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Effect of intermittent hydrostatic pressure on aging human chondrocyte cells

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***Abstract*— In this study, a hydrostatic pressure chamber (HPC) is designed and developed to host chondrocyte cell culture. External stimuli such as forces, pressure, vibration etc. are found to be significant factors on upregulating the relevant proteins for constructing the extracellular matrix (ECM) during culturing. The aim of this paper is to design a system which provides external stimuli during chondrocyte cell culture as well as to discover the relevant gene which can generate the repair and regeneration of aging cells. The system consists of a controllable HPC that provides intermittent hydrostatic pressure (HP) on the cultured cells. The chamber is capable of applying intermittent HP in the range: 0 to1 MPa, at a frequency of 0.5-1Hz. An investigation was undertaken to determine the improvement of human chondrocyte cells viz. of 3 sub-jects whose ages are 60 and above. The effect of HP on the aging cells is observed through the extracted ribonucleic acid (RNA) after the cell is treated with HP for two hours, each day, over four days. The experiments were conducted to observe the effect of HP on the level of collagen type I, collagen type II, and aggrecan. Results show that HP did little to help in upregulating the aggrecan and collagen type II in aged-chondrocyte cells. Further, it was found that the application of HP depended on the number of days applied. The results presented the possibility of ap-plying HP in regeneration of damaged cartilage in elderly.**

Keywords—hydrostatic pressure, chondrocyte, external stimulation

I. Introduction

Mechanical stimulation is considered one of the key factors which can bring about changes in tissue and cell during culture[1, 2]. In the past, a number of researchers have explored the effect of mechanical cues such as compression[3, 4], tension[5, 6], HP, fluid shear on the differentiation of cells, and the differentiation of mesenchymal stem cells(MSCs)[7, 8]. It is essential to understand how such external stimuli regulate osteogenesis and chondrogenesis of MSCs. Most cells can sense and respond to external stimuli, and these physical cues can influence the differentiation of MSCs. High HP applied to cells can lead to changes in cell morphology, microtubule assembly, and actin organization[9]. Many works [10-12] have supported the hypothesis that HP influences the ECM metabolism of articular chondrocytes. Ikenoue et al[11] studied the effect of the magnitude and duration of applied HP on the regulation of aggrecan and collagen type II in normal human articular chondrocytes. Results show the application of HP in the range: 1 to 10MPa, for a specific duration, increased chondrocyte protein expression.

The attempt to apply physical stimuli on in vitro cell/tissue culture starts from the development of bioreactors. A bioreactor is always the host where specific environmental conditions are set for particular cell types. Volkmer et al. [13] designed a perfusion bioreactor to culture tissue and engineer bone on a scaffold in both a static and dynamic environment. They also studied oxygen perfusion, which appears to help prevent cells dying. Egger et al. [14] designed a miniaturized perfusion bioreactor system to study the shear stress profile of a porous scaffold. Computational fluid dynamic (CFD) analysis was conducted in order to examine the flow and stress effect on osteogenic differentiation of human MSCs.

HP is acknowledged as pivotal in the regulation of articular cartilage tissue regeneration[15] and MSC differentiation. A critical role of HP is in the management of ECM turnover in articular cartilage[16, 17]. Static HP is applied to chondrocytes at different conditions. Simultaneously, HP helps increase glycosaminoglycans (GAGs) and collagen production[18]. The application of cyclic HP (CHP) on MSCs can lead to an increase in chondrogenic gene expressions such as aggrecan and collagen type II[19]. Under CHP of 10MPa, Carroll et al.[20] investigated the influence of in vivo CHP in order to determine the terminal phenotype of chondrogenically primed MSCs. Huang et al. [21] studied the effect of HP applied via perfusion bio-processors on the in vitro human bone marrow-derived MSCs. It was found that HP enhanced cell viability as well as improved osteogenic differentiation and maturation. It is noted that long term application of HP can boost the functional properties of cartilaginous tissues engineered using bone marrow-derived MSCs, by enhancing collagen and GAG accumulation[22]. In addition, CHP and cyclic tensile strain differentially regulate the expression of hypertrophic markers on primary chondrocytes[23]. Smith et al. [24] reported the intermittent effects of HP in bovine chondrocytes. Results show that pressure stimulates cells and regulate the mRNA level of aggrecan and collagen type II.

Besides external physical stimuli, culture technique is seen as a key factor during cell culture. HP can be applied to different forms of chondrocytes: monolayer, 3D culture, polyglycolic acid (PGA) mesh, and explants. In scaffold culture, composition, and architecture create different responses to mechanical stimulation of chondrocytes [25].

The chondrocyte is often time cultured in a high-density form such as pellets. Steven et al. [26] applied CHP to pellets and alginate chondrocyte culture and studied their response. The pellets of chondrocytes exhibited an anabolic response to dynamic HP while the alginate ones did not. Puetzer et al. [27] investigated the effects of CHP on chondrogenic differentiation of human adipose-derived stem cells (ADSCs) in three-dimensional (3-D) agarose constructs, maintained in a complete growth medium without soluble chondrogenic inducing factors. The finding highlights the importance of external stimuli along with the appropriate 3D culture for the engineering of cartilage tissue. This paper focuses on the application of HP on primary chondrocytes, as conducted on senior patients. The investigation aims to observe the effect of intermittent HP on improving the quality of aging chondrocyte cells. The applied HP is 1MPa of 1Hz frequency. The synthesis of the ECM is observed through the relevant structural proteins i.e. aggregan, collagen-I, collagen-II. These proteins are used to determine the changes which are affected by the applied intermittent HP.

II. METHOD

A. Design of pressure chamber

A pressure chamber was designed to apply intermittent HP in the range: 0 to1 MPa at 0-1 Hz frequency. The pressurized chamber can handle the culture cell through two 12-well plates with maximum dynamic pressure at 1MPa. A stainless-steel vessel (Figs. 1a,b) was developed. Its inner dimension measured (90x130x52) mm, which was the minimum volume capable for two 12-wells plates. By using a stainless-steel rack, two culture plates were aligned vertically, side by side, in the pressure chamber. As shown in Fig.1b, an O-ring and six toggle latches were used to seal the pressure inside.

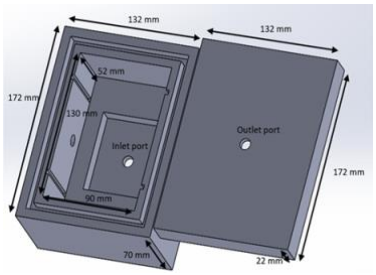

Figure 1a. A three-dimensional model of hydrostatic pressure chamber.

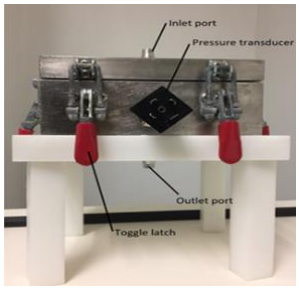

Figure 1b. A sealed device before connecting with pneumatic system.

The designed chamber was validated by Solidworks simulation based on cyclic pressure of 1 MPa for 100,000 cycles.

B. Pressurizing system

As shown in Fig.2, the chamber is connected to a pneumatic system. An air tank filled with in-house air is connected to a high-pressure air compressor (MCH EM6, Coltri). The quality of air in the chamber is sterile, and is achieved via 3 Mist separator filters (AF20, AFD20, and AFM20, SMC) and 0.2μm hydrophobic PTFE membrane filter (IPF, TS Filter).

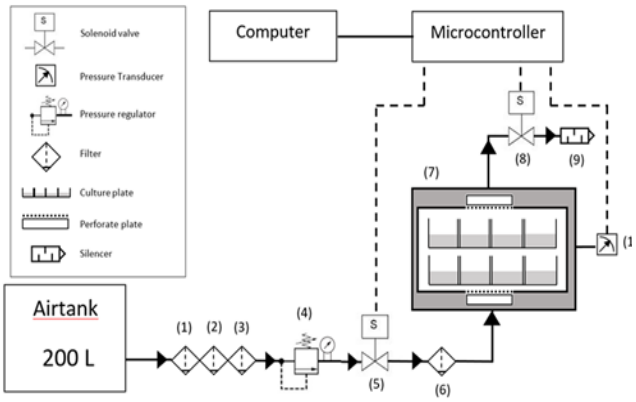

Figure 2. The schematic of the pneumatic system.

The system provided pressure by opening valve No.(4) so the bulk airflow would flow through the initial filtration system, which consisted of: (1) particle separator (2) mist separator, and (3) micro-mist separator. Then, the air passed through the solenoid valve No.(5). The signal from the microcontroller operated the valves and controlled the air that entered chamber No.(7). Before entering the chamber, the air passed through valve No.(6) to sterile the inlet air. Next, the outlet air was controlled by solenoid valve No.(8). Valve No.(9), a silencer, was used to reduce noises that occur while the air was being re-leased from the system. Valve No.(10), a pressure transducer, was used to measure pressure changes throughout the investigation, combined with an Arduino microcontroller. Thus, the pressure level was monitored and displayed on a computer.

Pressure was released into the chamber through a pressure regulator. Both the magnitude and frequency of the pressure were controlled by a solenoid valve (5404, Burket) through an Arduino microcontroller. The frequency could be varied up to 1Hz to create intermittent pressure. The internal pressure in the chamber was real-time, captured by a pressure transducer (NP620, Novus). A honeycomb plate and a polypropylene (PP) membrane were attached to the inlet and outlet port of the pressure chamber to prevent jet impingement. The flow direction of the chamber was designed to flow from the bottom to the top, and the polytetrafluoroethylene (PTFE) membrane was used to cover the top of the culture plates.

C. Chondrocyte cell culture

In the investigation, chondrocyte cells extracted from cartilages of three patients were cultured. The cells were in passage three. For each patient's cell, the cells were seeded in four 24-wells plates (2 plates for treatment group and two plates for control), each with a density of 100,000 cells/cm2.

Then, the cells were cultured inside an incubator for three days. In Fig.3, the process is shown. Treatment started on the fourth day after the chondrocytes were seeded on the plate. The treatment group was placed inside the designed HP system and intermittent HP of 1MPa and frequency of 0.5Hz for 90 minutes was ap-plied, accordingly. After each day's treatment, four wells of the treatment group and the control group were collected for real-time polymerase chain reaction (RT-PCR), within 1 hour after the treatment finished. Next, the rest of the plates were placed back inside the incubator. The process was repeated and the same procedures were carried out, for a duration of eight days. After the 8th day of the seeded chondrocytes, the experiment was completed. During the trial, control cultures were maintained outside the incubator and placed without receiving the treatment. Simultaneously, the same amount of samples were collected. The property of media was confirmed throughout all experiments to be around 6.8-7.1pH.

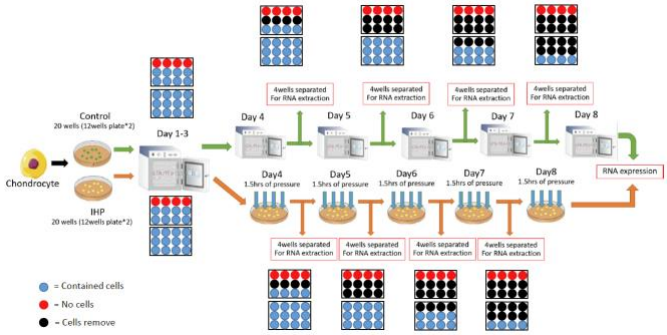

Figure 3. The diagram of experiment depicts the duration of giving hydrostatic pressure to the cell in each condition

III. Results

Collagen type II and aggrecan were used as biomarkers for the chondrocyte phenotypes 12. After collecting RT-PCR data from each patient, the data was normalized by the GAPDH gene, and the results were presented. Figs.4 (a-e) show a comparison of gene expression between control and treatment group for each day.

Patient No.1 is a 60 years old male patient who has undergone knee replacement surgery. Figs. 4 (a-e) present five days comparison between the applied HP chondrocyte cells, as compared to the control group. According to the results, as presented in Fig.4, the level of gene expression proved to be relatively the same between the two groups (control group and the treatment group). In order to increase the level of genes, it was found necessary to apply pressure along with an appropriate amount of treatment time. For example, after receiving treatment during the first two days, cells that received HP were seen to have raised the level of aggrecan and collagen type II. When HP treatment was carried out longer than two days, a significant drop in all gene expression was observed. However, during the 4th day and the 5th day, the level of all gene expression appeared to be about the same. It is clear that receiving treatment each day generated a decrease in lower expression of aggrecan. In contrast, the control group showed various changes.

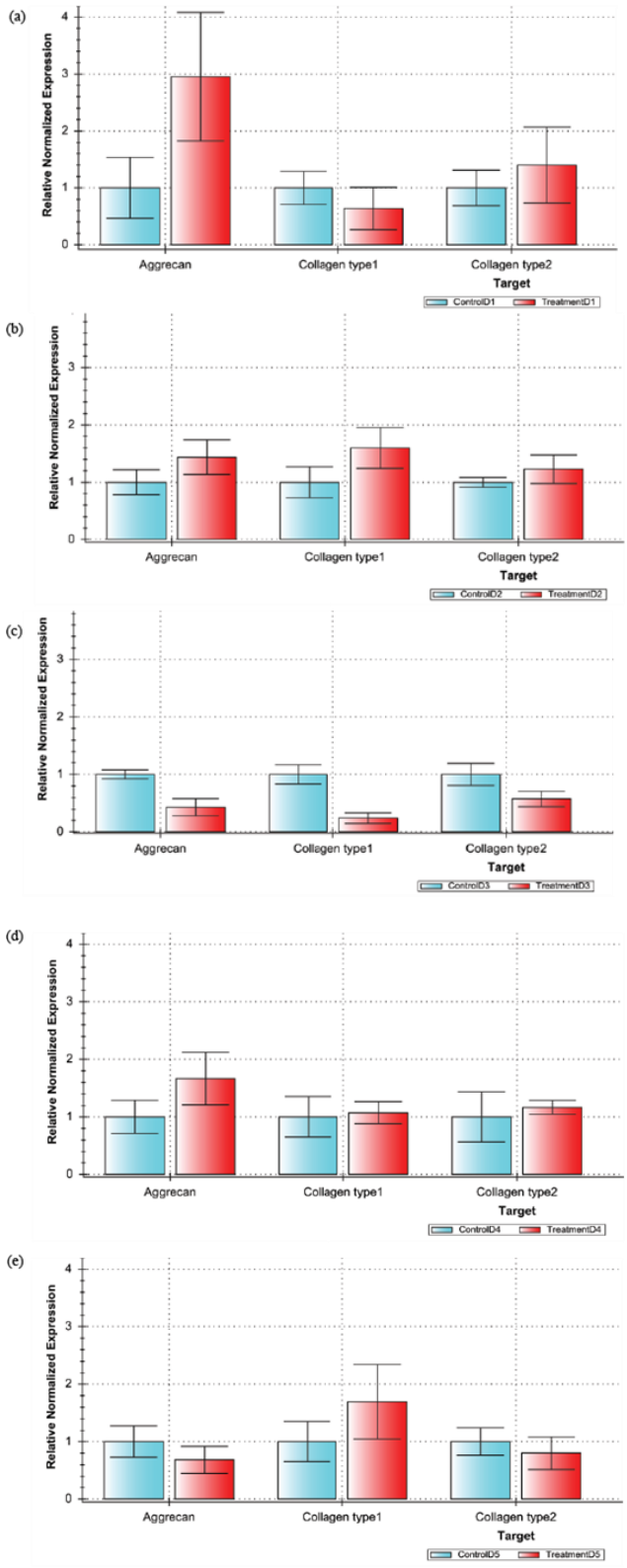

Figure 4. Patient #1 gene expression of the treatment and the control group during five days treatment (a) day 1, (b) day 2. (c) day 3, (d) day 4, and (e) day 5

Even though there was a significant drop in the treatment group on the third day, the cells that received treatment tended to have had the level of collagen type I increased. The control group, however, had seen a decrease in collagen levels. In Fig.4, treating the cell with HP did not significantly alter this

decreasing trend of collagen type II. Both groups appeared to record relatively the same collagen type II level.

Patient No.2 is a 79 years old female patient who has undergone total knee replacement surgery. In Figs.5 a-e, comparison between the treatment sample that received HP treatment up to 5 days and the control group is presented.

According to Fig.5, the application of HP had shown a significant increase in gene expression only when giving load. Both the control and treatment group were found to have shown a similar trend with regard to gene expression. It was noted that HP could maintain the level of aggrecan in the treatment groups (Figs.5). Both control and treatment groups shared the same trends. However, after 3days of receiving load collagen type I, the treatment group increased more than the control group. Similar to the results found in patient#1, treating the cells with HP did not tend to decrease collagen type II.

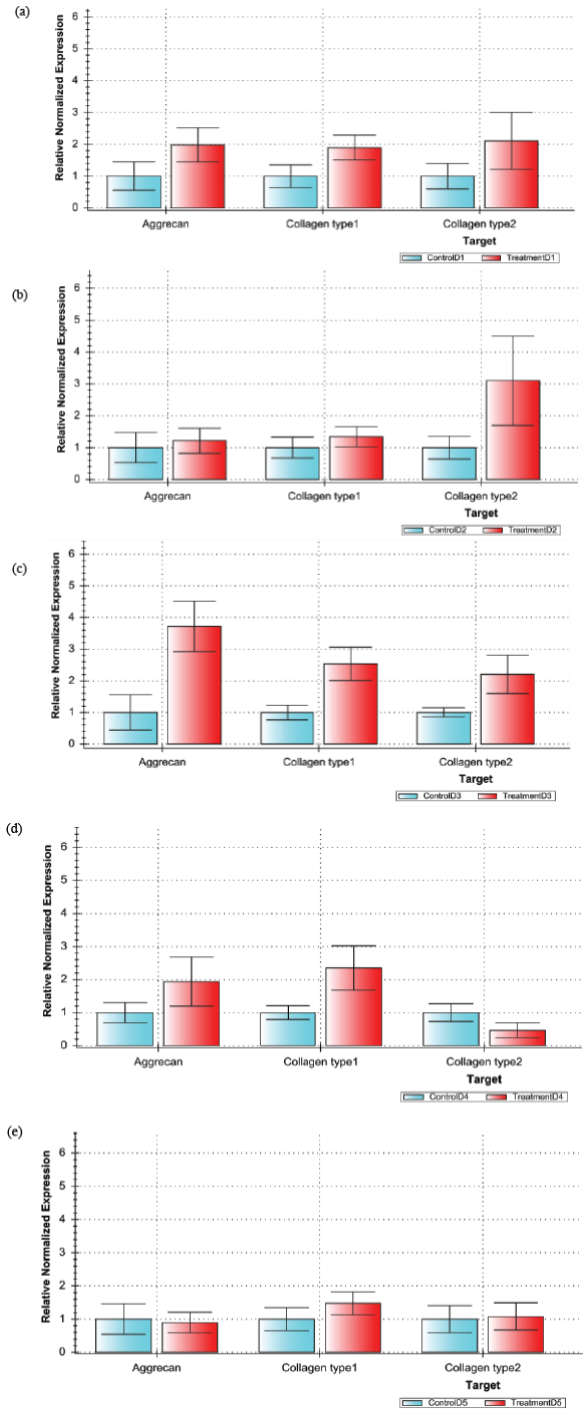

Figure 5. Patient #2 gene expression of the treatment and the control group during five days treatment (a) day 1, (b) day 2. (c) day 3, (d) day 4, and (e) day 5

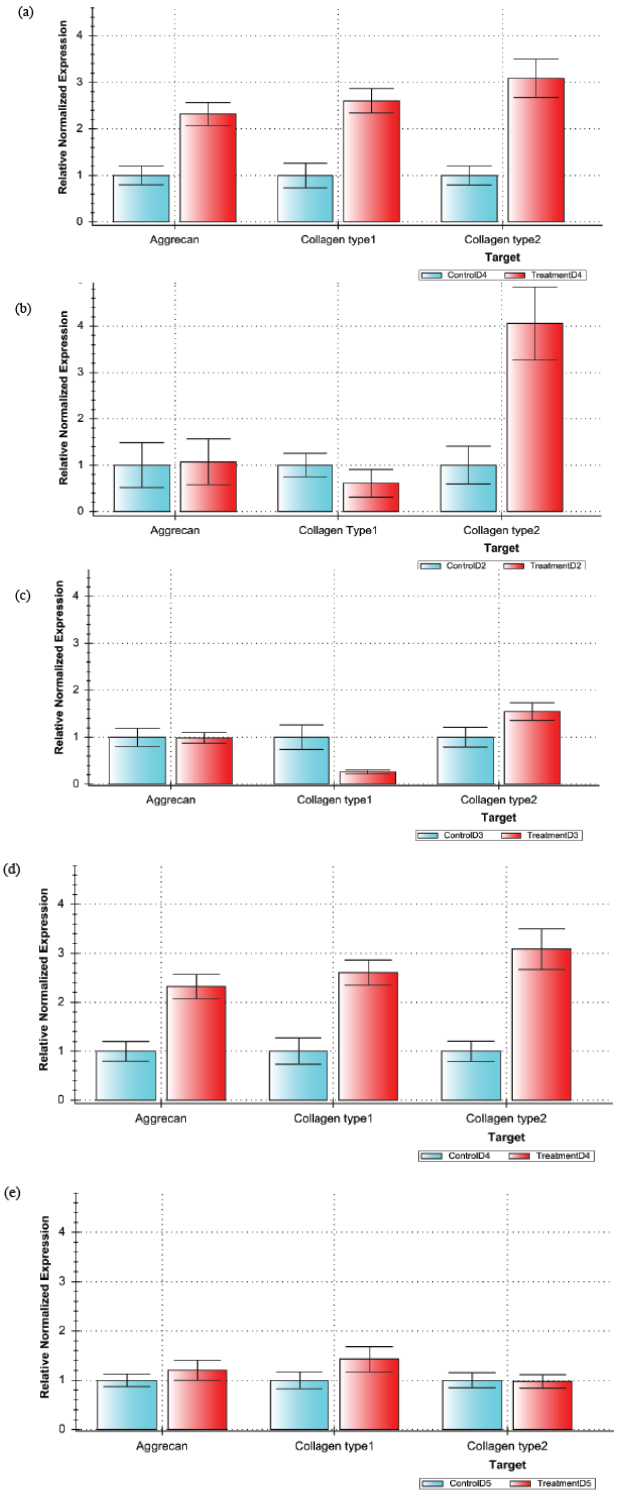

Figure 6. Patient #3 gene expression of the treatment and the control group during five days treatment (a) day 1, (b) day 2. (c) day 3, (d) day 4, and (e) day 5

Patient No.3 is a 71 years old female patient who has undergone total knee replacement surgery. In Figs. 6 a-e, comparison between the treatment sample that received loads up to 5 days and the control group is presented. As shown in Fig.6, the application of HP on chondrocyte cells also resulted in a significant increase in aggrecan and collagen type II levels. HP caused an increase in collagen type I. On days 2 and 3 of the investigation, collagen type II, in the control group, was found to develop. Yet, a day later, a significant drop was seen to take place. It is evident that collagen type II in both groups reached its peak after receiving treatment up to 2 days. The following day collagen type II continuously dropped.

It is noted that the observed protein expression found after HP had been applied in the aged chondrocyte cells were relatively similar to previous work [11]. The effect of HP on aged chondrocyte cell has not been previously investigated and therefore this experiment is the first to confirm the effect of HP on aged chondrocyte cells. Collagen type II and aggrecan are considered important factors for synthesis of ECM. Thus, intermittent HP is seen to promote the expression of collagen and aggrecan in primary chondrocyte cells. However, for each day of loading, it was found that the gene expression recorded a different result. Based on the results of our investigation, intermittent HP contributed to the upregulation of collagen type II and aggrecan depending on the day stimulation was applied.

Results, however, did not show the improvement in the relevant structural protein level, after the third day in which the stimulation was applied. The number of day or duration which HP applied on the cells hence plays significant role. The results appeared that the highest protein regulated during the first three day that cells received HP and stayed unchanged afterward. This outcome is in contrast to previous work by Ikenoue et al. [11] which applied HP on mono layer cultured of human articular chondrocyte cells. They found that small magnitude of HP of 1MPa of 4 hours played no role in upregulation of collagen type-II mRNA signal. While it on the other hand effect the increase in level of aggrecan mRNA level. The differential regulation of the individual proteins was observed in previous work, but not in our case. The hypothesis of HP stimulation on aged chondrocyte cells are there-fore relevant. The results confirm that intermittent HP is an effective stimulus for matrix protein synthesis in aged chondrocyte cells. However, the chondrocyte cells used in this work were from three senior patients male and female. Since quality of chondrocyte cells are different from person to person, male/female, and various health conditions. Hence, future studies should include the investigation of the effect of HP on specific group of sub-jects.

IV. Conclusion

This paper investigates the effect of HP on aging cells in elderly subjects. A bioreactor which can provide intermittent HP on the chondrocyte cells was developed. Results highlighted the fact that HP can help regulate collagen type II and aggrecan in primary chondrocyte cells. A bioreactor was successfully designed and developed that could ap-ply HP on the aging cells. Thereby, changes in protein expression in the chondrocyte cells of senior patients were observed. During the first three days, protein expression was enhanced and eventually stayed the same. Results, based on three senior patients whose ages are 60 and above, reveal that HP had little effect on upregulating the hyper-trophic markers of chondrocyte cells among seniors. In contrast to the results found by Ikenoue et al. [11], the applied stimulus demonstrated little effect on upregulating the ex-pression of aggrecan, collagen type-I, and collagen type-II. It is evident that since proper-ties of the cells range from person to person, more experiments are required to confirm that HP can be applied as part of the mechanical stimulation to promote cartilage repair and regeneration among senior patients.

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