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Designing and validation of an automated ex-vivo bioreactor system for long term culture of bone

Rupak Dua [a,*], Hugh Jones [b], Philip C. Noble [b]

[a] Department of Chemical Engineering, School of Engineering & Technology, Hampton University, Hampton, VA, USA
[b] Center for Orthopaedic Research, Innovation and Training, McGovern Medical School, UTHealth, Houston, TX, USA

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ABSTRACT

Several different bioreactors have been developed to study bone biology. Keeping a bone viable for long-term studies is still a challenge. We have developed an ex-vivo bone bioreactor that can keep the ex-vivo live bone viable for more than 4 weeks. Keeping a bone viable for over a month can be used as an alternative model for in-vivo experiments in animals. We hypothesize that the perfusion flow and mechanical load on the bone provide a real-time environment for the bone to survive. Cancellous bones were harvested from the bovine metatarsals and were placed in the dynamic culture with cyclic loading at regular intervals. After a period of week 4, the bone cores were retrieved from the bioreactor and tested for viability using calcein-AM and ethidium homodimer −1 fluorescent dyes and were compared with the cores that were placed in static culture with and without any loads on them and Day 0 bone core that acted as a positive control. The bone blocks were then fixed in 10% formalin, and bone mineral density was evaluated using a DXA scanner before staining them for H&E to study the morphological changes. Results revealed that the bone cultured in the bioreactor was viable as compared to the one in the static culture with and without constant load. Bone cores cultured in our ex-vivo bioreactor system also maintained their morphology and no statistical difference was found in the bone mineral density compared to positive controls and the statistical difference was found when compared with the cores cultured in static culture. This tool can be used to study bone biology for various applications such as bone ingrowth studies, to study the effect of drugs, hormones, or any growth factors, and much more.

1. Introduction

The study of bone biology in its 3D functional form is essential for the investigation of bone growth factors and hormones, inflammatory responses, antibacterial therapy, bone ingrowth studies, cancer metastasis, and many more (NODA and CAMILLIERE, 1989; Holen et al., 2015; Widmer and New developments in diagnosis and treatment of infection in orthopedic implants, 2001; Masri et al., 1998). However, the progression of any research to animal studies is hindered by significant costs and complications. 2-D cultures of osteoblasts offer an alternative, but this approach fails to replicate the native mechanical, morphologic, and multicellular environments that influence cellular response (Sorkin et al., 2004; Sherr and DePinho, 2000). Ex-vivo organ culture allows the potential to study bone in its functional state, but the maintenance of long-term viability can be challenging (Roux et al., 2010).

Perfusion media flow and mechanical stimulation are the two critical components for maintaining bone ex-vivo. Perfusion flow is carried out in the tissue through the network of porous channels. In our case, the ex-vivo bone used for the study has a well-developed network of channels providing natural perfusion conditions (Smith et al., 2018). Perfusion flow facilitates the transporting of nutrients and removes waste, thus preventing the localized buildup of lactic acid due to cell metabolism that can cause cell death (Freyria et al., 2005; Cartmell et al., 2003). The supply of oxygen and soluble nutrients continuously to the bone tissue is critical to keeping it viable (Ratcliffe and Niklason, 2002). Additionally, it is well established that in vivo, bone cells respond robustly to dynamic mechanical stimuli (Robling et al., 2001a; Rubin and Lanyon, 1984), leading to enhanced formation of mineralized matrix (Bancroft et al., 2002; Sikavitsas et al., 2003). However, their sensitivity to the stimulus declines quickly after its initiation (Turner, 1998). Robling et al. in one of their studies, highlight two critical points about the mechanosensitivity of bone. First, bone loading sessions need not be long to maximize bone formation. Second, extending the loading session beyond a few minutes does not provide any additional osteogenic effect (Robling

* Corresponding author at: Department of Chemical Engineering in the School of Engineering and Technology, Hampton University, Hampton, VA 23668, USA.
E-mail addresses: rupak.dua@hamptonu.edu (R. Dua), Hugh.L.Jones@uth.tmc.edu (H. Jones), philipnoble66@gmail.com (P.C. Noble).

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et al., 2001b) but instead leads to saturation of the bone cell's mechano-sensitivity. Hence, recovery periods are very important for restoring the mechano-sensitivity of desensitized bone cells.

Several bioreactors incorporating perfusion flow and/or mechanical stimulation have been developed for bone tissue engineering and bone biology-related studies as an alternative to animal studies (Smith et al., 2018; Jones et al., 2003; Janssen et al., 2006; Schnieders et al., 2013; Yu et al., 2004). Still, none can provide programmable fluid flow, mechanical loading, and media exchange all together in an automated fashion. Therefore, we have constructed an ex-vivo organ culture system that can keep the bone viable for long term in an automatic manner with little maintenance. Our ex-vivo organ culturing system provides the biochemical and mechanical environment necessary to maintain the viability of bone samples for over 4 weeks. Some of the salient features of our system include quadrangular shaped specimen chambers to induce laminar fluid flow, autoclavable polysulfone materials, programmable perfusion flow rate, a sterile media exchange system and programmable pneumatic actuators that can provide adjustable intermittent mechanical stimulus. This bioreactor system potentially provides a means to empirically test implantable orthopedic devices during the design process in a cost-effective manner. This system can serve as an indispensable tool in studying and developing orthopedic devices requiring fixation through ingrowth. It can also serve as a tool for several other applications such as studying the effect of growth factors and hormones on bone regeneration, studying cancer metastasis, and testing local antibiotic drug delivery systems to treat bone infections.

The purpose of this research paper is to describe the design and operation of an ex-vivo bone bioreactor system capable of keeping bone viable for at least 4 weeks. Its automatic loading and perfusion flow systems are described along with a preliminary study to evaluate and validate the bioreactor system as a whole by maintaining harvested bovine metatarsals.

2. Material and methods

2.1. Ex vivo bone bioreactor design

Figs. 1 & 2 show the assembling and the schematic of an ex-vivo bioreactor system. The developed ex-vivo bone bioreactor system is capable of batch testing eight specimens. The system consists of eight specimen chambers that mount easily on an aluminum base plate using wing nuts.

Each specimen chamber is made of polysulfone (McMaster-Carr, Atlanta, GA), which is MRI, X-Ray, and autoclave compatible. The internal shape of the specimen chambers is conical with a large diameter of 5/8 in. tampering at 60^{o} to 65^{o} giving it a quadrangular space for the promotion of laminar fluid flow to prevent any turbulence that hampers the growth of cells. Previous studies have demonstrated that turbulence or eddies within a bioreactor chamber can lead to the formation of high mechanical forces and potentially cause disruption of cell membranes or even death of cells (Brindley et al., 2011; Born et al., 1992).

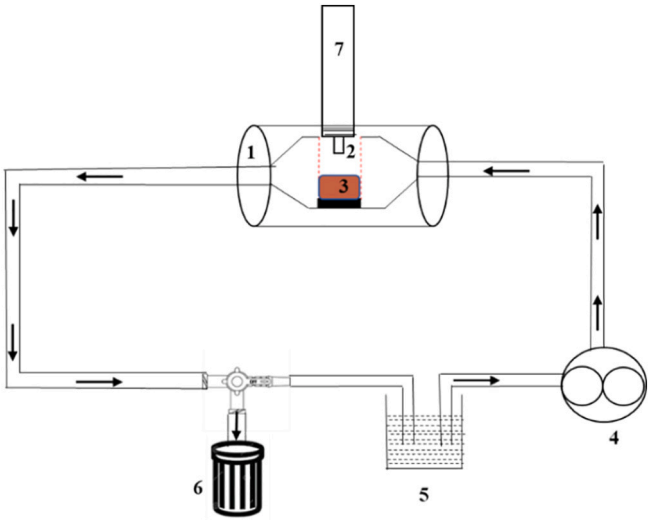

Fig. 2. Schematic view of the setup of one complete specimen chamber in an ex-vivo bioreactor system 1. Specimen chamber, 2. Polycarbonate holder, 3. Bone Core. 4. Peristaltic pump, 5. Media container, 6. Waste container, 7. Pneumatic Actuator.

Additionally, another study suggested (Hidalgo-Bastida et al., 2012) that a quadrangular shaped chamber is superior to the cylindrically shaped chambers that are most commonly used in other bio-reactor designs. The specimen chamber is also bi-valved, allowing loading and confinement of the test sample into cylindrical polycarbonate holders with holes directly under the later described pneumatic actuator. The two inner-locking components have a circular O-ring in-between them to prevent leakage when assembled. Each part has a port for the medium, one port for introducing the medium into the chamber, and the other allows exit.

2.1.1. Media exchange and removal

The design of the system enables the sterile exchange of media to avoid contamination, which is the major complication of long-term ex-vivo experimentation using bioreactors. Fresh media is introduced into individual vented media reservoirs via hypodermic injection ports using a hydrophobic membrane that provides gas exchange (50 ml centrifuge tube, Fisher Scientific, Waltham, MA medium is delivered to the specimen chamber by a multichannel peristaltic pump (Cole Parmer FH100M, Vernon Hills, IL) via platinum cured silicon tubing (Masterflex tubing, Cole Parmer). This tubing has a high permeability to both oxygen and carbon dioxide, thereby ensuring adequate gas equilibrium between the media and the surrounding ambient incubator air. Besides, this tubing is also low protein binding to avoid adsorption of proteins to the tubing itself.

Each specimen chamber has its own separate medium supply to avoid cross-contamination but one common collector for waste and used

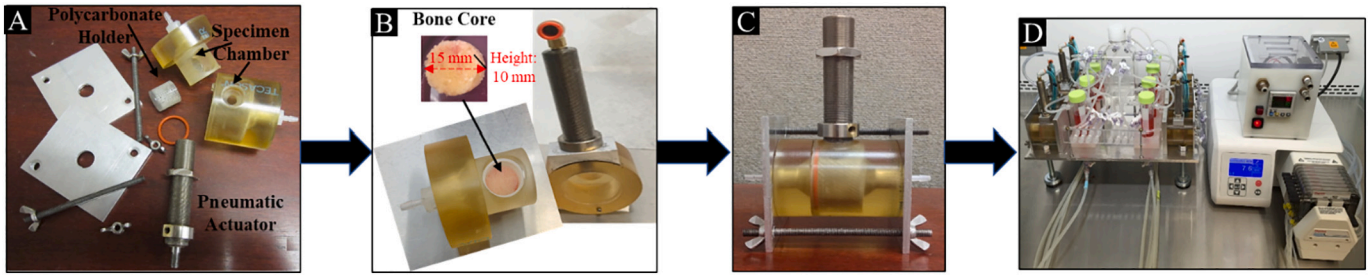

Fig. 1. Ex-Vivo Bone Bioreactor System. A) Individual components of a Specimen chamber, including specimen chamber, polycarbonate holder, pneumatic actuator, holders and wing nuts. B) Loading mechanism for a bone core (Diameter 15 mm and Height: 10 mm) into the specimen chamber C) Assembled Specimen Chamber. D) Complete assembly of an ex-vivo bone bioreactor system with 8 specimen chambers with a peristaltic flow pump, media containers and waste container.

medium. This also enables us to add or pull any one chamber from an experiment at different time points.

2.1.2. Flow rate

The bioreactor has a programmable perfusion flow rate. The medium is continuously circulated using an 8 multichannel peristaltic pump. This pump is programmable and capable of providing flow rates from 0.002 to 760 ml/min per channel. For our study, the flow rate was set at 1 ml/min. Previous literature has shown that high flow rates will induce shear stress to bone cells (Kapur et al., 2003), and that continuous application of shear stress can be detrimental to the health of the cells and may lead to the saturation of their mechanosensitivity (Robling et al., 2001b; Plunkett et al., 2009). It has been found that a flow rate of 1 ml/min will not introduce the effective shear stress for overstimulating bone cells (Bancroft et al., 2002; Jaasma et al., 2008).

2.1.3. Loading mechanism

Each specimen chamber is equipped with miniature pneumatic cylinders (Mead USA, Chicago, IL) with a ½ inch bore size, ½ inch rod diameter and stroke of 2 in. that draw air from an eight-outlet manifold supplied by a regulated programmable logic controller solenoid (AutomationDirect, Cumming, GA). For our study, the air pressure was set at 16 psi, generating a force of 18.4 lbf (81.8 N) on our bovine bone sample. These values were based on previous in-vivo studies done on the dynamic loading of rat bone using a peak threshold load 12.1 lbf (54 N) (Robling et al., 2000; Turner et al., 1994). In addition, the force applied in this system can also be adjusted by changing the input pressure to the pneumatic cylinder. The controller was programmed to load cyclically at 1 Hz with a 10 s recovery period in-between cycles for 15 min every 8 h (Robling et al., 2001b; Endres et al., 2009; Robling et al., 2002). This loading regime provided both the short and long-term recovery periods necessary for bone cells to restore their mechanosensitivity.

2.2. Bone samples

Cylindrical cores of trabecular bones explants (diameter:15 mm (0.59 in.); height:10 mm (0.39 in.)) were harvested from bovine metatarsals in a sterile manner. Briefly, the bovine metatarsals were retrieved from an abattoir on the same day of slaughter. Soft tissue was removed from the bone and a 15 mm hole saw was used to extract the core. A holding jig and a reciprocating saw were then used to cut the cores to length at 90 degrees. While cutting, the bone was continuously irrigated with 0.9% NaCl solution at 4 °C to counter heating and prevent bone debris from clogging the pores. After shaping, the bone cores were immersed in Phosphate Buffered Saline (PBS) (Sigma Aldrich, St Louis, MO) with 5% antimycotic solution (Sigma Aldrich). They then were washed twice with PBS at 37 °C for 10 min and a third time with PBS with 5% antibiotic solution for another 10 min. The cores were finally placed in a 6 well plate in 87% of DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) basal media containing L-glutamine, HEPES combined with 10% FBS and 3% antimycotic solution supplemented with 5 mM β-glycerophosphate, 5 mg/L ascorbic acid, and 0.12 g/L sodium hydrogen carbonate and placed in a humidified environment (37 °C and 5% CO_2) for 24 h to acclimatize.

2.2.1. Static culture of bone cores

Twelve of the acclimatized bone cores were divided into three groups of four (4) and allocated to static loading trials. The first group consisted of just the bone core placed in static culture (Group1). The second group consisted of unloaded bone cores placed in contact with a coupon (1 cm diameter) of porous-coated titanium fiber wire derived from cementless hip prostheses (Zimmer Inc., Warsaw, Indiana) (Group 2). The third group consisted of the bone core with a contacting metal fiber wire implant coating coupon with an applied static load of 3.3 lbs. (Group 3). All samples for the three different groups were placed in the 6-cell culture well plates. The media was changed every 7 days, and their pH was measured.

2.2.2. Dynamic culture of bone cores

Four of the acclimatized harvested bone cores ($n = 4$), after 24 h of being in medium, were exposed to cyclic loading in contact with the fiber-metal coupons in the specimen chamber of the ex-vivo bone bioreactor (Group 4). The bioreactor was placed in an incubator at 37 °C and all necessary tubing connections were made. Using the programmable pneumatic controller, cyclic loading of 3.3lbs was applied at 1 Hz for 15 mins with 10 s recovery periods between cycles every 8 h. The media was changed every 7 days using the injection ports and the valve system by opening the incubator. This change of media was done quickly to minimize effects to the temperature and gas equilibrium with the bioreactor. This period was a small fraction of the 168-h period between media changes.

2.3. Viability of bone core

A Live-Dead assay was performed on bone cores retrieved from the bioreactor and static cultures using Calcein AM/Ethidium homodimer (Life Technologies) staining according to the manufacturer's protocol. Briefly, samples ($n = 4$) from each group at week 4 were recovered, washed with PBS, stained with Calcein-AM and Ethidium homodimer in PBS solution, and incubated for 30 min. Each sample was then washed three times with PBS solution to remove the background fluorescence and subsequently visualize under the confocal fluorescent microscope (Olympus FV-1000, Olympus America Inc., Miami, FL) at excitation and emission wavelengths of 468 nm and 568 nm, respectively (Nieto et al., 2015).

2.4. Cell toxicity

The toxicity of each bone cores was evaluated by measuring the change in the pH of the medium that was collected every 7 days over the 28 days of incubation. The difference in the pH is an indication of the change in the microenvironment of the bone. Acidification has been shown to be associated with the onset of the apoptosis of the cells in culture (Simpson et al., 1997; Perani et al., 1998; Naciri et al., 2008).

2.5. Morphological assessment

The morphological changes of the bone cores over a period of 4 weeks were evaluated using histological H&E staining. A Day 0 bone core served as a positive control. In brief, the bones were harvested from the bioreactor and the static groups after 28 days. They were fixed in 10% formalin and were decalcified. The decalcified bone cores were embedded in paraffin, and 10 μm sections were cut using a microtome. The sections were subsequently stained with hematoxylin and eosin (H&E) according to the manufacturer's protocol (ScyTeck Laboratories, UT). The images were viewed under a bright field microscope, and representative images of each group's bone cores were visualized, and a comparison of the morphology of all the group samples was conducted.

2.6. Bone mineral density

The bone mineral density of each group was determined using a DXA scanner (Delphi, Hologic). Concisely, the samples were fixed in 10% formalin and washed with PBS thrice. A regional high-resolution x-ray with line spacing and point resolution of 0.0311 cm each was conducted for a scan area of 4 cm to cover the whole sample. The cross-sectional area of the cylindrical bone core was used to calculate the bone mineral density area mean in g/cm^2. This was further divided by the sample's actual thickness measured using calipers to get a value of bone mineral density in units g/cm^3. Each sample was scanned three times, and the average value was used in our calculation.

2.7. Statistics

All data are expressed as mean ± standard error of mean (SEM). Statistical analyses of the results obtained from the bone mineral density were performed using commercially available software (SPSS, IBM, version 20, Armonk, NY). A one-way ANOVA and post hoc Tukey test was used to compare means and to determine statistically significant differences ($p < 0.05$) between groups, respectively (Dua et al., 2014; Dua and Ramaswamy, 2013).

3. Results

3.1. Cell viability

Representative images from each group have been shown in Fig. 3. The viability of the bone cores was maintained after 28 days, with the bioreactor samples showing far fewer dead cells (Fig. 3). We found similar results in the Day 0 bone core, which was a positive control. Additionally, the cell's morphology can be seen more clearly in the bone cores retrieved from the cyclic loading bioreactor.

3.2. Cell toxicity

The pH of the medium (control) was found to be 7.32 ± 0.02. At week 1, the pH of all four groups was stable and ranged from 7.18 to 7.24. (Table 1). Over a period of 4 weeks, the value remained consistent for the bioreactor group samples, but there was a progressive decrease in the pH of all the other static groups at every time point. At week 4, the pH value dropped below 7 for the static groups and was acidic while it remained at 7.21 ± 0.01 for the bioreactor group. There was a statistical decrease in the pH value of group 1 when compared with the media of the bioreactor group in week 3 and week 4.

Table 1
Measurement of pH in the media ($n = 4$) during incubation of bone cores over 4 weeks for four groups, means ± SE.

Timepoint of sampling	Group 1 Media	Group 2 Media	Group 3 Media	Group 4 Media
Week 1	7.12 ± 0.06	7.20 ± 0.08	7.24 ± 0.02	7.24 ± 0.02
Week 2	6.90 ± 0.05	7.08 ± 0.15	7.23 ± 0.01	7.21 ± 0.01
Week 3	6.81 ± 0.02	7.08 ± 0.14	7.20 ± 0.02 *	7.20 ± 0.01*
Week 4	6.68 ± 0.03	6.92 ± 0.11	6.93 ± 0.14	7.21 ± 0.01*

"*" indicates that the difference between the group compared with Group 1 was significant ($P < 0.05$).
Group 1 - Bone core in static culture; Group 2 - Bone core in contact with a wire mesh scaffold in static culture; Group 3 - Bone core with a contacting metal fiber wire implant coating coupon with an applied static load of 3.3 lbs. in static culture; Group 4: Bone core in Dynamic culture in an ex-vivo bone bioreactor.

3.3. Morphological assessment

H & E histology images at 20× objective magnification revealed that when compared to Day 0, nuclear shrinkage was occurring in the static groups. The bioreactor group appeared similar to the positive control (Fig. 4).

3.4. Bone mineral density

The bone mineral density of the bone cores for four groups was measured at Day 28. Statistically significant differences were found between the bone cores cultured in the bioreactor with dynamic loading and the bone cores cultured in static culture. There was also a statistically decrease in the bone mineral density of the bone cores cultured in

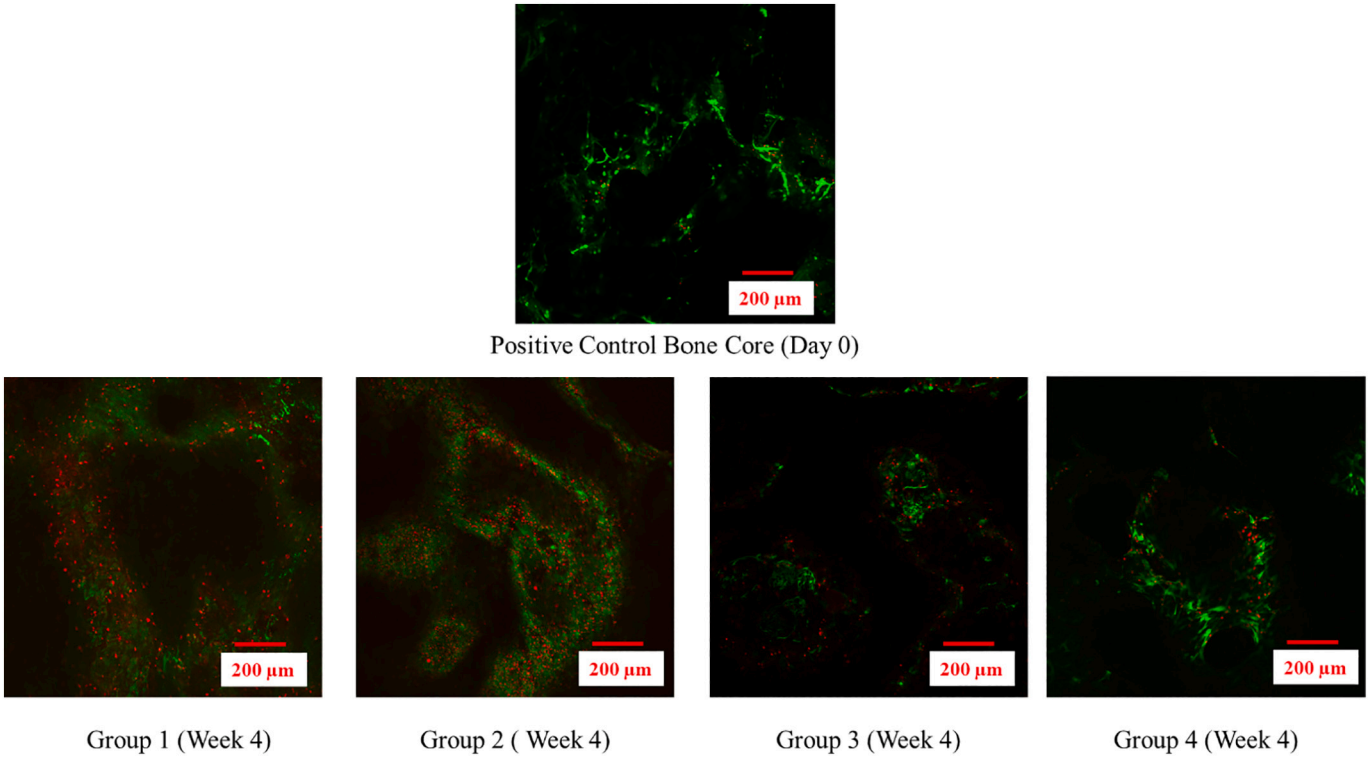

Fig. 3. Live Dead Assay of bone cores for different groups. Bone core in static culture. Group 1 consists of bone core in static culture. Group 2 consisted of bone cores with a contacting metal fiber wire implant coating coupon. Group 3 consisted of the bone core with a contacting metal fiber wire implant coating coupon with an applied static load of 3.3 lbs. Group 4 consisted of bone cores under dynamic loading in bioreactor. Green color indicates live cells while red color indicates dead cells.

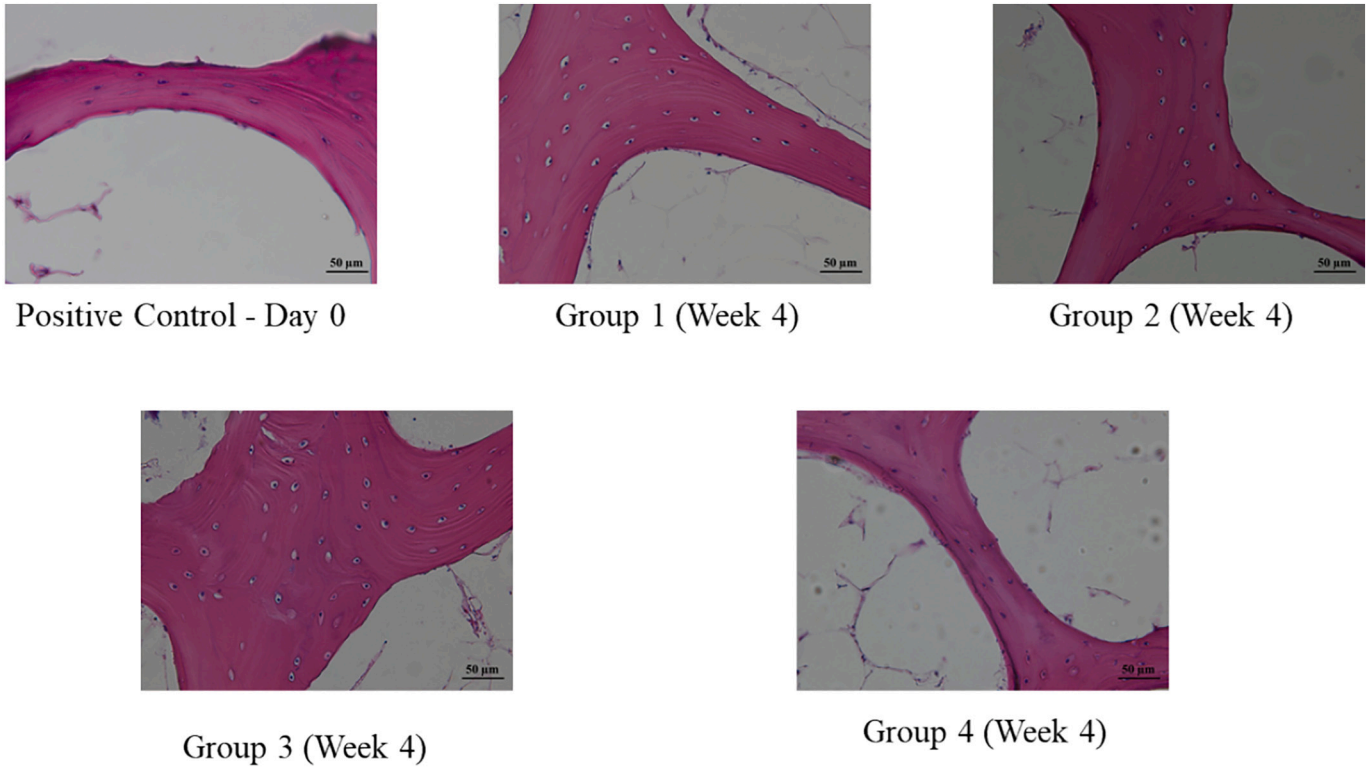

Fig. 4. H&E Staining for different groups. Blue dots indicate the nucleus of the cells.

static culture compared with the Bone mineral density values of bone cores at Day 0. However, there was no significant difference found in the bone mineral density of the control group at Day 0 and cores that were cultured in the bioreactor for 4 weeks (Fig. 5).

4. Discussion

Bioreactors have been extensively used in tissue engineering and regenerative medicine applications. However, very few groups have used bioreactors for long term organ culturing. Some of the more prominent work with bone bioreactors has been described here: Bancroft et al (Bancroft et al., 2002) used a perfusion flow bioreactor to increase mineralized matrix deposition in 3D tissue engineering scaffolds by inducing shear stress based on the fluid flow. Another study conducted 3-D computational modeling of media flow through scaffolds in a perfusion bioreactor and found that an average surface shear stress of 5×10^{-5} Pa corresponds to increased cell proliferation. In contrast, higher stress leads to upregulation of bone marker genes (Porter et al., 2005). Davidson et al *(Davidson et al., 2012)* investigated the use of perfusion flow bioreactor for keeping a murine femur viable for 14 days and suggested a novel method for organ culture in vitro. Another group developed a bioreactor platform capable of providing direct perfusion to tissue constructs and proper culture conditions to fabricate primary tissues of any design with minor adjustments (Smith et al., 2018; Sego et al., 2020). These bioreactor systems were solely based on fluid-induced shear stress.

Another group (Schulz et al., 2008) developed a bioreactor for tissue engineering of chondrocytes constructs based on loading and perfusion flow together, while others (Orr and Burg, 2008) developed a modular bioreactor incorporating both perfusion flow and hydrostatic compression for tissue engineering applications. Jones et al (Jones et al., 2003) developed a culture loading system to keep the bovine trabecular bone viable for extended periods (20 days) (Smith et al., 2000). They demonstrated the effectiveness of their bioreactor system for maintaining the viability of bone specimens and their system had been used

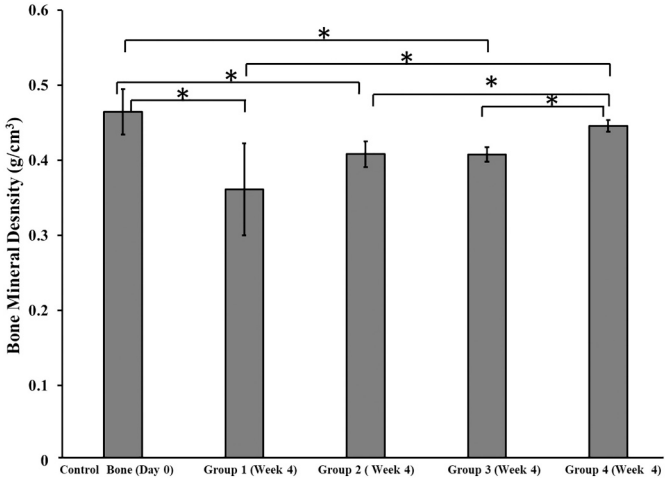

Fig. 5. Bone Mineral Density for different groups. The "*" indicates that the difference between the groups was statistically significant ($p < 0.05$).

for various bone-related studies (Schnieders et al., 2013; Rupin et al., 2010; Richards et al., 2007; Davies et al., 2001; Koller et al., 2003). However, there is no ex-vivo bone bioreactor system with controllable cyclic loading and perfusion flow in an automatic manner that can be used for long-term culture of an ex-vivo bone. With this idea in mind, the objective of this study was to develop a bone bioreactor system that is able to keep bone alive ex-vivo for than 4 weeks.

In the present report, we have shown the development of a user-friendly ex-vivo bone bioreactor with preliminary data that demonstrates the system's effectiveness to keep bone viable in organ culture by providing cyclic loading and perfusion flow in a controlled manner. From the live-dead fluorescent studies, we observed that the bone cores that were cultured in the static medium had more dead cells when

compared with the bone cores cultured in the bioreactor group. It has been found that in static organ culture, the transfer of nutrients to the center of the tissue does not take place, leading to cell death (Phillips et al., 2006). We also found that the pH was stable in all groups at week 1 but with a slight decrease in value compared to the control medium's (7.32 ± 0.05) (Table 1). This decrease in the pH value potentially indicates the production of lactic acid from cellular metabolism (Tanner et al., 2018). Over time, the pH value dropped further in the static culture groups due to the buildup of lactic acid from cell metabolism and release of nucleic acid from dying cells under conditions where there is no fluid flow. The much lowering of pH in the culture medium causes local acidification, and this acidification has been associated with a decrease in cell viability (Perani et al., 1998; Naciri et al., 2008; Davidson et al., 2012). The cell death linked to acidification was further confirmed from the H&E staining results at week 4 in the static group that revealed nuclear shrinkage of the cells in the static groups indicating necrosis while the bone cores in the bioreactor group maintained the same bone cell morphology as seen in control bone specimens prior to testing. In contrast to the static culture specimens, the pH in the bioreactor group remained stable at approximately 7.2 throughout the duration of testing. This indicates that the fluid flow was sufficient to provide nutrients and remove waste to and from the bone cores without the local buildup of lactic acid leading to the viability of the bone cells. From the bone mineral density (BMD) results, we found that the bone cores that were cultured dynamically in the bioreactor maintained the bone mineral density over time. Further, there was no statistically significant difference in the bone mineral density value of bone cores at Day 0 and cultured in a bioreactor. In contrast, the BMD was statistically significantly reduced in bones cultured in all static groups compared with the bone cores at Day 0 or cultured in the bioreactor (Fig. 5). This may be due to the fact bone resorption had taken place leading to the loss of the mineral content in the static culture. The lack of mechanical stimulation and flow of nutrients in static groups may have contributed to the death of cells, thereby increasing the acidity of the media and bone loss.

Even though our preliminary results seem promising, one of our study's limitations is that the testing was done only for 4 weeks. We need to conduct an extended study to determine its ultimate capacity for maintaining viability as we hope to use this tool for many bone-related studies while decreasing the use of animal sacrifice for research. One of the other limitations of the study was the lack of quantification related to viability and histological analysis. The trabecular bone's porous nature makes it challenging to focus the full area of the bone under the microscope, limiting our ability to obtain quantitative information from the images, and we were only able to obtain the qualitative data as presented in this study. Another limitation of the study was that we didn't have the age or gender of the bone coupons used, which may have a different response. However, for this particular study, age or gender may not be critical here because our main focus was to keep the bone viable for long term culture irrespective of age or gender.

5. Conclusions

This is the first attempt to provide a physiologic environment through cyclic mechanical loading and perfusion flow to organ cultured bone in an automated fashion so to maintain its viability and morphology for long term studies. The described ex-vivo bone culturing bioreactor system can serve as an indispensable tool for studies normally conducted in-vivo, but without the associated costs and ethical concerns.

Author roles

Dr. Rupak Dua, Hugh Jones, and Dr. Philip C. Noble designed the study. The data was collected and analyzed by Dr. Dua and Mr. Jones. Dr. Dua wrote the first draft of the manuscript. All authors interpreted the results, reviewed, and approved the final manuscript. Dr. Dua takes responsibility for the integrity of the data analysis.

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Declaration of competing interest

None.

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