biotek, Epoch) at 570 nm.

2.8. Spectrophotometric biochemistry assays

Quantification of sulphated GAG in PGS sheets was performed with DMMB (1,9-dimethylmethylene blue) assay (Sigma, USA). For this purpose, required amount of 1,9-dimethyl-methylene blue zinc chloride double salt was dissolved in 250 mL of a solution composed of 40 mM NaCl (Sigma, Germany) and 40 mM glycine (Fluka, Honeywell, Bucharest, Romania) in 0.1 M HCl (BDH AnalaR). Chondroitin sulphate sodium salt from bovine trachea (Sigma, USA) was used to prepare appropriate standards (0–80 mg/mL) for analysis. On day 12, the sheets were removed from the culture and digested enzymatically by using Proteinase K (Sigma, USA) solution prepared with a concentration of 1 mg/mL in 100 mM ammonium acetate (BDH Analar, UK) for overnight at 60 °C. Digestion solutions (40 μL) were reacted with test solution and absorbance were directly read at 525 nm using plate reader. Obtained values were normalized to the dry weight of each sample. The same solution was used to determine the DNA content by using Quant-iT PicoGreen kit (Molecular Probes, USA). Appropriate standards (0–1 μg/mL) were prepared by diluting DNA stock solution (2 mg/mL DNA) with Tris-Edta (TE) buffer before the experiment. Accordingly, 50 μL digested sample solution were reacted with 50 μL PicoGreen test solution and subsequently fluorescence intensity was measured at 520 nm using plate reader.

2.9. Real-time polymerase chain reaction (PCR)

Real-time polymerase chain reaction was performed to determine the effect of mechanical stimulation on the gene expression of tenocytes. Prior to the isolation step, the scaffolds were incubated in 1u/mL collagenase (Sigma, USA) for 6 h at 37 °C to facilitate separation of the

Table 1
Primers used for gene expression analysis.

Gene Name (Human)	Forward Primer (5′- 3′)	Reverse Primer (5' – 3′)
COLIII	AGCGGTTCTCCAGGCAAGGAT	AGTGATCCCAGCAATCCCAAG
COLI	ATCGACAGTGACGCTGTAGG	CCAGCAGATCGAGAACATCC
TNC-1	GGTACAGTGGGACAGCAGGTG	RGAGAAGGATCTGCCATTGTGG
TNC-2	GAGACATCTGTGGAAGTGGA	CGTACTCAGTGTCAGGCTTC
TNMD	ATTCAGAAGCGGAAATGGCACTGA	TAGGCTTTTCTGCTGGGACCCAA

cells from the tissue scaffold. After the supernatant was obtained, the RNA isolation was performed. The total RNA of the cells on scaffolds were extracted using the Zymo Research Quick-RNA Mini Prep (USA) kit. After DNAseI treatment, concentrations of the obtained RNAs were determined using a UV-Vis spectrophotometer (Nanodrop 2000/2000c, Thermo Fisher Scientific) device. Complementary DNA was synthesized from the isolated RNAs using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) using PCR device (Veriti, Applied Biosystems). qPCR analysis was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, USA) in Viia7, Applied Biosystems instrument with QuantStudio Real Time PCR Software Version 1.2 software. The PCR conditions were 50 °C for 2 mins, 95 °C for 2 mins and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. β-Actin gene was used as a reference gene and comparative CT method (2^{-ddCt}) was used to analyse the results. The primer sequences of tenascin gene used in the experiment are listed in Table 1.

2.10. Statistical analysis

Each experiment was performed in triplicates. Student T-test and ANOVA tests were performed for statistical analysis using GraphPad Prism software version 6 (GraphPad Software, La Jolla, USA). p values at least less than 0.05 were considered significant. Level of significance were represented as follows: $p > 0.05$ as not significant, $p \leq 0.05$ as *, $p \leq 0.01$ as **, $p \leq 0.001$ as ***, and $p \leq 0.0001$ as ****.

3. RESULTS

3.1. Chemical characterization

Polymerisation was confirmed by ^{1}H-NMR analysis (Fig. 2A). Calculations on the integration of peak areas were performed to reveal the monomer ratios of the final product. The actual monomer mole ratio was calculated for glycerol:sebacic acid according to the literature as 21:79 (n/n) [20].

FTIR analysis was performed with the aim of determining the structure of the PGS scaffold following the cross-linking step (Fig. 2B). The C–O and C═O bonds can be observed at the 1150 and 1730 cm^{-1} peaks of the prepolymer elastomer, respectively. The peaks between 2928-2851 cm^{-1} belong to the -C-H stretching peaks of the -CH_2 groups. The peaks between 1411-1464 cm^{-1} result from the bending absorption of the -CH_3 methyl groups. At 925 cm^{-1}, a C–O in-plane bending vibration peak is visible. The sharp peak at 1730 cm^{-1} is evidence of cross-bonding with the -C═O stretching peak. The FTIR results of the cured PGS samples are in accordance with the literature and the peaks at 2927-2852 cm^{-1} show alkene (-CH_2) groups, the peak at 1734 cm^{-1} show C═O group, the peak at 1455 cm^{-1} show methyl group (-CH_3) and finally the peak at 1416 show the O–H group. As the duration increased, it was seen that the absorption at 1730 cm^{-1} bands had increased. This indicates that the C═O bonds at 1730 cm^{-1} are increased with the prolonged duration [27].

Fig. 2C is the DSC thermogram showing the thermal properties of the PGS elastomer. According to the thermogram, the T_g value which is obtained with the curve drawn with the 1st heating, indicated the glass transition temperature of the polymer (T_g: -29.10 °C). The reverse peak seen in the cooling phase indicates the crystallization temperature of the polymer. The PGS is a semi-crystalline polymer and the crystallization temperature (T_c) obtained was 23.57 °C. The peak value in the heating process gives us the T_m, the melting temperature, which was 0.84 °C. The observed values of DSC thermogram were consistent with the literature [19].

3.2. Degradation behaviour and mechanical characterization

In order to determine the degradation rate, the PGS sheets were placed in Ringer's solution containing 1% AA at pH 7.4 and were stored at 37 °C and the degradation rate was examined on the days 7, 14, 21, and 28 [21]. For each defined day, 4 samples were evaluated and the data is given in percentages (Fig. 3A). The PGS sheets were degraded 20% in the first 7 days on average. There was a considerable difference between day 7 and day 14 scaffolds ($p \leq 0.001$), although the sheets appeared to be stable between the days 14 and 21. Yet, after 3 weeks, degradation rate was increased significantly ($p \leq 0.0001$), leaving sheets with a 30% degradation. The degradation graph shown in Fig. 3A is arranged according to the results of Eq. (1) given below. M_0 represents the dry weight of the PGS sheet, while M_t represents the vacuum-dried weights of scaffolds obtained from each defined degradation point of day.

$$M_{loss}\ (\%) = \frac{M_0 - M_t}{M_t} x \quad 100 \tag{1}$$

Mechanical tensile test results from triplicate measurement indicate tensile strength (σ), elongation at break (ε), and Young's modulus (E) values as 0.26 ± 0.0326 MPa, 260.94 ± 17%, and 0.1567 ± 0.00647 MPa, respectively. The obtained values were found to be in accordance with the literature [20]. Fig. 3B shows the graph of the mechanical tensile tests conducted on the PGS sheets. These results indicate that the PGS scaffold exhibits similar elasticity with the natural tendon structure [18,28].

3.3. Live/dead staining assay

Live/dead staining was applied on the 4th, 7th, and 12th days of both static and dynamic cultures and the confocal microscopy images were examined (Fig. 4). It was revealed that PGS sheets showed no cytotoxic effect on human tenocytes (cultured both in static and dynamic environment) throughout 12 days. Besides showing any cytotoxic effect, cell proliferation could clearly be observed throughout the culture period in all scaffolds. Human tenocytes were proliferated starting from day 4 to day 12 in both conditions. However, the proliferation rate seemed higher in dynamic culture as a result of cyclic strain that enhanced the proliferation of cells compared to static condition. While cell distribution was predominantly homogeneous under cyclic strain, the cells were prone to be aggregate in static culture. Easily distinguishable cell clusters can be seen at Fig. 4 A-C-E. On the other hand, the cells were broadly single under cyclic load (Fig. 4B–D-F)

ImageJ Software (v1.410) were used to semi-quantitative evaluation of the effects of different culture conditions upon cell density. Selected live/dead stained confocal images of different culture conditions throughout the culture period were adjusted to proper color threshold and the cell density in given areas from 6 different image were quantitatively analysed (Fig. 5). The cell density under bioreactor

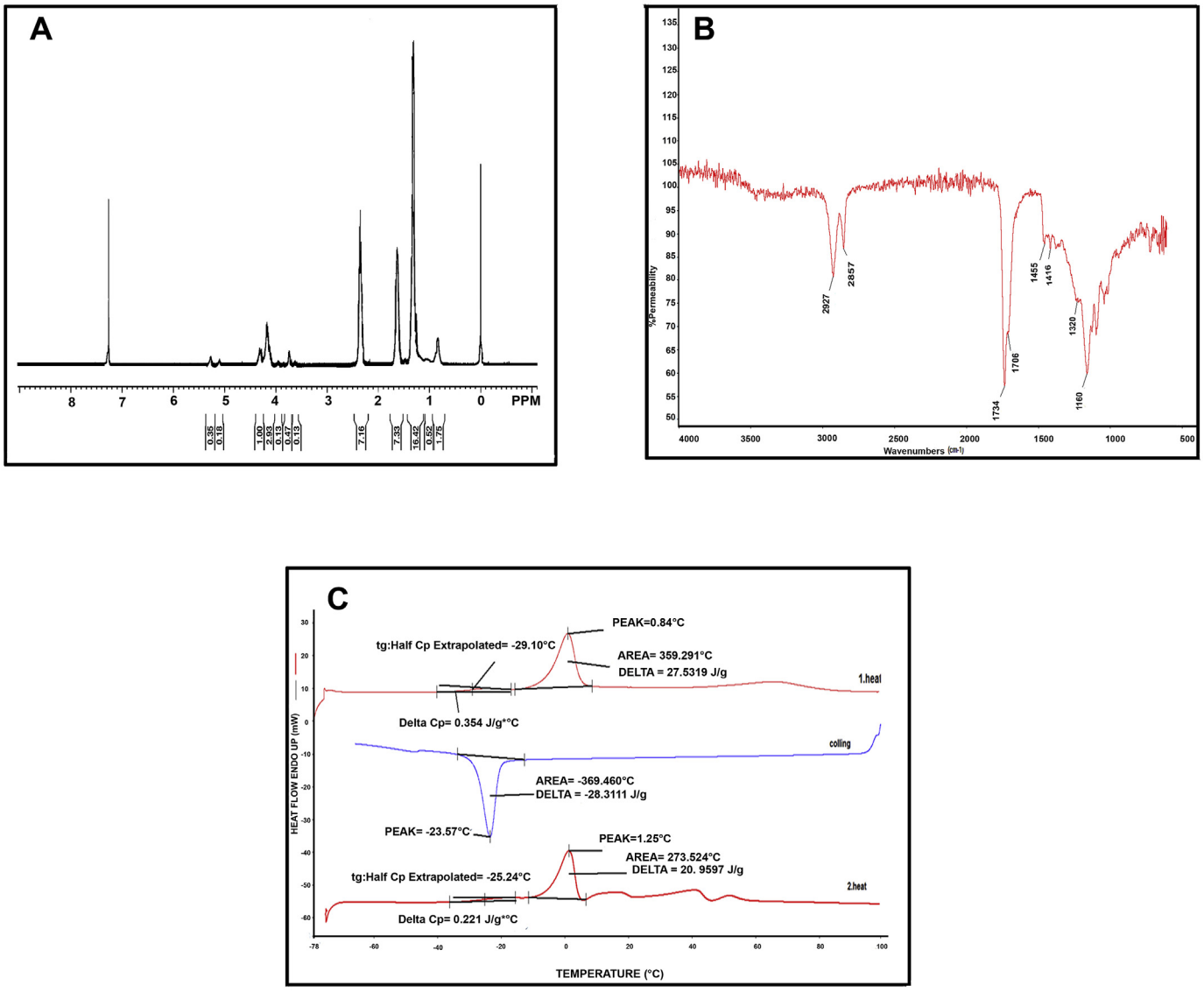

Fig. 2. A) ^{1}H-NMR spectrum, B) FTIR spectrum, and C) DSC thermograms of the PGS sheets.

conditions was significantly higher than the static group throughout the 12 days of culture period, especially on 7th and 12th days of cultivation ($p \leq 0.0001$).

3.4. Scanning Electron Microscopy examination

Structural and behavioural characteristics of human tenocytes cultured on PGS sheets in dynamic and static cultures were investigated with SEM at 4th, 7th, and 12th days (Fig. 6). Prior to mechanical induction, all cell seeded sheets were subjected to static culture for 2 days to ensure that the cells would not be affected from the dynamic tensile force and not detached. SEM images on the 4th day of culture show the mechanical pulling force of 30 min which was applied 2 times have led to cells in the dynamic culture to be more homogenous than the cells in static culture (Fig. 6 A&B). SEM images of PGS sheets at day 7 (Fig. 6C& D) show that the distribution of cells in the dynamic culture constructs

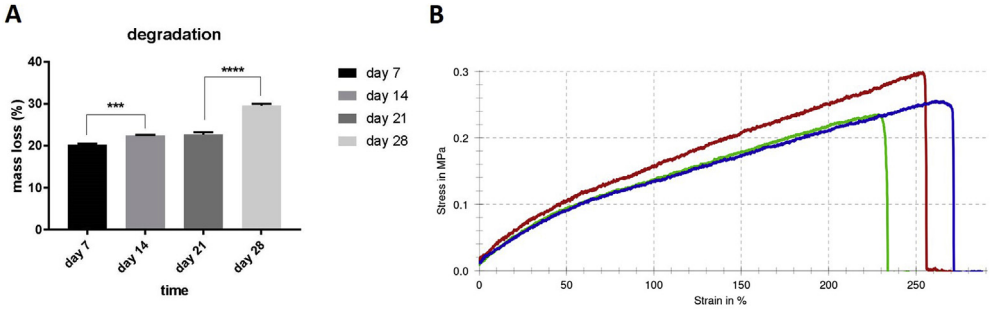

Fig. 3. A) Degradation behaviour, and B) Stress-strain graphs of PGS sheets.

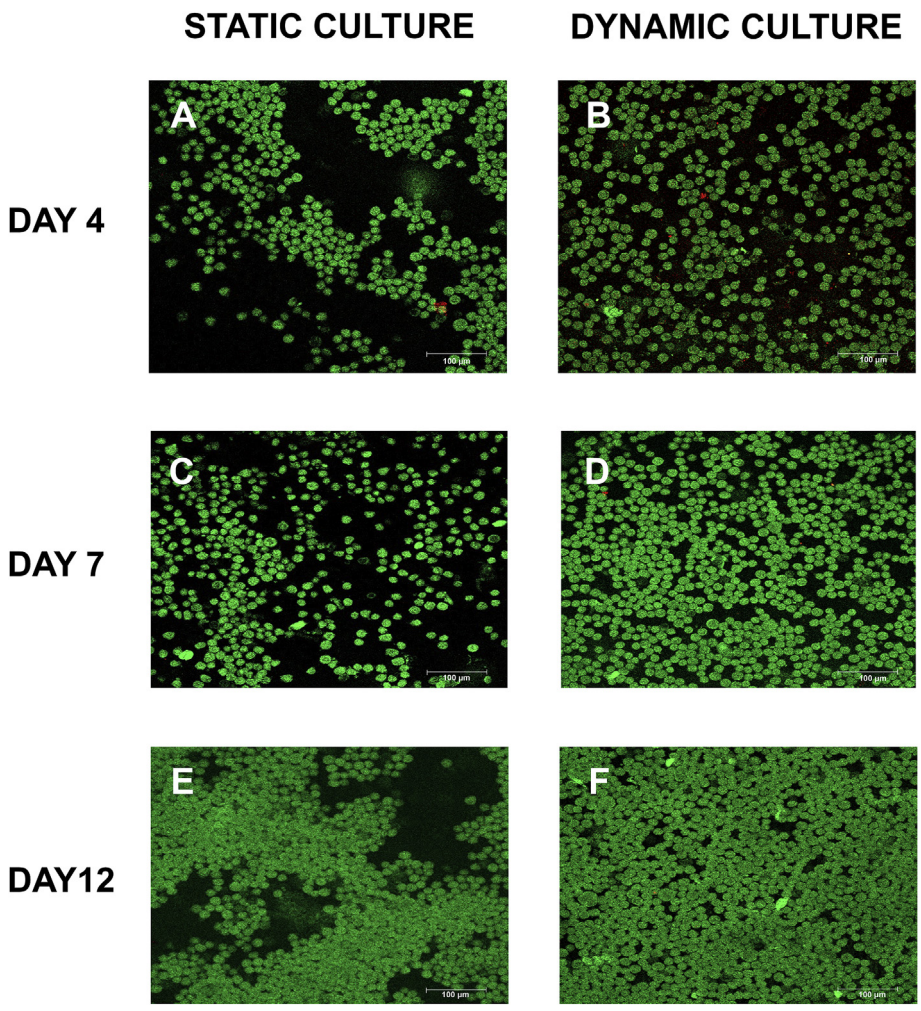

Fig. 4. Confocal microscopy images of live/dead staining of human tenocytes seeded on PGS sheets cultured in static (A, C, E) and dynamic (B, D, F) conditions (scale bar = 100 µm).

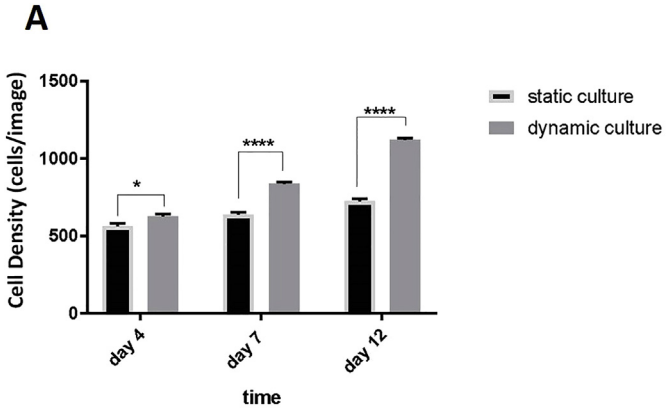

Fig. 5. The density of cells (A) under the static and dynamic culture conditions (Semi-quantification of cell density via ImageJ Software (v1.410), n = 6).

exposed to dynamic tensile force for 60 min per day after the 4th day were more homogenous compared to day 4 and it can clearly be observed that the cells on the PGS sheets were starting to interact with each other to initiate ECM secretion. In the static culture group, the cells showed advanced clustering behaviour indicating that the tendency of cells to form tissues in culture have decreased.

SEM images of day 12 indicate that the intercellular communication have increased and the cells were on the brink of forming a precursor tissue with their ECM secretions. In the static culture, the clustering behaviour caused by the lack of exposure to mechanical load led the cell clusters on the sample to be sparse. It can be argued that the prolonged static culture may have led to cell clustering with the reduction of potential tissue forming (Fig. 6E).

3.5. Cell viability assay

Human tenocytes were cultured with extract medium obtained through the 24-h incubation of PGS sheets at 37 °C (5 mg/mL) [29]. Any change in the extract medium (toxic residues, pH, etc.) affects the activity of the cells. Fig. 7 shows cell activity results performed via MTT assay. First day of the assay, the cells exhibited quite similar activity rates for different concentrations of extract medium. However, in the second day of the assay, the cells in control group which has no PGS elution (0 mg/mL) possessed the highest activity. Cell activity results in percentages for different elution concentrations were 87%, 92%, and 84% for 1.125 mg/mL, 2.5 mg/mL, and 5 mg/mL elution ratios, respectively. The cell activities were proceeded to increase on the following day. Yet, the increase in cell activity for control group was less than the other extract mediums.

These results may be related with over expansion of cells in plain medium which reduces the activity of cells. Cell activity results in percentages for different elution concentrations for the day 3 were found to be 116%, 107%, and 103% for 1.125 mg/mL, 2.5 mg/mL, 5 mg/mL, respectively. Overall, despite the lack of significant difference

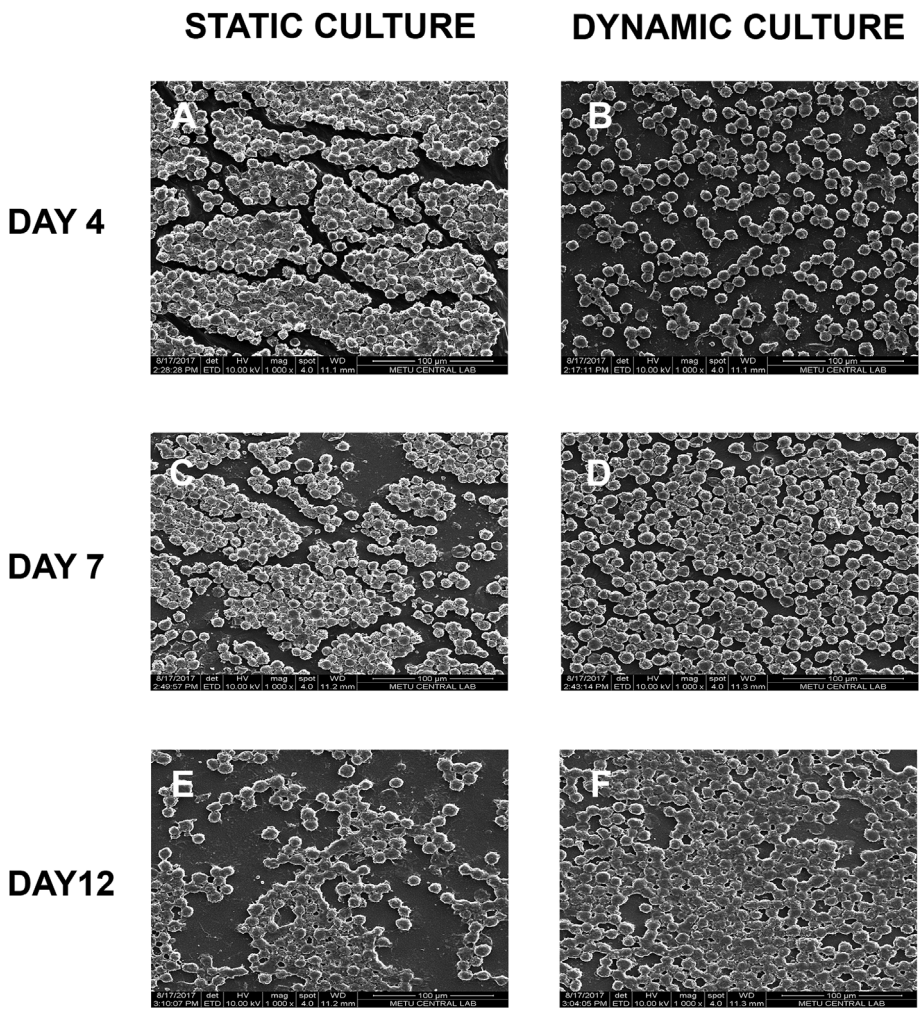

Fig. 6. Scanning Electron Microscopy (SEM) images of human tenocytes seeded and cultured in static (A, C, E) and dynamic (B, D, F) conditions (scale bar = 100 μm).

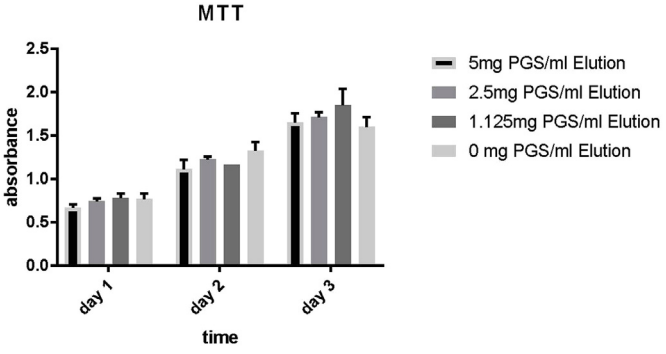

Fig. 7. Dose dependent cytotoxicity of PGS sheets.

between different elution concentrations, the cell viability was increased considerably each day, revealing convenience of all concentrations for *in vitro* tenocyte culture studies ($p \leq 0.01$).

3.6. Real-time polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed to measure the response of the tenocytes to the applied cyclic strain on a molecular level compared to stationary cell culture experiment. According to the results obtained from the analysis, applied strain values (3% and 6%) were found to have an effect on gene expression of tenocytes. Mechanical forces are fundamental regulators of tissue homeostasis and are important for the normal functioning of musculoskeletal and connective tissues. Tissues bearing high tensile loadings such as ligaments and tendons normally express tenascin (TNC) [30]. Tenomodulin (TNMD) expression has also been shown to have a role in tendon development and tenocyte proliferation, but its synthesis can only be clearly identified in advanced-stage cell cultures [31]. Tenomodulin gene expression was also assessed within the scope of the study in the early stages of a culture and was found only at a trace amounts, with lower expression than other genes in the culture (data not shown).

Relative gene expression levels of TNC was studied with two set of primers to cover all variants as TNC I and TNC II, which are known to be highly expressed in tenocytes [32]. Both variants were found to be upregulated in dynamic and stationary culture conditions, especially in the 12th days of cultivation period. For TNC I gene expression levels, no significant difference was observed in the first 7 days of 3% strain group compared to static cell culture ($p > 0.05$) (Fig. 8A). For 6% strain group, gene expressions seemed to be elevated more, compared to 3% strain group and an increase of 65% was shown in samples of 6% strain group on day 7 ($p \leq 0.01$). On day 12, mRNA levels of TNC I were nearly doubled in 6% strain group ($p \leq 0.05$). Comparing TNC I expression levels of tenocytes on 6% strain group to static cell culture on 12th day, a tremendous increase was observed ($p \leq 0.001$).

Regarding TNC II gene expression levels, 3% strain group did not show a significant difference compared to static control, likewise TNC I variant. mRNA levels of TNC II were shown to be increased 2.5% and

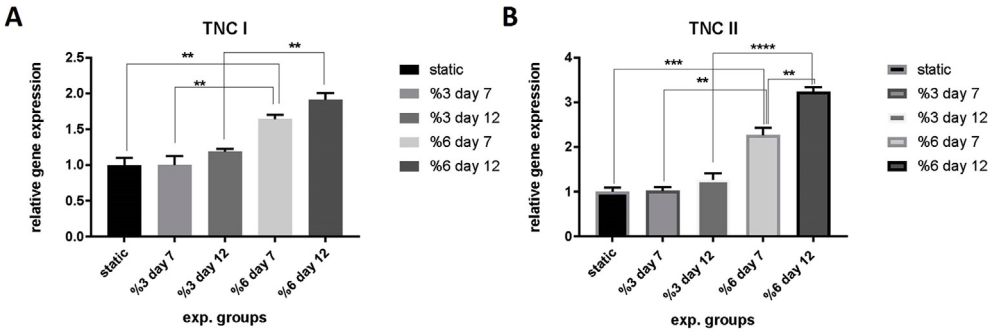

Fig. 8. Results of real-time PCR analysis: relative gene expressions of TNC I (A) and TNC II (B) genes are represented.

27.3% for 3% strain group on day 7 and 3% strain group on day 12, respectively and elevated to higher levels in 6% strain group on day 7 and 6% strain group on day 12 with an increase of more than 80% ($p \leq 0.01$). On day 7, cells on 6% strain group displayed a 2.2-fold increase compared to static group ($p \leq 0.001$). Difference between TNC II gene expression of 3% and 6% strain groups on day 12 was found to be more significant compared to TNC I variant ($p \leq 0.0001$) (Fig. 8B). Overall, TNC mRNA levels were shown to be increased with mechanical induction and increasing cultivation periods.

Gene expression levels of COL III and TNMD was also studied but no considerable difference was observed due to limited cultivation days. Regarding mRNA levels of COL I, although a slight increase was found in day 12 in cell cultures, it was not statistically significant (data not shown since coating of PGS sheets with collagen-I solution to increase cell attachment efficiency would make the comparison of the expression levels of the COL I gene between stationary and dynamic culture samples erroneous).

3.7. Spectrophotometric biochemistry assays

Quantification of GAG content was performed with cell seeded PGS constructs cultured 12 days under static and dynamic conditions (6% strain). Quantification of GAG content is shown in Fig. 9A. The amount of GAG in the mechanically induced group during 12 days was 230.3 μg per sheet, while the amount of GAG in the static culture group was 166.6 μg per sheet. Comparison of the results showed that the amount of GAG secreted by the human tenocytes subjected to mechanical induction was significantly higher than the amount of GAG secreted by the tenocytes in static cultures ($p \leq 0.05$). In the dynamically induced PGS sheets, 38% more GAG content was detected than the statically cultured one [33,34].

The quantification of DNA content shown in Fig. 9B. The amount of DNA per PGS sheet subjected to mechanical induction was 923.9 ± 31.84 μg, while the amount of DNA per sample in stationary culture was found to be 712 ± 3.60 μg, (29% increase). The results were found to be statistically different as well and consistent with the GAG measurement results given in the Fig. 9A ($p = 0.0027$). Hovewer, the endpoint GAG/DNA ratio (Fig. 9C) was not significantly different between dynamic and static cultures conditions ($p = 0.4331$).

4. Discussion

Tissue engineering constructs (TECs) have been developed to achieve full repair of tendon ruptures. To that extent to produce a clinically functional engineered tendon substitute, biochemical and biomechanical properties should meet to its nature. Biomaterial-based tendon regeneration takes the advantage of many scaffolding techniques by using many different synthetic and natural polymeric materials such as polyesters [poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA)], collagen derivatives and polysaccharides (chitosan, alginate, hyaluronan) [35]. While polyesters suffer from hydrophobicity limitations that prevent the cell adhesion; collagenous scaffolds exhibit lower biomechanical behaviour for tendon regeneration in spite of their high biocompatibility. In this study we offered elastomeric PGS sheets as tenocyte guidance platform under cyclic loading and potential prospective candidate as tendon tissue constructing material. For this purpose, PGS elastomers were synthesized via microwave irradiation and dehydrothermal crosslinking and human tenocytes were seeded onto PGS sheets were exposed to biaxial strain throughout the 12-day culture in a cyclic strain bioreactor.

The mechanical tensile test for the PGS sheets revealed a percentage of elongation of around 260%. The results were found to be in accordance with the literature [20]. PGS elastomer is a promising candidate for tendon tissue engineering with its flexibility and durability. Weight loss was calculated as 20% over the first 7 days. During the following days, the degradation rate increased over time but the acceleration of degradation was not as high as it was observed during the first 7 days. Since PGS elastomer exhibits surface degradation, the structure of the scaffold maintained its integrity along the remodelling process [35]. Considering the long-term regeneration period, it is

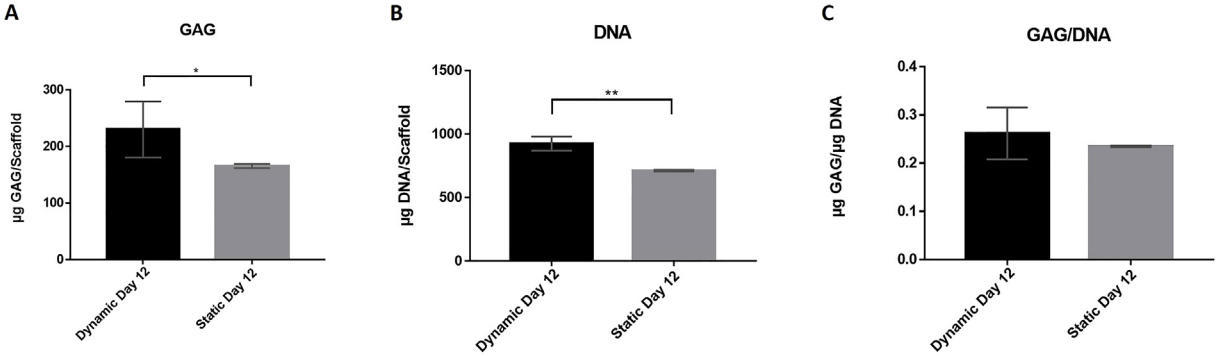

Fig. 9. Endpoint GAG content performed with DMMB assay (A), and DNA quantification analysis performed with PicoGreen test kit (B) and GAG/DNA ratio (C) reveal the effects of dynamic induction on the GAG and DNA amounts.

favourable to preserve the scaffold integration during biodegradation process.

The sheets prepared were initially subjected to a stationary culture for 2 days so that cells were not detached as a result of the dynamic tensile forces. It was observed that the application of cyclic mechanical stimulation for 30 min at 0.33 Hz at 6% strain in the first two days and 60 min in the following days induced the precursor tendon formation. When the results of SEM analysis and live/dead assay were compared, it was observed that the results of both analyses supported each other. Confocal images indicated that mechanical stimulation was not adversely affect the attachment and the viability of the cells. Appropriate cell adhesion to the materials surface is a crucial step for ongoing cell spreading, proliferation and differentiation processes. Initial cell adhesion onto PGS sheets could be achieved following short term static culture. Followed periodical cyclic loads did not detached the cells from PGS surface and that is also an indicator of strong cell adhesion. Furthermore, proliferation of tenocytes onto PGS sheets occurred all along culture period according to cell viability assay and confocal imaging. It has been reported that PGS itself is cell adhesive, cytocompatible and also support cell proliferation [36]. Hovewer, the surface characteristics appear rather low supportive on cell spreading could not been observed properly following days of culture. The reasoning behind the fact that the normally spindle-shaped tenocytes have shown a more round-shaped structure onto this material might be related with surface properties of the elastomer including hydrophilicity-hydrophobicity, surface charge and roughness [22,37]. Relatively low hydrophilicity of PGS mainly arise from the doped sebacate ratio in polycondensation process determines the quality of cell attachment and growth. Increased sebacate ratio results with decreased cell attachment and growth. Guo.et al. reported that PGS exhibits better cell adhesion and growth with more hydrophilic composition [36]. To gain better cell-material interaction the composition ratio of glycerol and sebacic acid can be modify. Proper cell phenotypes and cell-material interactions also can be enhanced via surface modifications like coating the surface with adhesion proteins or proper grafting with RGD sequences [38]. Kerativitayanan et al. have developed a mechanically stiff nanocomposites from poly(glycerol sebacate) to enhance the bioactivity and proper the cell phenotype onto PGS by covalently reinforcing PGS network with two-dimensional (2D) nanosilicates [37]. When the SEM images of 4th day was examined, it was observed that the application of mechanical tensile force 2 times for 30 min led the cells in the dynamic culture to show a more homogeneous distribution compared to the cells in stationary culture. The samples on their 7^{th} day were found distributed more homogenously compared to those at the 4th day of the culture. When the images were observed, it was seen that the cells on the PGS sheets seemed started to interact with each other and to secrete ECM. In stationary culture group, the cells showed a clustering behaviour as day 4th. It is clear that the tenocytes on day 12 of dynamic culture became even more homogeneous compared to the 7th day of culture and that they were starting to form a precursor tissue with their intercellular interactions and their secretions of ECM.

The density of cells were analysed semi-quantitatively via imageJ software demonstrated that cells gradually increased throughout the culture period in both static and dynamic environments. But, significant changes occurred between different culture conditions at the days 7 and 12 ($p \leq 0.0001$). Distinct increase rate were observed in dynamic condition when compared with static.

In vitro cytotoxicity assay showed that the highest cell viability rate were observed in 1.125 mg/mL elution group with 116% at day 3. The cell viability rates of the other elution groups (2.5 mg/ml, 5 mg/ml) were 107% and 103% at day 3 respectively. When the results between the groups and the literature are compared, it is found that PGS sheets prepared in this study was not cytotoxic. The reason for the decline observed on the third day in the control group might be due to the over-proliferation of the cells. That is, the activity of cells decreased due to excessive proliferation (Fig. 7).

The results obtained with the PCR analysis clearly showed that cyclic strains (3% and 6%) were effective on expression of tenogenesis-specific genes. TNC gene is known to be highly expressed in tendon and is responsible for the ECM formation [39]. It was also shown that the TNC genes are regulated by mechanical loading. They provide elasticity to tissues subjected to heavy loading and the expression levels can be increased with mechanical stimulation [24,40]. It was also reported that the TNC protein is a key player in ECM organization of tenocytes specifically by arranging collagen fibrils [41]. In this study, multiple variants of TNC were analysed and found to be increased significantly with mechanical stimulation. It was clearly shown that tenocytes cultured with 6% cyclic strain conditions increased their TNC expression levels remarkably on both 7th and 12th days of culture ($p \leq 0.01$). Regarding intragroup results, cultivation period was shown to be effective for higher gene expression levels that cell that have been cultured for 12 days were shown to express TNC higher than cells cultured for 3 days. In addition, 3 days of cell culture was shown to be inadequate for tenocytes to increase their TNC mRNAs, as any significant difference could not be observed, regardless from cyclic strain level applied on scaffolds ($p > 0.05$). With the increase in TNC gene expression, it can be concluded that the application of mechanical force induced the tenocytes to form a precursor tissue and that they structured their ECM secretions to this end [42,43]. The genetic expression upon tendogenesis may be also enhanced via creating porous PGS scaffolding could be lead to achieve proper cell phenotypes and migration of the cells through medial layer of scaffold may result with enhanced matrix deposition.

Increase in GAG levels correlates with the gene expression analysis, revealing TNC protein function and the application of mechanical induction promotes the tenocytic gene expression to form precursor tissues and shapes the ECM structure. In addition, this increase in TNC gene expression also correlates with proliferation results and previous studies showing isoforms of tenascin-C potentially induce tenocyte proliferation [44].

Qin et al. also reported that cyclic strain induces the cell proliferation and matrix deposition.

The negatively charged regions of GAG chains contribute to the mechanical stability of the tissue by binding to positive regions on the collagen molecules. The amount of GAG in the tissues varies according to the type of force that affects the tendon, and it is known to be synthesized in higher amounts in the tendon areas that are subjected to compression [45]. In this study, the amount of GAG secreted by tenocytes subjected to mechanical forces was about 38% greater than the amount of GAG secreted by the tenocytes in static culture (Fig. 9A). It can be observed that the application of dynamic force promotes precursor tissue formation by increasing tenocyte proliferation (Fig. 9A). The obtained tenocyte GAG yield is in accordance with the literature [33]. According to the results of DNA analysis, it was found that dynamic group possessed 29% more DNA compared to the stationary group (Fig. 9B). The reason behind this could be the increased cell proliferation observed in the dynamic force group, compared to the static ($n = 3$). Yet, the endpoint GAG/DNA ratio (Fig. 9C) was not significantly different between dynamic and static cultures conditions ($p = 0.4331$).

To the best our knowledge, it is the first time that the PGS elastomeric sheets were used as a guidance platform for tendon tissue engineering. Even though the tissue culture was in its early stages, when all the results were evaluated, the hypothesis that the precursor tissue formation has commenced in the group where dynamic forces were applied, is confirmed.

Even we did not used a porous scaffold and any growth factors known for tendon regeneration process such as insulin-like growth factor-I (IGF-I), transforming growth factor beta (TGF beta), and vascular endothelial growth factor (VEGF) [46], we proved that PGS is a suitable material for tendon tissue engineering and only mechanical tensile induction could be sufficient to promote the tenogenic gene

expression. By addition of these growth factors to the bioreactor culture together with suitable surface modification and porosity may takes a step further by means of tendon formation.

5. Conclusion

In this study, the PGS-based sheets were used with the tenocytes for the first time under dynamic *in vitro* conditions in order to investigate the potential of the material as a tendon regeneration scaffold. Two different mechanical induction group were evaluated: 3% and 6% groups were compared with the stationary condition. The results reveal the effects of mechanical induction on tenocytes seeded on PGS sheets. The best cell behaviour and proliferation was observed in the group subjected to 6% mechanical stimulation. The PGS scaffolds prepared and the mechanical stimuli scheme applied in this study may be a source for prospective tendon tissue engineering studies. \

Statement of significance

Induction of the cell response via mechanical stimuli in tendon tissue engineering can be achieved by using elastomers. The elastomer to be used should match the mechanical properties of the target tissue. Here we demonstrate the use of poly(glycerol-sebacate) (PGS) elastomeric sheets in dynamic tenocyte culture in a cyclic strain bioreactor. The results show an increase both in the cell proliferation and gene expression. The elastomeric PGS sheets therefore can provide a suitable platform for the cultivation and tissue engineering of tendon cells. In addition, the sheets obtainable via a facile route by using a household microwave and a vacuum oven can be used in many other soft tissue engineering applications by simply changing the cross-linking time.

Declaration of Competing interest

The authors declare that there is no conflict of interest.

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