THE PARAMETRIC STUDIES OF HUMAN FETAL MESENCHYMAL STEM CELLS IN BI-AXIAL BIOREACTORS

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Summary

Successful bone tissue engineering strategy relies on robust cell culture technique as well as stimulating cellular microenvironment. For the current clinical treatment, the expansion of cells *in vitro* has been a promising alternative method for the bone tissue engineering strategy. In order to enhance culture efficiency, bioreactor systems are widely researched for the different clinical purposes. In this thesis, it is hypothesized that, bioreactors can enhance efficiency of human fetal mesenchymal stem cells (hfMSCs) in terms of proliferation and differentiation by parametric studies in biaxial bioreactor systems for the bone tissue engineering. The hypothesis includes: first, the osteogenesis of hfMSCs can be improved by mechano-induction effect; second, chemical stimulation can safely enhance the proliferation and differentiation of hfMSCs; and third, for the bioreactors application, cell seeding method can play a significant role supporting the progress of *in vitro* cell culture.

Firstly, the mechanical stimulation effect on the bone tissue scaffolds was investigated. The aim is to trigger signal transduction pathways and mechano sensitive genes to provide this stimulus on scaffolds in dynamic culture. Using the torque wrench to induce the loading on special fabricated pins, scaffolds were under this stimulation. The results showed that the continuous compressive force can enhance the osteogenesis ability of bone tissue. The levels of calcium and alkaline phosphatase (ALP) under the external loading were found to be higher than the control group.

Secondly, effective cell seeding approach is required before the construction of tissue engineered bone graft (TEBG), however, current static cell inoculation technique has been harassed by several limitations, such as the inhomogeneous proliferation and the low efficiency of seeding. Biological glues can provide homogenous cellular distribution as well as improve the osteogenic differentiation of inoculated cells.

Therefore, the aim of this study is to investigate the effect of fibrin glue on hfMSCs, and to prove that this can retain more cells in porous scaffolds and enhance the mineralization. Tisseel gel was used to mix with the cells suspension and seeded directly on scaffolds. The results shows this allows cells to have a more homogenous proliferation and reduce the possibility of contamination. More importantly, this can be applied for *in vivo* experiments as it can promote specific extracellular matrix (ECM) proteins.

Thirdly, zinc can be a nutritional element for bone growth retardation. Zinc has also been demonstrated that it can play a physiologic role to stimulate bone protein synthesis, promoting the regulation for bone formation. The experiments have shown aim that for the bone tissue engineering, the additional of zinc can increase the differentiation and the proliferation for hfMSCs *in vitro*.

Overall, the research demonstrated the whole optimization for the existing bioreactor systems. The results of this research can be used to further the study of bone tissue bioreactors and might optimize the parameters in growth of bone grafts.

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Chapter 1: Introduction

1.1 Background

Different types of bioreactor are used for cell proliferation on a large or a small scale in order for therapy concepts and individual patients, respectively [1]. Compared with static culture methods, the dynamic bioreactor should have more advantages in terms of controlling environmental conditions: pH, temperature, oxygen tension and shear stress. Moreover, bioreactors can induce the external forces, which to some extent can mimic the physiological environment and help to regenerate the grafts with high efficiency. Thirdly, the transportation of metabolic products can be realized by bioreactors. The tubing and the filtering controlled by motors can carry out the function of the blood flow and the circulation of bodies. Moreover, the enclosed bioreactor system can avoid the possibility of contamination during the static culture in incubators when changing medium.

The bone tissue bioreactor involving the expansion of cells on scaffolds before implantation is a promising alternative for the clinical treatments. In the United States, there are about one million bone grafting surgeries performed [2]. Usually, before the implantation, the cells should be cultured *in vitro* aiming to get a high proliferation and sometimes even differentiation to satisfy the requirement of the patients themselves. However, the feasibility of cell-based strategies needs to be overcome due to some limitations in vitro culture techniques, and this can be solved by perfusion bone tissue bioreactors which can convectively transport nutrients to cells and continuously mix medium.

In addition, *in vitro* cell growth the scaffolds provide the adhesion for the cells but as the concentration of the medium decreases toward the core of scaffolds as well as the

oxygen concentration, especially for some large scale of scaffolds. At the center of the scaffolds cells survival rate is quite important and because if the levels of concentrations drop below the minimum to sustain cell growth, the efficiency and the success of scaffolds' implantation will be substantial affected [3]. Therefore, the design of bone bioreactor in terms of supporting and expanding the cell population and the homogenous proliferation can be a proper solution for this issue existing in static culture. Bioreactors' perfusion systems in particular expose cells to shear stress and enhance the progress of nutrient transport.

Another aspect of bone bioreactors is that this microenvironment creates the *in vivo* condition which is more like the bone's *in vivo* environment [4]. Bone tissue is a kind of dense connective tissue and mimicking the anatomy principles, the variety parts of the bones may suffer from different kinds of the mechanical stimulations and usually this is a multi-forces loading together. Wolff's Law, developed by Julius Wolff in the 19th century suggests that bone can adapt to the external loading it is placed under [5]. According to Wolff's law [6], when bone is under compressive load, it can remodel by itself over a period and can become much stronger in order to resist this loading. Therefore, bone in a healthy person or animal will remodel in response to the loads it is placed under. However, if the loading on a bone decreases, the bone will become less dense and weaker as there is no stimulus for continued remodelling that is required to maintain bone mass. In this project, taking into account this requirement, we utilise this selectable control machine. In our daily life, the mechanical loading condition is basic modulator for bone apposition rate, because with those different kinds of force, the expression of osteogenic genes can be induced. For osteoblasts, they can be directly activated by mechanical forces and the proliferation and matrix synthesis can be to a large extent enhanced. Meanwhile, the indirect effect can be obtained by growth factors and the release of nitric oxide (NO) [7]. This whole process of the transformation is "mechano transduction", which means converting the physical stimulation into cellular response. For instance, the most common loading is fluid shear, the fluid shear can exert effect on osteoblastic differentiation [8, 9]. the cells exposed to shear can

produce more collagen, calcium and components of bone extracellular matrix [9]. Meanwhile, the osteocytes can play the role of mechano sensors and transmit the mechanical information to adjacent cells through the intercellular communication network [10, 11]. Therefore, the role of bioreactors is not only providing an environment to culture cells or organs, but establishing the signal pathways for the nucleus [12].

1.2 Objectives

The generation of effective tissue engineered bone grafts requires efficient exchange of nutrients and mechanical stimulus. The application of mechanical load on cell-seeded scaffolds also stimulates bone tissue formation and may determine the phenotype of the engineered tissue. The definition of an appropriate mechanical environment stimulating bone formation requires scaffold microstructure optimization and, on the other hand, the determination of mechanical loading conditions able to generate within the scaffold the expected mechanical stimuli. These two considerations are necessary to control the mechanical pathway to stimulate bone tissue formation using porous scaffolds.

The objectives of this study can be the improvement of the efficiency of the biaxial bioreactors by several different methods. The can be realized by external loading on scaffolds, cell seeding optimization and the additional chemicals. Therefore, after the improvement, culture period can be shortened and stability of the proliferation and the differentiation will be enhanced.

Specifically, in the research, we can find the compressive force may stimulate the bone formation in terms of several different ways, such as cells proliferation, calcium content, ALP activity and gene expression. Apart from this, the optimization by the medium and the cell seeding method are also the aims of my research.

1.3 Scope

Chapter 1 covers the background of this proposal which illustrates the need for synthetic bone grafting material in conjunction with the enhancement of rapid bone formation. Chapter 2 summarizes the relevant literature study including bone and various bone repair treatments, MSC and hfMSCs,; implant-associated bioreactor systems; Chapter 3 presents the preliminary study on the mechanical stimulation on bone scaffolds; Chapter 4 shows the fibrin experiments for the optimization for cell seeding methods . Chapter 5 gives zinc sulfate as a chemical stimulation at the present work and Chapter 6 makes a conclusion; Chapter 7 provides some future recommendations for this research.

Chapter 2: Literature Review

2.1 Bone tissue engineering

Bone tissue engineering has been a critical issue for the current clinical need. Langer and Vacanti first defined tissue engineering as the interdisciplinary field which helped people's life towards the development of biological grafts. Bone tissue engineering to replace damaged bone represents an alternative. A tissue engineering method concludes some steps, seeding, culturing on the scaffolds and implantation after the period of culture [13].

Before the scaffolds are implanted into bodies, constructs containing cells are often cultured *in vitro* to increase the feasibility. During the progress of cell-based tissue engineering strategies, bioreactors systems are used to improve the nutrients transfer and provide the condition for cells.

2.1.1 Bone anatomy

The human skeletal system consists of 206 individual bones joined by connective tissue and can provide the metabolic supply and biomechanical support during daily life. Specifically, bone performs several functions in the body systems, such as providing site of muscle attachment for locomotion, generation of white and red cells for oxygenation, retaining reserve stores of calcium and the protection of vital organs. Therefore, pathologies of bone can affect different range body functions.

The bone of the human skeletal system can be divided into cortical bone and cancellous bone (Fig.2.1). The proportions of cortical and trabecular bone may be different at various locations. Compact bone consists solid and with spaces for blood vesssels and canaliculi. Like a sponge, cancellous bone has a honeycomb of bars, plates and rods with different sizes. Cancellous osteocytes are less in matrix embedded and they are more influenced by marrow cells [2, 14, 15].

Long bones are divided into three sections. The epiphysis is between the bone and the growth plate which is covered with cartilage. The metaphysic is the transistion from the wide part to the tubular section, and it is called the diaphysis. Cancellous bone is mainly found in the metaphysic, but cortical bone comprises diaphysis. Cortical bone usually forms layers over cancellous and it can improve mechanical properties.

The outer bone sheath is called the periosteum which covers both cancellous bone and cortical bone. The periosteum is continuous except the region near joints and the outer layer can be more fibrous and can connect bones at joints. The inner layer can be more vascularized and contains cells which are capable of becoming osteoblasts.

According to the organization, bone can be divided into woven bone and lamellar bone. Generally, woven bones can be found in the development of embryonic skeletal,

early fracture healing or the longitudinal bone growth. The process of bone formation can lead to a perisosteal layer around the middle of the long bone. Woven bone can be formed directly with the orientated and loosely collagen fibers. At this stage, mineral deposition is low and the mechanical properties are also weak. When bone healing occurs, a woven bone may rapidly form and then serve as a scaffold for bone remodeling and can be replaced by lamellar bone. In contrast, the other kind of bone is lamellar bone, which has stronger mechanical strength, because of its stably bundled collagen fibers and orderly oriented, high mineral deposition, but forms a much slower pace compared with woven bones.

On the large scale, both woven and lamellar bone can be found in cortical bone and trabecular bone. When the lamellar bone dose breaks, it can occur along cement line instead of osteons. Therefore, this can provide deflection of crack and the crack in the osteon cannot be quickly spread across the entire bone.

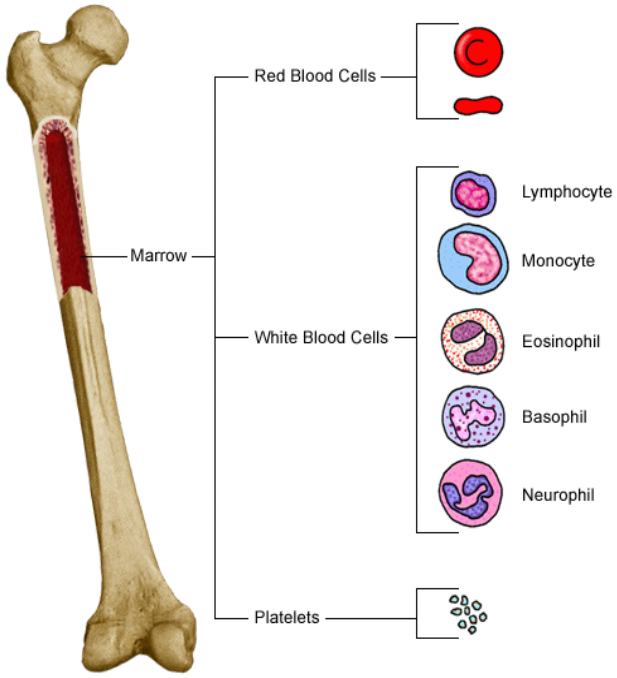


Fig.2.1. The anatomy of bone

From table.2.1, the functions and different types have been showed in the extracelluar environment [16].

Table.2.1. Functions of immobilized and mobile marcromelecules in extracellular environment [16]

Type of macromolecules	Bone extracellular matrix constituents	Function
Immobilized macromolecules (synthesized by osteoblast)	Collagen	Contain peptide motif (DGEA) which is a putative α2β1 integrin binding site for osteoblast
	ECM protein – osteonectin, osteocalcin, osteopontin, Proteoglycans, glycoproteins (eg: fibronectin and vitronectin)	Contain chemotactic or adhesive properties due to containing Arg-Gly-Asp (RGD) sequence which is specific to the fixation of cell membrane receptor like intergin
Mobile biochemical cues	Transcriptional and growth factors	Facilitates osteoblast proliferation, differentiation and subsequent bone formation and regulation
	Systemic and local complimentary hormones	
	Phospholopid membrane-bound matrix vessels (ALP)	Initiate biomineralization process

2.1.2 Bone fracture healing

It is a common phenomenon that bone fractures and this usually requires some performed orthopedic procedures. Every year in the United States, around 6.8 million cases come from the medical field. Fracture can occur due to trauma, when the external loading exceeds the range of loading which bone can bear and adapt during the growth and development. Meanwhile, osteoporosis and bone tumor or such pathological conditions can cause the fractures [17].

Bone fracture repair methods are usually quite similar and may involve three stages: inflammation, repair and remodeling. Fracture at first may result in the formation of blood clot. From the wound site, the cellular debris of phagocyte can be removed and the fibrin clot can release chemo attractants. This can be the inflammatory phase and it can be the shortest progress of the whole three. During the first stage, the repair

process can begin when the migration of osteoblasts from the periosteum begin to cover the blood clot. When the fracture is unstable, the MSCs differentiate into different cells as a temporary bridge.

Bone fracture healing can be a very remarkable process and it can lead to the complete regeneration of damaged bones to the original anatomy and function, unlike the other soft tissue healing, this formation is formed without scar. Moreover, bone fracture is a highly regulated the complex process and involves a series of biological events.

2.1.3 Bone tissue engineering scaffolds

In the body, tissues consist of several types of cells and a matrix. This matrix is a natural three dimensional scaffold for cells which can provide a special environment and the mechanical architecture. This matrix also can be a reservoir of nutrients, water, cytokines and growth factors. Therefore, when the regenerative medicine is needed, three dimensional scaffolds can provide not only mechanical functions but also the function as a supportive matrix for cell proliferation, consequent bone in growth and extracellular matrix deposition. Furthermore, working as a template for vascularization and scaffolds can actively contribute to the regenerative process by enhancing the cells proliferation and osteogenic differentiation.

There are several requirements usually for the bone tissue engineering scaffolds:

Biocompatibility

Biocompatibility can be the ability of a biomaterial to perform the function with respect to the therapy. This can generate the most beneficial tissue response. Therefore, the scaffolds should be non-antigenic, non-mutagenic and non-toxic. The scaffolds should be biocompatible and the fabrication process cannot affect the biocompatibility of the scaffold.

Porosity and pore interconnectivity

The porosity is very important as an open-pore structure with microstructure can allow the cells growth and also provide the space for cells. The porosity can directly influence the transition and diffusion of cells. During the culture period, the metabolic waste and the nutrients in the matrix need to be exchange and the porosity is very important. The uniform cell distribution, cell survival, proliferation can be ensured by this.

Pore size

The pore size should be with proper size. It is a critical parameter for the fabrication for scaffolds. If the size is too small, the proliferation of cells can occlude the pores. The cellular penetration and extracellular deposition and even the vascularization can be affected. Meanwhile, if the pores are too big, the mechanical properties will be reduced and the fibrous tissue can penetrate easily and invade into the scaffolds. Therefore, the size of scaffolds should be considered according to the specific conditions for the selection. For the bone tissue engineering, the parameter should be between 300-900μm and this size can satisfy the cellular migration, extracellular production.

Surface properties

First, surface properties such as chemical and topographical properties can control the cellular adhesion, proliferation and differentiation. Compared with the smooth surface, the rough topographical surface property can be more osteoconductive. The rough surface allows stronger adhesion of the fibrin matrix and facilitates the migration of cells to the surface of materials. Then, the chemical properties can make the cellular migration and differentiation easier. For bone tissue engineering, osteoconduction and osteoinduction are important factors.

Mechanical properties and biodegradability

For bone tissue engineering, mechanical properties can be very important as after implantation, the tissue needs to grow and the bone itself needs external loading to recover, due to Wolff's Law. If this mechanical stimulation decreases or disappears, the cells cannot be affected by the mechanoinduction. The mechanical properties of scaffolds must be high enough for maintaining the defective space for bone formation and allow the early mobilization.

Biodegradability is an important factor determining the whole research and medical application. *In vivo*, the period of the degradation and resorption rate should match the natural tissue replacement.

To alleviate the shortcomings, PCL-TCP, as a bioactive composite material comprising of a PCL and TCP has been developed. This kind of materials combines the advantages of PCL and TCP, allows the subsequent scaffolds to integrate with bone spontaneously and possess osteoconductive and osteoinductive properties. Moreover, the acidic release from the polymer can be neutralized by the alkaline calcium phosphate.

Overall, the scaffolds selection for bone tissue engineering is the first step and also be a very fundamental step. A series of properties needs to be considered to further the application.

2.2 Mesenchymal stem cells

2.2.1 MSC and hfMSC

Mesenchymal stem cells (MSC) are known as colony forming unit or marrow stromal cells for the cells therapy. At about 1960s, they were first found and isolated from adult bone marrow. Currently, it has been showed as the promising and excellent cell

source for the tissue engineering application. They have many advantages compared with other cell source from the bone tissue engineering. Firstly, MSC and differentiated MSC are non-immunogenic and can be used for suitable for allogenic applications. Then, the isolation of MSC can be easily handled and the osteogenic differentiation is well defined. Thirdly, MSC can generate greater amount of bone tissue than other kinds of cell source. In addition, the cryo-storage does not seem to affect the osteogenic potential of MSC [18].

MSC have been showed to have a heterogeneous population of cells. Colony size, proliferation rate, multipotency and cellular morphology have been observed to differ in single MSC strains.

Human fetal MSC (hfMSC) demonstrates some plastic adhesion properties: they can self-renew without showing the altered phenotype, differentiate down multiple mesenchymal lineages and have a similar immunophenotype. Apart from the characterizations of MSC, hfMSCs show that some other properties. They can differentiate with greater proliferation with low immunogenicity. This makes them as a suitable source for bone tissue engineering.

2.2.2 Clinical applications of hfMSC/MSC

Moreover, hfMSC can provide intrauterine gene for the cell therapy and Le Blanc et al proved the first successful clinical application with human fetal MSC.

In summary, MSC can be a promising cell source for the implantation during the tissue engineering development. hfMSC can be a much more suitable cell source compared with MSC. They can be utilized as an ideal cellular source off the shelf for bone tissue engineering. They can have lower risk for immunogenic and bacterial contamination compared with MSC. The proliferation rate and differentiation rate is

potentially higher than MSC. This can decrease the culture duration and patients can benefit from its use much earlier.

2.3 Bioreactor systems

2.3.1 Comparison of various types of bioreactors

The bone engineering of 3D tissue constructs contains cell seeding and maintenance, proliferation and formation. As shown in Fig.2.2, there is an overview of bioreactors Those different types of bioreactors have different advantages and disadvantages, and the most basic and simple culture systems can only conclude a petridish, a multi-well plate or a T-flask. The spinner flask can be a simple bioreactor system to achieve medium mixing, composing a glass media reservoir and often have porous covers for the gas exchange, for the regenerative medicine [19].

This kind of bioreactors are used for bone cells as they have been proved that they can improve the rate of cell growth and the expression of early osteoblastic marker

ALP and calcium content compared with static culture [20].

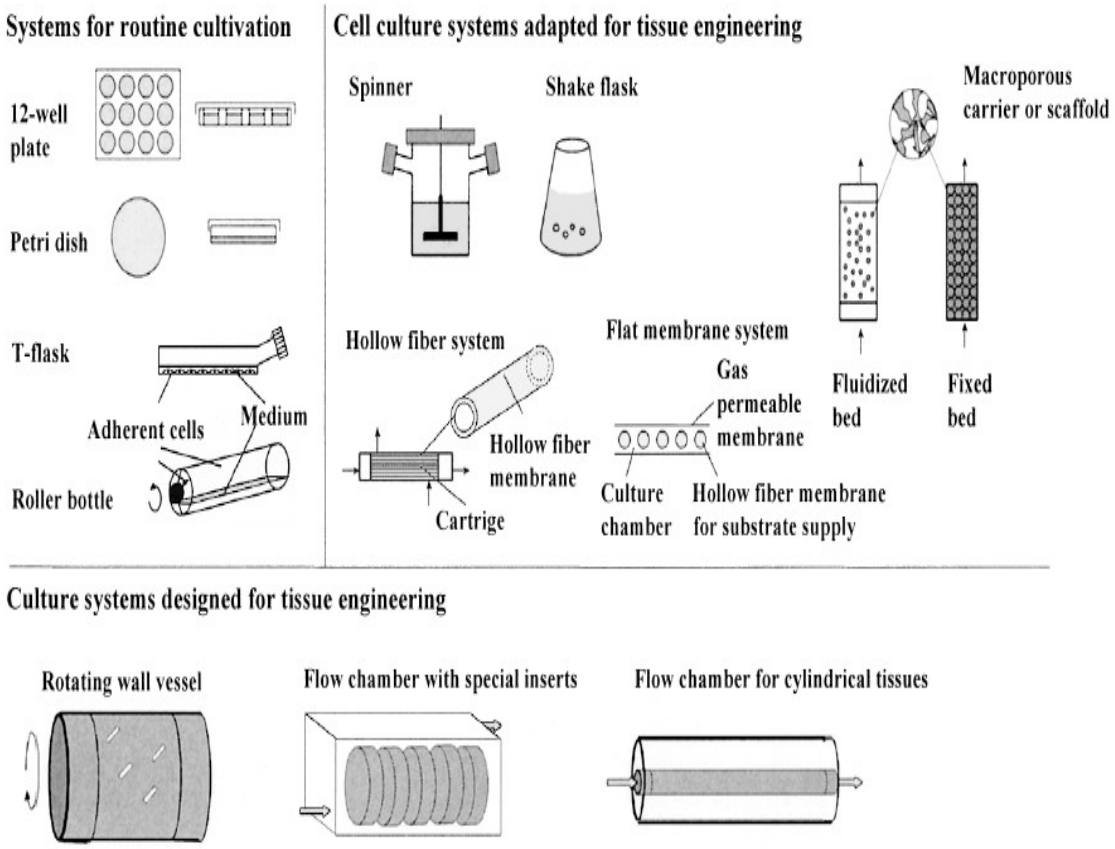


Fig.2.2.Cell culture systems used in tissue engineering [20]

Perfusion bioreactors have been chosen for use in this project, and will be elaborated upon in the ensuing sections.

Perfusion bioreactors design

The characteristic of perfusion bioreactors is the pump system to perfuse media through scaffolds directly (Fig.2.3). This design can use an array of scaffolds materials including titanium, hydroxyapatite (HA), ceramics etc. According to the literature review, a study using rat marrow stromal cells and this perfusion bioreactor with the continuous flow rate of 0.3-3.0 ml/min can be seen to increase both osteoblastic differentiation and calcium matrix deposition [21]. In their work, a

titanium fiber mesh scaffold and osteopontin was tested as a late osteoblastic differentiation marker. Because of the peaks in the expression, it can be proved that from the viewpoint of biofluids, the fluid flow can increase the rate of cell differentiation. Besides, the presence of extracellular matrix on titanium scaffolds can enhanced osteoblastic differentiation with shear stress.

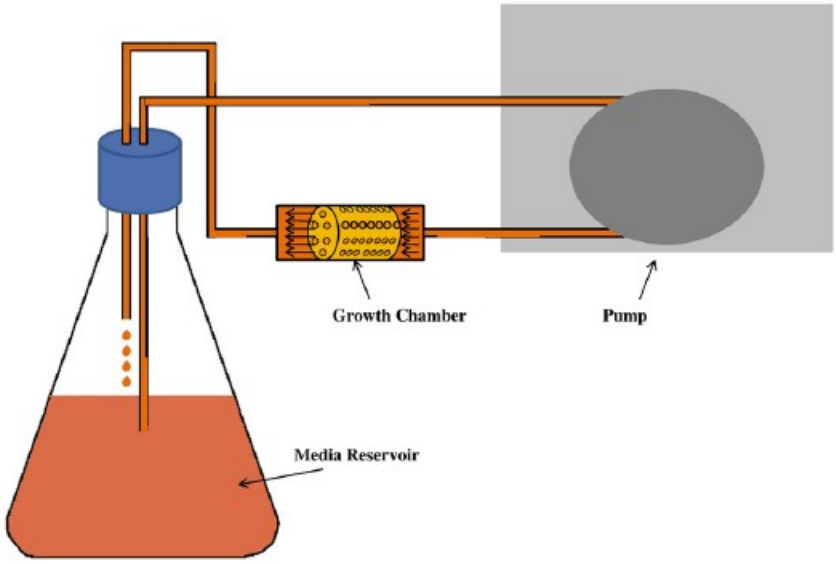


Fig.2.3 Schematic of perfusion bioreactor. Medium is directly perfused through porous scaffolds into a growth chamber [19].

Shear stress is an important factor in perfusion bioreactor systems, as it can enhance the efficiency of the transportation and the flow of nutrients due to the role in the increased matrix production, and the large volume of medium can to some extent induce this effect. In order to distinguish this issue, the contribution of shear stress and nutrient availability needs to be examined separately.

Biaxial perfusion bioreactor

In this research project, the type of bone tissue bioreactor used is biaxial perfusion bioreactor. MSCs are widely used as a source for osteogenic differentiation applications [18, 22]. In our preview research, hfMSC can obtain the high frequencies and long proliferation time compared with MSCs, and our experiments also use this kind of cells. But the difficulty encountered is the penetration problems when the scaffolds are cultured in medium, especially for some large scale piece of scaffolds. By the challenge of maintaining cellular viability at the center of those larger grafts, the application of common bioreactors is quite limited. This is due to the reason that the limits of the nutrients and waste products' diffusion is about 1-200 μm. Besides, the homogenous seeding and extracellular matrix distribution are concerned about the condition of larger scaffolds.

While the majority of bioreactors are uni-axial in design, our biaxial bioreactor bioreactors can solve those existing problems more effectively because it can rotates in 2 independent orthogonal axes in order to improve the biofluid flow. We reported that the biaxial bioreactor maturation step could enhance homogenous cellular proliferation, osteogenic differentiation and ECM distribution [23]. Moreover, it can maintain high cellular viability for the scaffolds (Fig.2.4).

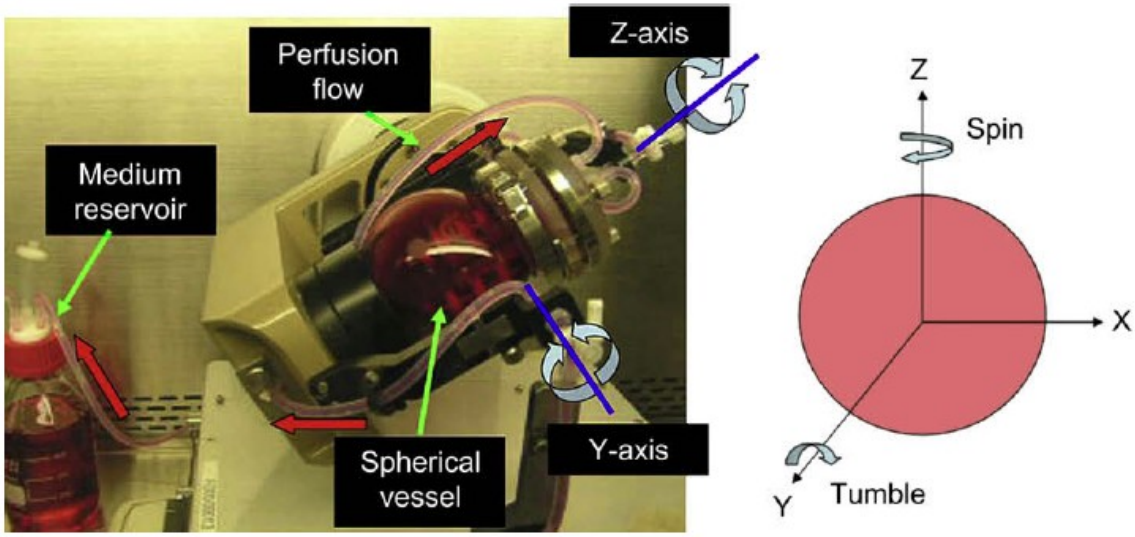


Fig.2.4 Biaxial perfusion bioreactor [23]

This kind of biaxial bioreactor can provide the stable motion by programmed motor and the rotation speed and the fluid rate of the medium can be adjusted and controlled. The chamber made of glass is stable during sterilization and is quite suit for the culture condition.

2.3.2 Mechano induction

Currently, mechanical stimulation has played a significant role in the biomedical field. For instance, when mechanical stress is applied to removable partial dentures, fixed partial dentures, or implants, it is transmitted to the residual ridge. Bikle et al suggested that biomechanical signals are essential for bone homeostasis, growth, adaptation, healing and remodeling; a lack of such signals leads to bone loss.

In Wolff's law, stress shielding refers to the reduction in bone density (osteopenia) as a result of removal of normal stress from the bone by an implant (for instance, the femoral component of a hip prosthesis). This is because by Wolff's law, bone in a healthy person or animal will remodel in response to the loads it is placed under. Therefore, if the loading on a bone decreases, the bone will become less dense and weaker because there is no stimulus for continued remodeling that is required to maintain bone mass; bone remodeling is influenced by mechanical loading.

Usually the development of the most loads on the bone should be multiple directions, and the force applied on the bone during walking can be a good example.

The design for the chamber introducing stresses

A.Continuous compressive force

The continuous force on the scaffolds can be an initial step for the whole outline of our mechanical design. This basic foundation research is a test for the properties of our TCP-PCL materials. Compared with the origin chamber without this compressive load, this continuous force can to some extent optimize the culture condition in vitro.

B.Cyclic compressive force

Cyclic stress is quite common during our daily life. Regarding to the example of walk, the mechanical force is cyclic as every step is extremely similar to the one before. Meanwhile it is also multiaxial because the joints and muscles are pushing and pulling the bone in different directions.

In order to realize the function of this cyclic compressive force chamber, the usage of motor to control the frequency and the quantity of the stress is important. We plan that with the addition of the cyclic force, the period of the exposure time under this is strictly accurate according to the different clinical applications and the bone biomechanics.

C. Enhanced shear stress

The fluid shear can exert effect on osteoblastic differentiation, which means the osteogenic signal expression of MSCs can be influenced. Because bone cells, in particular osteocytes, are quite sensitive to shear stress, which can be a common phenomenon related to mechanical adaptation of bone. Meanwhile, the osteocytes can play the role of mechanosensors and transmit the mechanical information to adjacent cells through the intercellular communication network. Therefore, via this optimization, the cells in vitro can be more effective and more adapted to the patients' requirements.

Based on our initial biaxial bioreactor, the combination of the pins and the holder of the bioreactor system can keep pins rotating. Because the origin bioreactor's chamber

will rotate along X axis, when the pins now are fastened to the base, the motion of the chamber can let the scaffolds have a relative movement in the medium.

D. Ultrasound effect on scaffolds (just my own idea, not sure about the FEAS)
Since continuous-wave ultrasound has been applied to the fields for diagnosis and treatment, how to combine this with compressive force can be the future test for our research.

2.3.3 Cell seeding methods

Cell seeding efficiency has been a significant factor affecting the therapy result of the tissue engineering. It should also be the first and basic step to determine the experiment results. For cells such as adiposites which have the relatively low proliferation ability and rate, the process of seeding may be a critical solution for the issue [24]. It has been showed that the cell distribution is quite important for the development of engineered tissues and especially when the tissue bears a loading. If the construct is uniform, its resistance or some other mechanical properties will be much better. In the bone tissue research, this point seems like to be more critical.

According to the existing cell seeding methods, they can be divided into two groups: static, which means passive, and dynamic, which suggests active and they have been widely used in different types of cells.

Static seeding methods
Static seeding methods involve loading a small volume of cell suspension, which can be seeded into the scaffold. More culture medium will then be added to the culture dish after a stipulated period of time, such as 1 day or longer. This method is easy and simple to handle and perform. The effect of this is that majority of ECM might be secreted on one side of tissues because of the lower regions of the scaffold during seeding.

Dynamic seeding methods

Usually for the dynamic seeding method, spinner flasks may be used with the fixed scaffolds and immersed in a stirred cell suspension. This takes about 3 days and the cells can circulate and attach to scaffolds. This method can attain high seeding efficiency while also producing the non-homogeneous distribution of cells. If other alternative seeding techniques are applied to improve the efficiency, they relatively require more advanced or specialized equipment. Therefore, considering this factor, static seeding methods should still be used frequently in tissue engineering due to simplicity and application [25].

Specifically, there are some methods widely used in the current tissue engineering research. Every technique may have both advantages and disadvantages. From the ideas and the mechanism, the overview of the cell seeding can be showed.

The hydrophobic culture strategy is a novel method. Cells are cultured with chitosan films or gelatin microspheres on a PDMS-coated plate instead of tissue culture plate. According to literature review, the cell attachment efficiency can be significantly increased. Compared with normal method, cell counting experiments with gelatin microspheres in the novel PDMS-coated system resulted in the efficiency of 89.9% cell attachment after 1 day cultivation, while the cell attachment efficiency in the normal plate was only less than 1% [25].

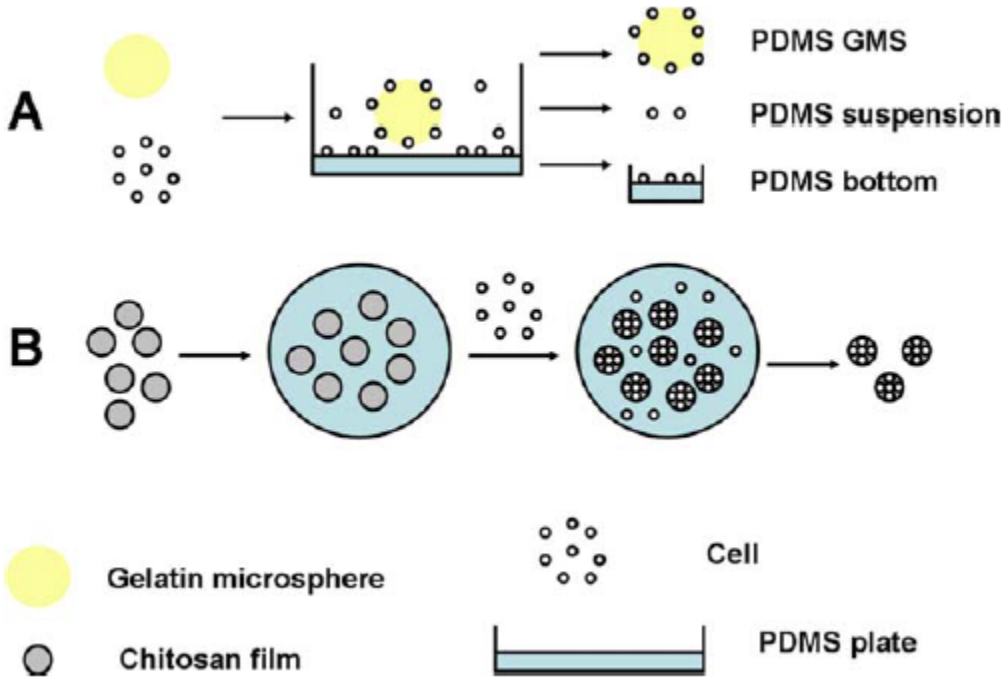


Fig.2.5 The representation of the hydrophobic culture strategy can be showed. The cells were cultured with gelatin microspehres. (A) Cells are suspended in the medium and cells are attached to the bottom of culture plate, but "GMS" means that cells are attached on the surface of gelatin microspheres. In the 2D model, cells can be cultured on chitosan films (B) [25].

It is known that this requires no external loading and can be applied to various different materials. In their application, chitosan films and gelatin microspheres in solution are light-transmittable and can be used for daily observation under a light microscope.

Another method is to coat the scaffolds with the micro porous outer layer, and the results demonstrated that the adhesion could increase from less than 10% to 43% after the out layer coating. The conclusion is that coating of porous scaffolds with micro porous outer layer facilitates effective cell seeding for the scaffolds [26].

Dynamic rotational seeding

Dynamic rotational seeding is also an important method, which can encompass various systems in a graft, and the graft is rotated in the medium suspension. The rate ranges from 0.2rpm to 500rpm and the culture periods from 12h to 72h. The efficiency can range from 38% to 90%. High-speed rotational systems can increase seeding efficiency, graft wall penetration and morphology. However, for the low-speed rotational systems, the effect on the cell morphology cannot be seen at lower cell concentrations [27].

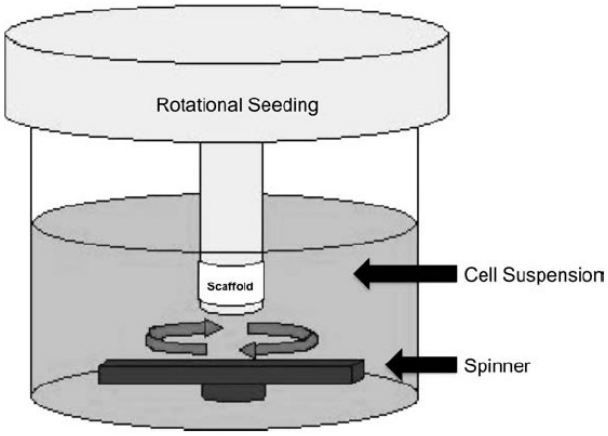


Fig 2.6. Rotational seeding. Scaffolds fixed to the needle are placed in a spinner flask with the cell suspension. The rotation of medium within the spinner flask drives cells into scaffolds [27].

Vacuum seeding

Vacuum seeding method needs the external or internal pressure to force the cell suspension through the micro pores of a TEVG. The efficiencies range from 60% to 90%. Another advantage of the method is the use of a disposable seeding device to reduce the risk of the contamination during the culture progress (Fig.2.7) [27].

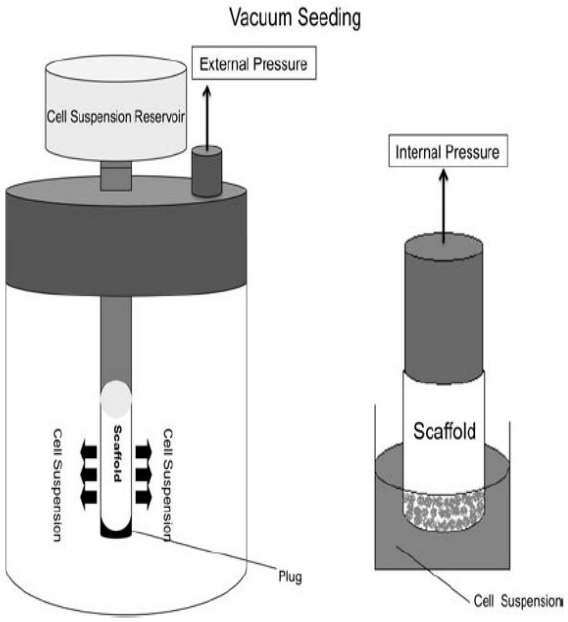


Fig.2.7 Vacuum/pressure seeding. As cells travel through scaffolds, they become lodged in the pores and then seeded into the graft. A cell suspension is forced through a scaffold by either internal or external vacuum pressure [27].

2.3.4 Chemical effect-Zinc Sulfate

During the culture progress, chemical signaling plays a significant role for the tissue engineering. In the culture medium, different chemical additions may have different effect on cells growth and differentiation. The application of those chemicals for enhancing efficiency and avoiding the negative effect is a critical issue.

Zinc is known as an important nutritional factor. For bone tissue engineering, zinc is needed for the growth, maintenance and development of bones [28-30]. If zinc deficiency happens, bone growth retardation may happen. Besides, zinc is physiologically important for bone homeostasis.

In humans, zinc intake has been correlated positively with bone mineral content in skeleton. This is through the multiple mechanisms which involve both increase of osteoblastic bone formation and decrease of osteoclastic bone resorption.

Osteoblasts are derived from stromal cells in pluripotent bone marrow, mesenchymal stem cells which commit to the osteoblasts by the influence of environmental factors. These include a potent recruiter of sites of bone resorption and the potent osteoblasts commitment factor. Meanwhile, bone-resorbing osteoclasts can be derived from the precursors which circulate within the macrophage population and can differentiate under the effect of osteoclastogenic cytokine. During the research, dietary zinc can be reported to decrease expression of acid phosphates. Further, Zinc can amplify the antiosteoclastogenic properties and *in vitro*, by decrease the induction of an osteoclastic gene program.

Firstly, in the biosynthesis of protein involving the enzymatic activation of the acids with adenosine triphosphate, this is followed by the conversion from amino acids to amino ribonucleic acids (RNA). The presence of zinc in the medium can increase the incorporation of leucine into acid-insoluble residues. Zinc also has a direct stimulatory effect on the protein synthesis in bone cells in vitro experiments.

Zinc can also enhance the anabolic effect of vitamin D_3 in bone components in the femur *in vivo*. The administration of vitamin D_3 can produce a substantial increase in the bone DNA and alkaline phosphatase activity. Therefore, the increase of alkaline phosphatase activity can enhance the administration of zinc and vitamin D_3. The increase of bone DNA level can also because that treatment. The experiments findings showed that the combination of zinc and vitamin D_3 can lead to a synergistic effect on the mineralization and the stimulation.

In vitro, zinc can also been shown to have the hormonal effect during the bone formation and the calcification in the bone tissue engineering culture. The research has showed that the presence of dihydroxyvitamin D_3 produce a significant increase in alkaline phosphatase activity, calcium level and DNA. The anabolic effect of estrogen of bone components in tissue culture can be enhanced after the culture with the additional of zinc sulfate [31].

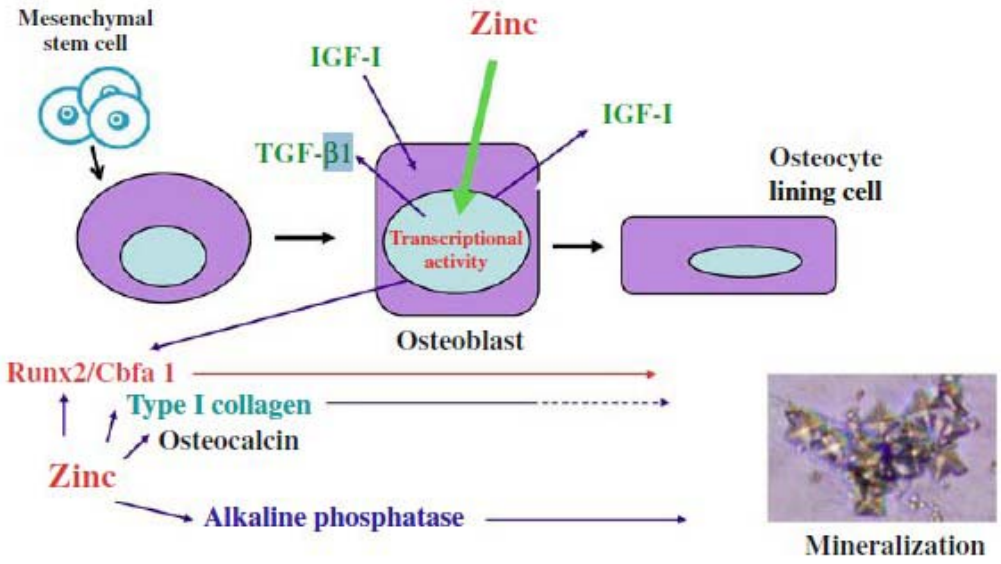


Fig.2.8 Zinc can stimulate cell proliferation, cell differentiation and cell mineralization in osteoblasts. Zinc can stimulate gene expression of various proteins including Runx2, type I collagen, alkaline phosphates, and osteoclacin in the cells.

Moreover, zinc can increase production of IGF-I and TGF- β1 in the cells. Zinc can also enhance the protein in synthesis due to activatin tRNA synthetase in osteoblastic cells [26].

From Fig.2.8, the mechanism of zinc action in stimulating osteoblastic bone mineralization and formation can be summarized [28]. Bone formation can be promoted by zinc stimulation in terms of cell proliferation, differentiation and mineralization. From the molecular mechanism, zinc action can be utilized to stimulate gene expression of different proteins. Zinc can increase growth factors of cells. Besides, zinc can enhance protein synthesis. Zinc can also increase the production of TGF- β 1 and IGF-I for osteoblastic cells.

For bone fracture healing, it can be considered as an optimal process. The reason for bone fracture healing is complex and fracture healing can be divided by several processes. It is recognized that during the progress of bone fracture repair, a number of growth factors and the cognate receptors should be at elevated levels around the fracture site. During the bone fracture healing, zinc can be showed that it can stimulate cell growth. According to experiments, protein content in the femoral-diaphyseal tissues can be markedly increased and many protein molecules can be produced in the bone tissues. When this diaphyseal tissue with fracture was cultured, in bone alkaline phosphates activity can be increased significantly and DNA content is caused. With zinc compound, the increase can be enhanced substantially.

Specifically, bone fracture can occur in osteoporosis, where chemical factors can stimulate the bone fracture. The most common method is oral administration of zinc acexamate, this can directly cause the increase of calcium content, DNA contents in the tissues, acid phosphatase activities.

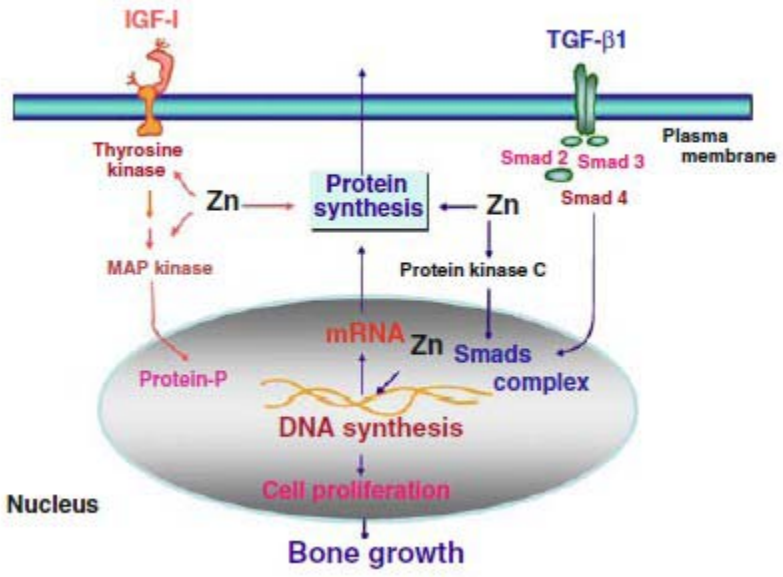


Fig.2.9 Role of zinc in the bone growth stimulation. Zinc increase protein synthesis at the translational process due to activating aminoacyl-tRNA synthetase in osteoblastic cells. Zinc can avtivate MAPK kinase or protein kinase C that is related to signaling in gene expression, and it may directly enhance gene expression[28].

From Fig.2.9, the mechanism of the stimulation of zinc on the bone growth can be demonstrated. During this translational process, zinc can increase the protein synthesis which is due to the activating aminoacyltRNA synthesis due to the activation of aminoacyltRNA in osteoblastic cells. Zinc activates the protein kinase C or MAPK kinase which is related to signaling in the gene expression. This can enhance gene expression directly. DNA synthesis in cells can also be stimulated. IGF-I and TGF- β 1can be increased.

Overall, there are increasingly evidences that zinc finger transcription has played a critical role in osteoblastic and osteoclastic cells differentiation.

Chapter 3: Mechanical stimulation on the bone tissue scaffolds

3.1 Introduction

Bioreactors are widely used for improving mass transfer of oxygen and nutrients *in vitro*, circumventing problems are associated with static culture. In addition, the mechanical stimulus exerted by fluid shear stress has been shown to promote higher cellular proliferation of osteoprogenitor cell types and osteogenic differentiation of MSC. Bioreactors allow tight control of culture conditions necessary for priming stem cells for osteogenic / vasculogenic differentiation. Our BXR bioreactor has demonstrated higher mass transport, proliferation, more robust osteogenic differentiation and even cell distribution within scaffolds compared to other commercially available bioreactors. Current bioreactor studies focus on the influence of fluid shear stresses upon dynamic culture but the study of physical forces under in vivo physiological conditions upon different loading modes is often neglected.

In our daily life, the mechanical loading condition is basic modulator for bone apposition rate, because with those different kinds of force, the expression of osteogenic and chondrogenic genes can be induced. For osteoblasts, they can be directly activated by mechanical forces and the proliferation and matrix synthesis can be to the large extent enhanced. Meanwhile, the indirect effect can be obtained by growth factors and the release of NO [7].

This whole process of the transformation is “mechanotransduction”, which means converting the physical stimulation into the cellular response. For instance, the most common loading is fluid shear, the fluid shear can exert effect on osteoblastic differentiation [8, 9]. The cells exposed to shear can produce more collagen, calcium and components of bone extracellular matrix [9]. Meanwhile, the osteocytes play the role of mechano-sensors and transmit the mechanical information to adjacent cells through the intercellular communication network [10, 11]. Therefore, the role of our bioreactors is not only

providing an environment to culture cells or organs, but establishing the signal pathways for the nucleus [12].

This approach of using mechanical bioreactors during cell culture means that various loading conditions can be applied directly onto scaffolds whilst the cells are being dynamically cultured. By introducing a defined mechanical environment, we aimed to trigger signal transduction pathways and mechano-sensitive genes to provide mitogenic and osteogenic stimulus in bone tissue.

The study also looked at the influence of nutrient supply, dissolved gas concentration levels and enhancement of intercellular signaling (nitric oxide and prostaglandin signaling) to maximize osteogenic differentiation of cells within the 3D construct.

The BXR system will also be modified with improved properties to meet cGMP requirements. In addition, additional fittings and equipment (sensors) will be attached onto the bioreactor system to allow for the perfusion of varying oxygen concentrations into the culture media to study the influence of oxygen tension in the cellular microenvironment.

The continuous force on the scaffolds can be an initial step for the whole outline of our mechanical design. This basic foundation research is a test for the properties of our TCP-PCL materials. Compared with the origin chamber without this compressive load, this continuous force can to some extent optimize the culture condition in vitro. Cyclic stress is quite common during our daily life. Regarding to the example of walk, the mechanical force is cyclic as every step is extremely similar to the one before. Meanwhile it is also multi-axial because the joints and muscles are pushing and pulling the bone in different directions. This has been investigated by the relevant *in vivo* tests [32].

3.2 Materials and methods

3.2.1 Isolation and culture of hfMSCs

Bone marrow derived of hfMSCs can be isolated for the cell culture. Specifically, single-cell suspensions can be prepared by flushing the marrow cells out of femurs and with gauge needle, the D10 medium can be prepared with Glutamax(GIBCO, USA) supplemented with 10% fetal bovine serum (FBS), 50mg/ml streptomycin (GIBCO, USA) and 50U/ml penicillin, and they can be referred to as D10 medium. During the culture period in static conditions, the D10 medium can be changed every 2 days or 3 days and the cells which are non adherent on flasks were removed by pipette. HfMSCs were trypsinized and washed by PBS at the concentration of 10^4/ cm^2 at sub-confluence. HfMSCs at passage 3 can be cultured for both characterization and the research. Both results and data presented here are the representative of the experiments.

3.2.2 Scaffold manufacturing and surface treatment

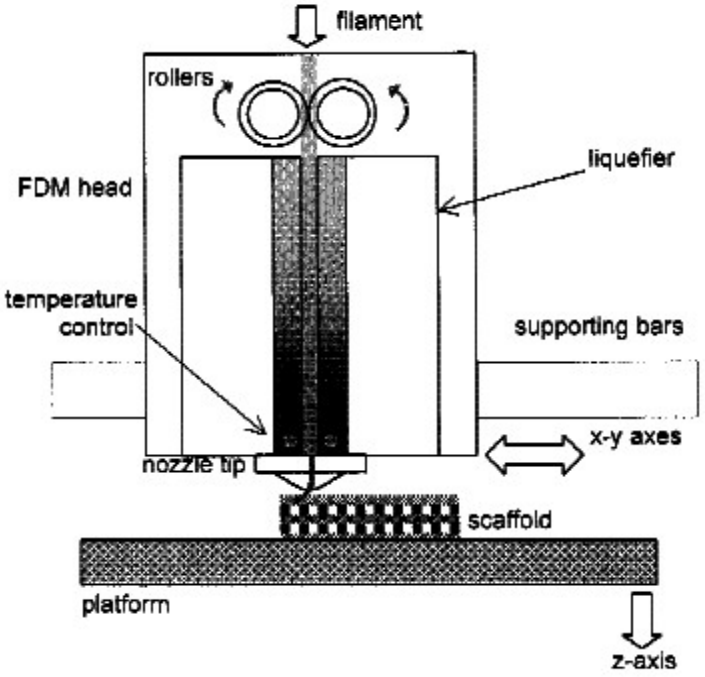


Fig.3.1 . Schematic diagram of the FDM process [33].

The current techniques for our research group can be rapid prototyping (RP) technology known as fused deposition modeling Fig. 3.1.(FDM). This can offer the ability and potential to design and fabricate the bioresorbable and highly reproductible 3D scaffolds with about 70% porous [33]. From the Fig. it can be showed that a schematic representation of the FDM process. The FDM method involves the melt extrusion of the materials through the heated nozzle and deposition at thin solid layers on the platform. The nozzle can be positioned on the surface of a build platform at the first stage of fabrication.

Fig.3.2 shows the layer is made of raster roads deposited both in the x and y directions. A fill gap can be programmed between the roads and provide the horizontal channels.

FDM is usually used to fabricate solid models (Fig.3.2.).

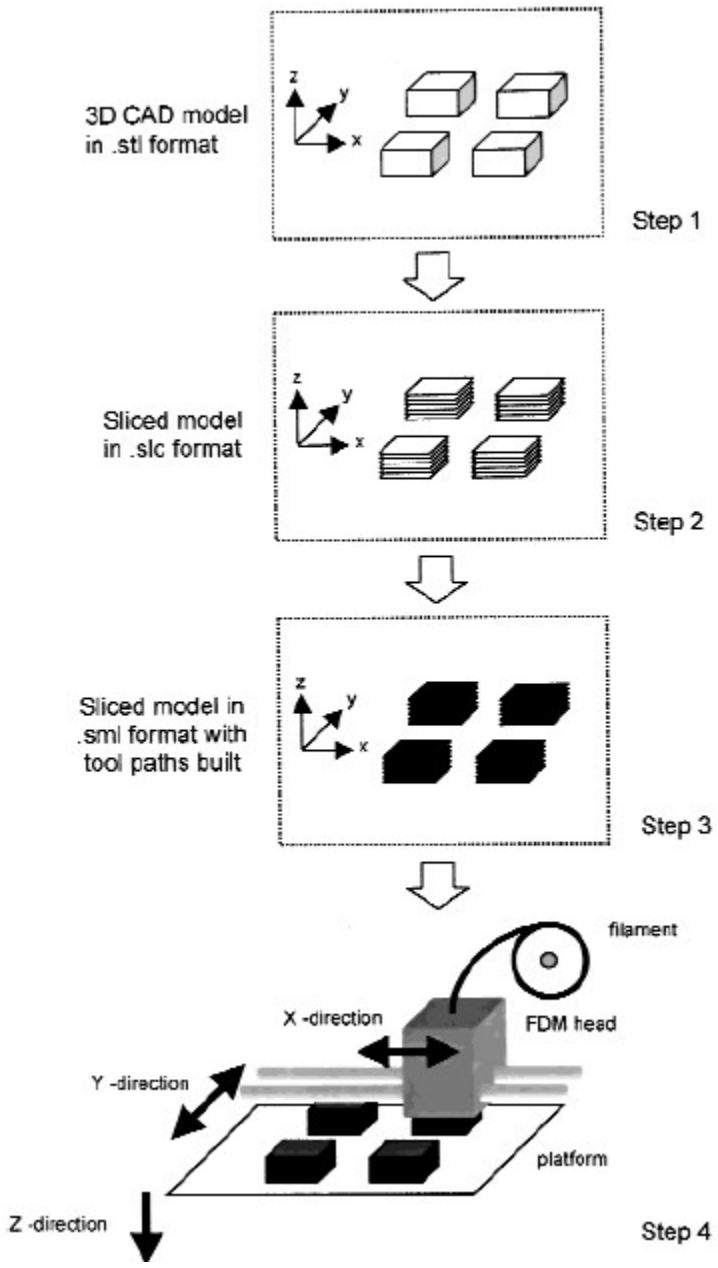


Fig. 3.2 Sequence of the data preparation for FDM model fabrication. The first step: Importing of computer-aided design (CAD) data in STL (stereolithography) format into QS. Then slicing of the CAD model into horizontal layers and conversion into SLC format. The third step is the creation of a deposition path for each layer and the conversion into SML format for downloading to the FDM machine. Step 4 is the FDM-fabrication process with a filament modeling material to build the actual physical part in the additive manner layer by layer [33].

For our research, a lay-down pattern of 0/60/120 °can be utilized to provide a honeycomb pattern of triangular pores with the porosity of 70% and the pore size was about 0.523mm. According to the mechanical stimulation experiements, tricalcium Phosphate-Polycaprolactone (TCP-PCL) scaffolds (n=3) with the dimension: 10mm OD * 3.2mm ID *10mm height, and cylindrical shape is easy for the pins to go through.

The scaffolds need surface treatment in 5M NaOH for 3h in order to enhance the hydrophilicity. After 3 times rinsing with PBS, they can be sterilized in 70% ethanol for 24h and then rinsed twice in PBS. The scaffolds can be transferred into 37 C° for drying.

3.2.3 Seeding hfMSC to PCL-TCP scaffolds

HfMSCs were re-suspended and subsequently seeded onto Tricalcium Phosphate-Polycaprolactone (TCP-PCL) scaffolds (n=3). The density of hfMSCs in every scaffold is 1.5 million in a dropwise manner. Before adding the D10 medium, the scaffolds with cells should be stored in the incubator for 3h in 6 well plates. After 3h, the additional 3ml of D10 medium could be added to each well and the cellular scaffolds were incubated in at 37 C° and 5% CO_2 for week and change medium for 3 times. During this period, the cells can adhere on scaffolds much better.

3.2.4 Pre test by gauze

The cell seeded scaffolds were firstly incubatored in CO2 (5%) incubator for predetermined time, one week and then randomly divided into two groups:

(A) Cellular scaffolds without gauze effect in static culture

(B) Cellular scaffolds with gauze effect in static culture

All of them were placed into 12-well plate and cultured with bone medium. At day 7, the scaffolds were harvested for the microscope assay and Pico green assay. Then at day 14, scaffolds could be harvested at the second time point for analysis.

3.2.5 Continuous compressive force vs. control group

The cell seeded scaffolds were firstly incubatored in CO2 (5%) incubator for predetermined time, one week and then randomly divided into two groups:

(A) A continuous group with 500 gf•cm moment and loaded with bone medium in the modified developed a biaxial bioreactor (Fig.3.4). For the first two weeks, the moment is 500 gf•cm for every scaffold via pins for compressive groups. From week 3 to week 4, this moment can be increased by 700 gf•cm.

(B) For the control group, the scaffolds were cultured in the same condition only without the compressive force in the same chamber. Then they were harvested at the time point of week four and characterized by assays.

The biaxial rotation bioreactor system has a spherical vessel for the volume of 500ml for culture, where the cellular-scaffolds are anchored to the lip of bioreactor by pins. The difference between two groups in the control group does not have the external loading while the experiment group has the mechanical stimulation. In the biaxial bioreactor system, the continuous replenish medium and the real time monitoring can control the oxygen, pH and the temperature Fig.3.3. The spherical vessel and 500 ml

reservoir are connected by tubing for the perfusion system with medium. The entire bioreactor was placed in an incubator with humidified atmosphere at 5% CO_2 and 37C°. Gas exchange was enabled through a special membrane into the vessel (Fig.3.4).

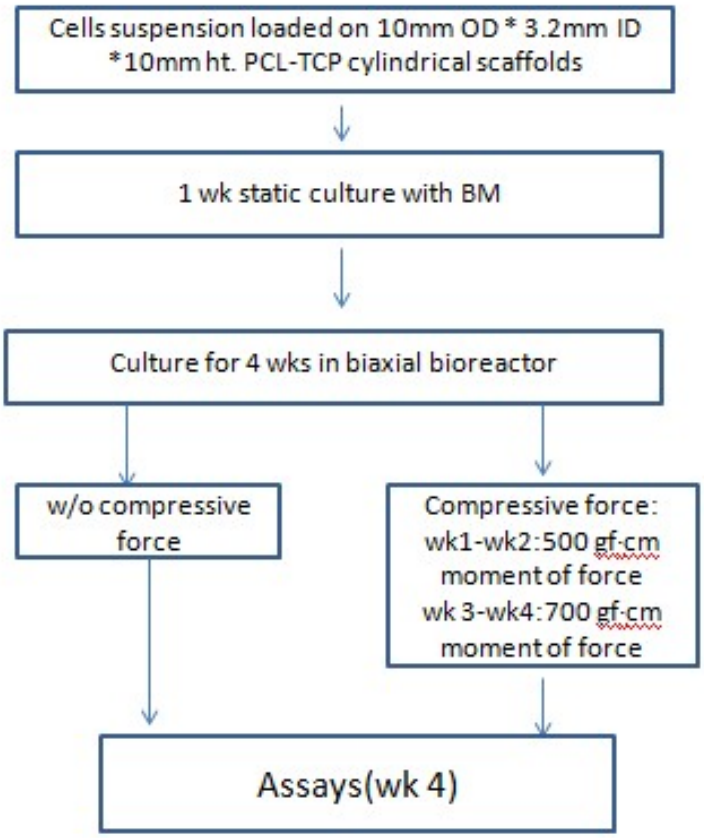


Fig.3.3 The experiment design

Fig.3.4. 1A. The design of the compressive attachments

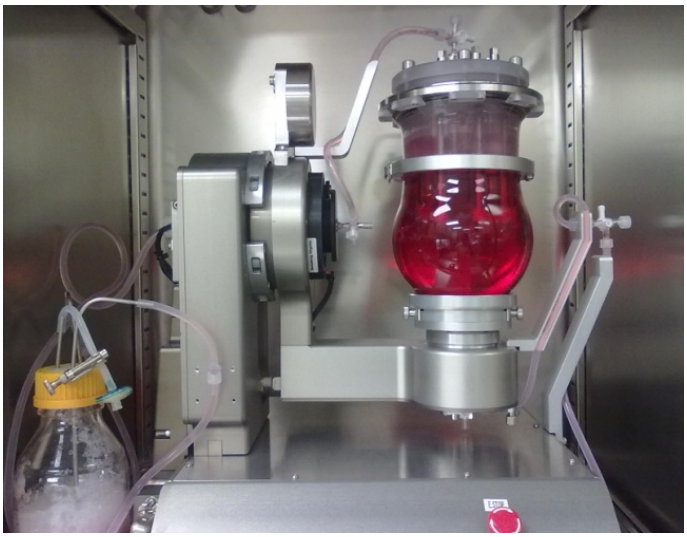


Fig. 3.4.1B.Novel biaxial bioreactor system

Cellular adhesion, viability and proliferation of hfMSC cellular scaffolds

The morphology of the cell in 3D culture, extracellular matrix (ECM) production and cellular adhesion were examined everyday by contrast light microscope (PCLM) over

28 days. Usually scaffolds were examined in two perpendicular panels, in planar (top) and side view points, because this can provide the more over information of the whole scaffolds.

Fluorescein diacetate/propidium iodide (FDA/PI) staining can test the qualitative analysis of cell viability in 3D was performed, where FDA stains viable cells green, and PI stains necrotic and apoptotic cell nuclei red. Scaffolds were bisected at half to expose to the centre of the scaffold to achieve a core view of the scaffold, stained with FDA/PI as previously described, and viewed under a confocal laser microscope (Olympus, FV300 Fluoview, Japan). Cellular scaffolds were examined in both planar view and side view on day 28.

The total cell number in the 3D cellular scaffold on days 28 (day of transfer to the bioreactor or static culture conditions, as illustrated by Fig. 1A) was estimated by quantifying the dsDNA content of every scaffold using a Pico- Green dsDNA Quantification Kit (Molecular Probes, USA). The total dsDNA was extracted from each cellular scaffold by incubating the cellular scaffolds in 0.4 ml enzymatic cocktail (consisting of 0.1% collagenase A (Roche) with 0.1% Trypsin mixed in PBS) at 37 ℃ for 3h, with vortex every 30 min then followed by three cycles of freeze and thaw; and assayed by following the manufacturer's instruction.

The proliferation of the hfMSC inside 3D scaffold was interpreted by the changes of dsDNA amount.

Comparison of osteogenic differentiation and mineralization in 3D scaffold culture

ALP activity assay

The intracellular ALP activities of hfMSC cellular scaffolds under two culture conditions were compared on days 28. Cell lysates were tested for ALP activity using SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec USA) and the ALP activities were normalized to the total protein content determined using the Bradford assay (Bio-Rad Laboratories, US) as previously described.

Calcium content assay

The calcium content of the hfMSC cellular scaffolds on day 28 was assayed as previously described. Specifically, the calcium deposition is dissolved in 1.0 ml 0.5 N acetic acid and determined by a colorimetric assay using calcium assay kit (BioAssay Systems, USA). And control cell-free empty scaffolds cultured as above were used as a negative control to offset the elution of calcium from the tricalcium phosphate component in the scaffold.

3.3 Results and Discussion

The results from the static culture with gauze can show that the small amount of force may not be easily inducted via scaffolds to cells. TCP-PCL scaffolds have high stiffness and thus the deformation of scaffolds is very difficult (Fig.3.5 and Fig 3.6). The preliminary experiments can provide the basic result and proved that the mechanical stimulation needed bioreactor systems to assist this and achieve this objective. From another view, this also demonstrated the importance of bioreactors systems not only due to the condition to supply nutrients but also the mechanical support or some other stimulation which hardly can be achieved by static culture.

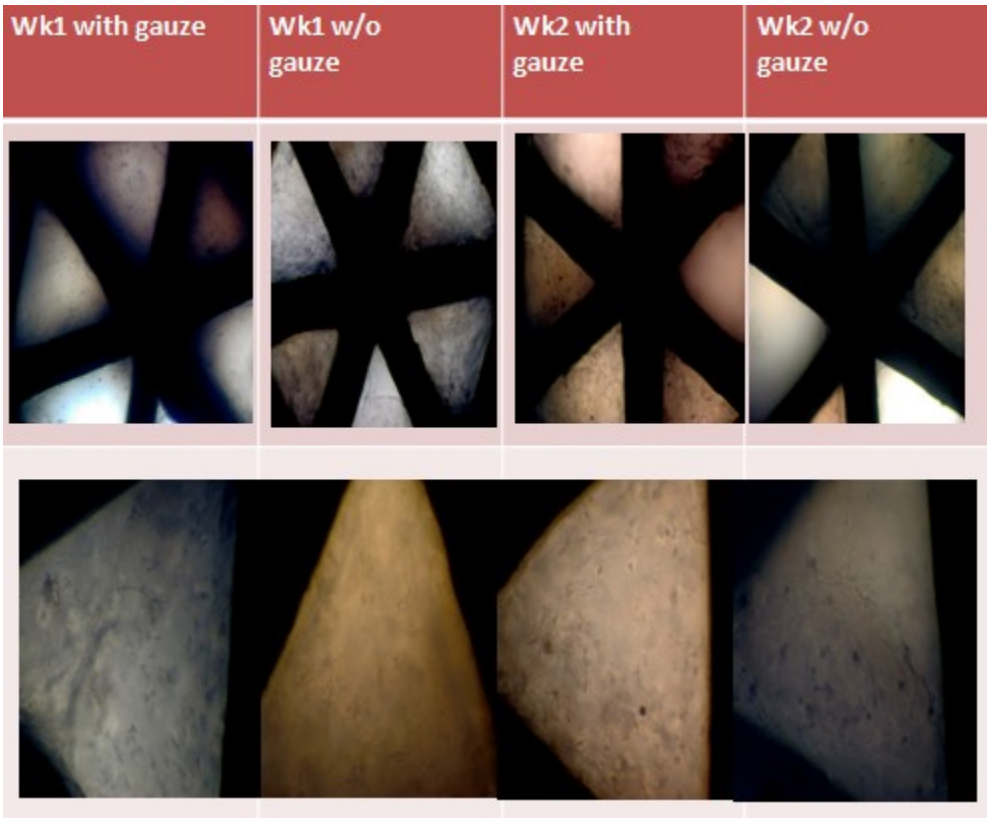


Fig.3.5 Microsopy images of pre –experiments by gauze.

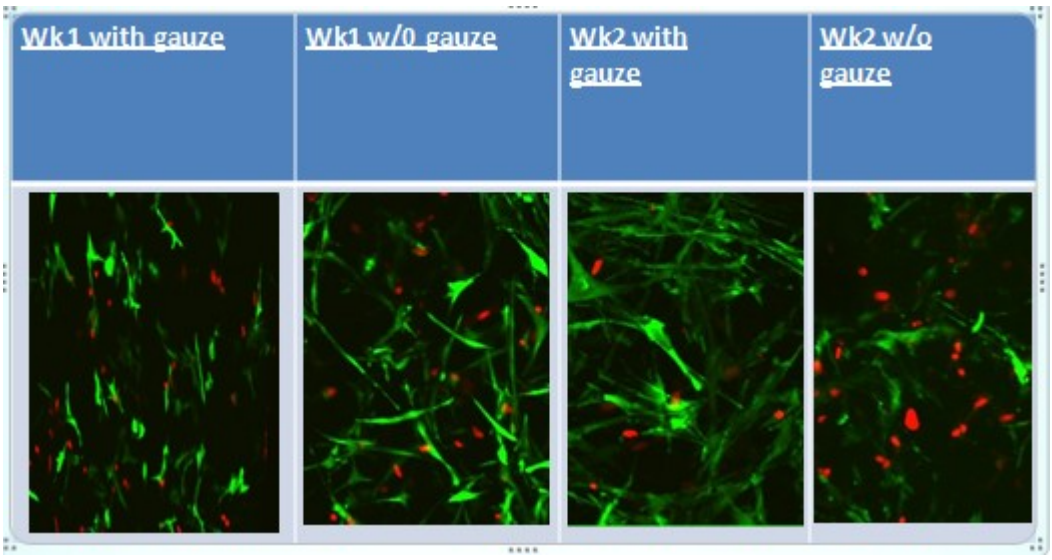


Fig.3.6. Confocal of pre experiments by gauze

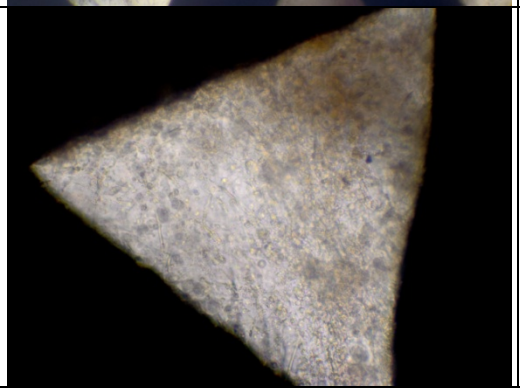
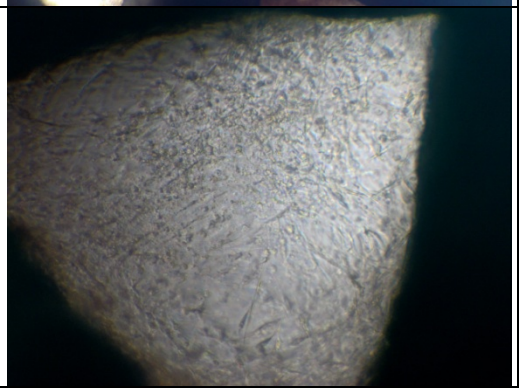
	Control group	Compressive group
50 X		
200 X		

Fig.3.7 Microscopy images

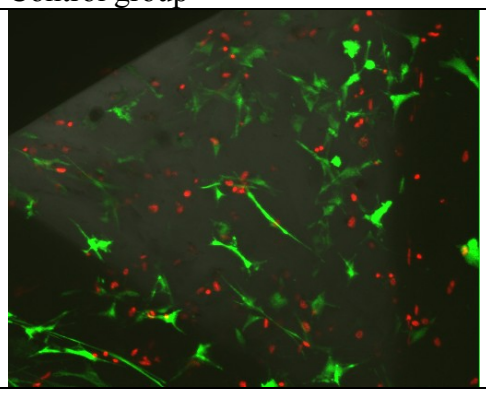
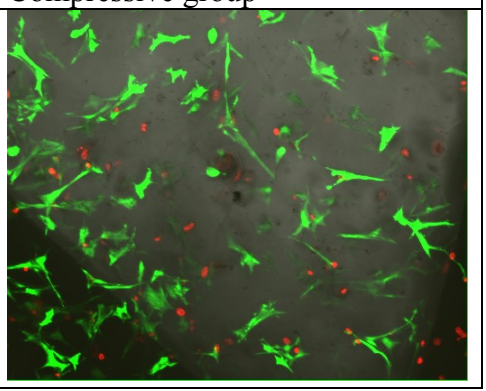
	Control group	Compressive group
Con-focal		

Fig.3.8 Confocal images

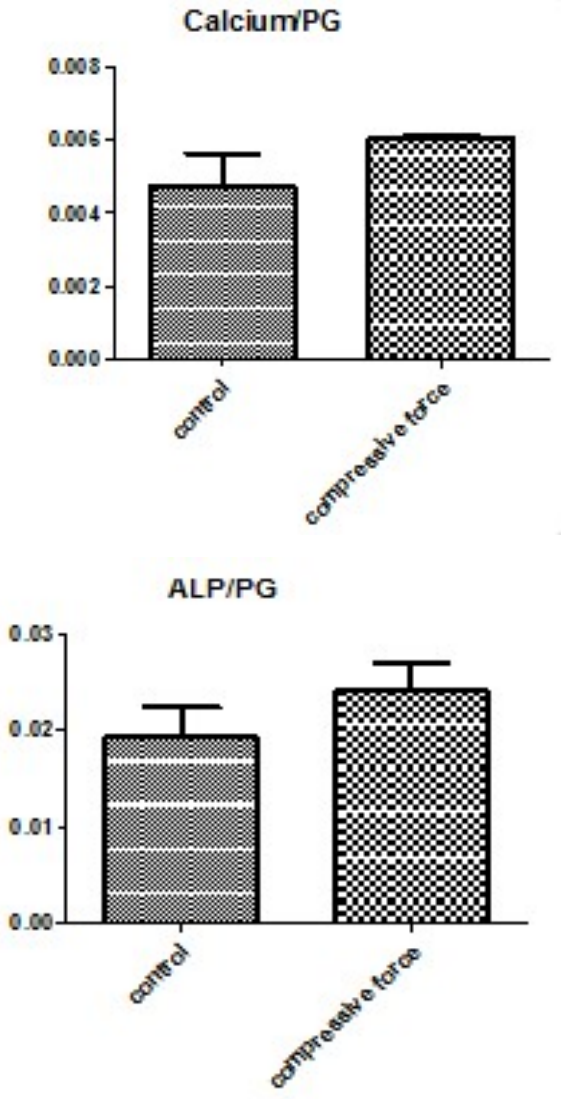


Fig.3.9 The ratio of Calcium/PG and ALP/PG after 4 weeks culture

From Fig.3.7 and Fig.3.8, the morphology of cells and their proliferation do not show obvious difference, suggesting that from the Qualitative experiment figure, but from Fig.3.9, the scaffolds under continuous compressive force could obtain the higher level of the ratio of Calcium concentration/ Picogreen dsDNA level (Quant-iT™, USA) by assays and as the same as the result of Alkaline phosphatase level ((AnaSpec USA))/ Picogreen dsSNA level after four weeks of culture, compared with the scaffolds without external loading. The reason of the difference is the mechano

induction, when the scaffolds under the compressive force, the cells adhered on scaffolds can be inducted by this signal. From the view of the cells, the growth factors will be affected and the level can be changed, which may directly result in the osteogentic ability. This showed the enhanced osteogenic differentiation in the compressive group. In this study, we demonstrated that mechanical stimulation on scaffolds resulted in higher cellular differentiation and the ability of osteogenesis. Overall, the mechanical external loading plays positive role for the growth and differentiation for hfMSCs. The design of the chamber is novel and it still needs further experiments involving more kinds of mechanical forces.

3.4 Conclusion

The use of the compressive force is quite novel and can to some extent improve the culture efficiency and more osteogenic induction. It can shorten the period of culture, demonstrating great potential in coupling with bioreactor for clinical application of the bone defect cases. Mechanical stimulation is an important initiator of osteoblastic differentiation and activation.

The experiment of the continuous compressive force is only the first step. By the result of this, we can at least prove the effect of mechanical stimulation for the regenerative medicine. Then we will further the loads boardly into the cyclic compressive force, cyclic torsional loading and the combination of both, and the magnitude and frequencies applied to cell culture should be tested and choosed specific to the tissue for the remodelling process.

Chapter 4: Fibrin cell seeding method to enhance the efficiency of bioreactors

4.1 Introduction

Currently, regenerative medicine has been a promising technique for medical applications and tissue engineering has directly influenced the development of the clinical trials. Usually the efficiency of the regenerative grafts relies on the cell type and culture, scaffolds and the microenvironment. Based on our previous study, the wide use of biaxial bioreactor was proven as advanced equipment for mimicking the requirements for the growth of the grafts, such as temperature, oxygen, the fluid of medium with nutrients and even the existing mechanical stimulation in human bodies. However, the optimization of the biaxial bioreactor systems should be our issue and trend for the further research.

The efficiency of the cell seeding should be the first factor we are concerned about. According to protocols of the experiments, the whole process of the culture period compromises the proliferation of hfMSCs in dynamic conditions (usually in culture flasks), after cell seeding by the traditional method in scaffolds, they still need one week with the goal of maximizing constant adhesion of the cells and the in 3D PCL-TCP scaffolds in a incubators, which also increases the possibility of the contamination due to medium change, compared with the enclosed bioreactor systems. Moreover, the originally employed method is directly pipetting the cell suspension onto the outside of our polymer scaffolds, and this seeding efficiency is only about 10%-25% [34]. Therefore, the importance of the cell seeding method is obvious in terms of tissue engineering.

Generally, a variety of seeding techniques has been utilized during the past three decades, but the existence of several limitations still affects different clinical applicability.[35] Overall, the seeding methods can be divided into three groups:

passive seeding, dynamic seeding and hybrid methods [36]. Static methods involve traditional pipetting cell suspension method and coating scaffolds with biological glues. The motivation of using the gules such as fibronectin and fibrin is not only trapping cells on 3D scaffolds but also facilitating cell attachment to the scaffolds or the matrix [37]. The shortcoming of passive seeding method is that the homogeneous cell distribution sometimes cannot be satisfied while for the glue method for the vivo experiment, partial animals need anticoagulants which can relieve acute thromboembolic events and some other failure. For the dynamic seeding methods, in an effort to enhance the efficiency, many researchers have investigated various dynamic methods: rotational seeding, which involves a rotational system ranging from 0.2rpm to 500rpm and the culture period can be as low as 12h and up to 72h. Scaffolds fixed to a needle are placed in a spinner flask and around the scaffolds is cell suspension. They showed that high-speed rotational system can either increase the seeding efficiency or improve the morphology.[38] [39] Apart from this methods, there still exist other methods such as vacuum seeding, sheet based cell seeding, electrostatic cell seeding and magnetic cell seeding etc.[27, 40-42]

Those different solutions for the cell seeding have their own pros: for the sheet-based cell seeding, it can eliminate the requirement for other materials acting as ECM and anticoagulation therapy; for electrostatic cell seeding, to achieve morphological maturation as demonstrated by SEM is more easier before implantation to animals; for magnetic cell seeding, the efficiencies can be obtained over 99%.[27, 42, 43]

Therefore, the aim of this study is to investigate the effect of the fibrin glue on the growth of hfMSCs, and we hypothesizes that this kind of biological glue can grasp larger amount of cells and due to the mimicking microenvironment of ECM by fibrin the mineralization would be enhanced and the calcium content should be higher.

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4.2Materials and methods

Cellular adhesion, viability and proliferation of hfMSC cellular scaffolds

The Tisseel gel was prepared by a mixture of thrombin and fibrin gel and diluted by Phosphate-Buffered Saline (PBS) 5 times. HfMSCs were re-suspended into the fibrin gel and subsequently seeded onto the Tricalcium Phosphate-Polycaprolactone (TCP-PCL) scaffolds.The morphology of the cell in 3D culture, extracellular matrix (ECM) production and cellular adhesion were examined everyday by contrast light microscope (PCLM) over 28 days. Usually scaffolds were examined in two perpendicular panels, in planar (top) and side view points, because this can provide the more over information of the whole scaffolds.

Fluorescein diacetate/propidium iodide (FDA/PI) staining can test the qualitative analysis of cell viability in 3D was performed , where FDA stains viable cells green, and PI stains necrotic and apoptotic cell nuclei red. Scaffolds were bisected at half to expose to the centre of the scaffold to achieve a core view of the scaffold, stained with FDA/PI as previously described , and viewed under a confocal laser microscope (Olympus, FV300 Fluoview, Japan). Cellular scaffolds were examined in both planar view and side view on day 28.

The total cell number in the 3D cellular scaffold on days 28 (day of transfer to the bioreactor or static culture conditions, as illustrated by Fig. 1A) was estimated by quantifying the dsDNA content of every scaffold using a Pico- Green dsDNA Quantification Kit (Molecular Probes, USA). The total dsDNA was extracted from each cellular scaffold by incubating the cellular scaffolds in 0.4 ml enzymatic cocktail (consisting of 0.1% collagenase A (Roche) with 0.1% Trypsin mixed in PBS) at 37 ℃ for 3h, with vortex every 30 min then followed by three cycles of freeze and thaw; and assayed by following the manufacturer's instruction.

The proliferation of the hfMSC inside 3D scaffold was interpreted by the changes of dsDNA amount.

Comparison of osteogenic differentiation and mineralization in 3D scaffold culture

ALP activity assay

The intracellular ALP activities of hfMSC cellular scaffolds under two culture conditions were compared on days 28. Cell lysates were tested for ALP activity using SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec USA) and the ALP activities were normalized to the total protein content determined using the Bradford assay (Bio-Rad Laboratories, US) as previously described.

Calcium content assay

The calcium content of the hfMSC cellular scaffolds on day 28 was assayed as previously described. Specifically, the calcium deposition is dissolved in 1.0 ml 0.5 N acetic acid and determined by a colorimetric assay using calcium assay kit (BioAssay Systems, USA). And control cell-free empty scaffolds cultured as above were used as a negative control to offset the elution of calcium from the tricalcium phosphate component in the scaffold.

4.3Results and discussion

From Fig.4.1, it showed that 3D scaffolds may affect the extraction of cells from scaffolds, with collagenized tripsin, cells still cannot be 100% dissolved in it. This showed that scaffolds can to some extent grasp cells more effectively and fibrin should be a proper alternative for cells adhesion. However, how to dissolve fibrin to obtain the correct level of essays should be an issue.

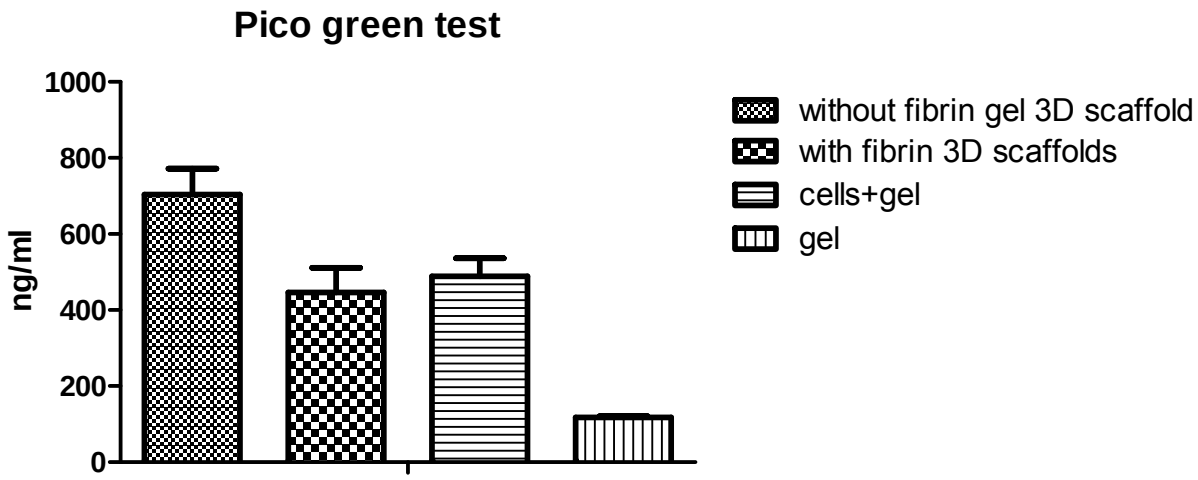


Fig.4.1 The pre test of ds DNA about the fibrin 3D scaffolds

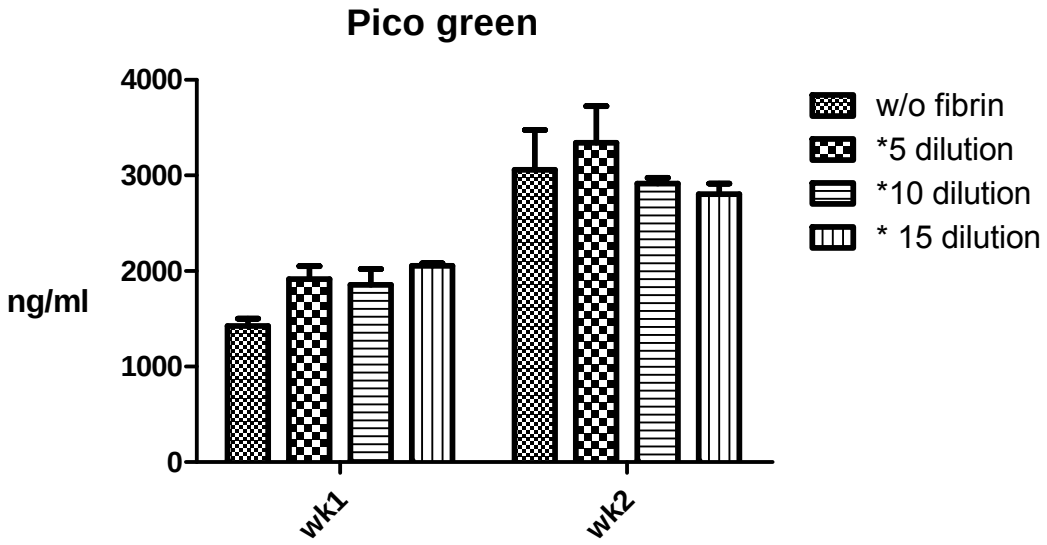


Fig.4.2. (A) ds DNA concentration of different dilution

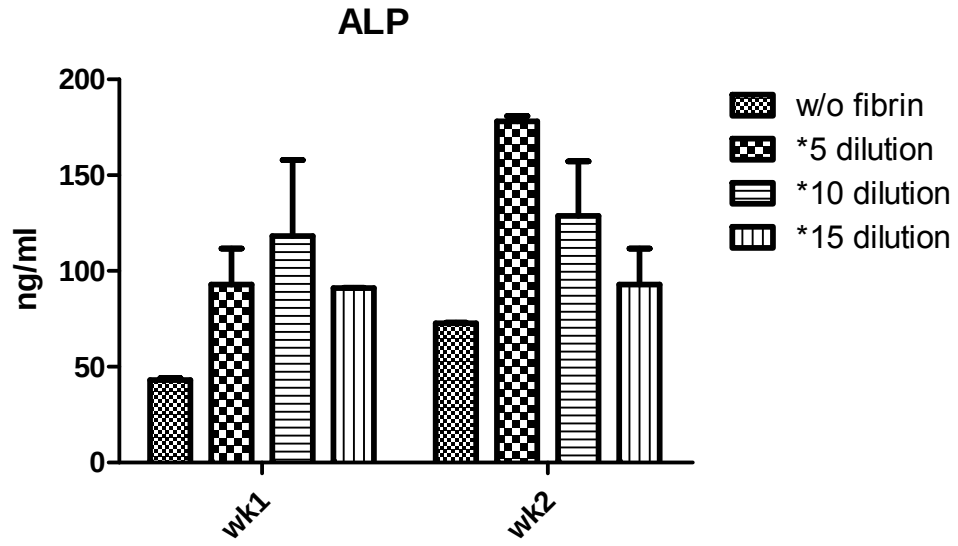


Fig.4.2 (B) ALP activity of different dilutions

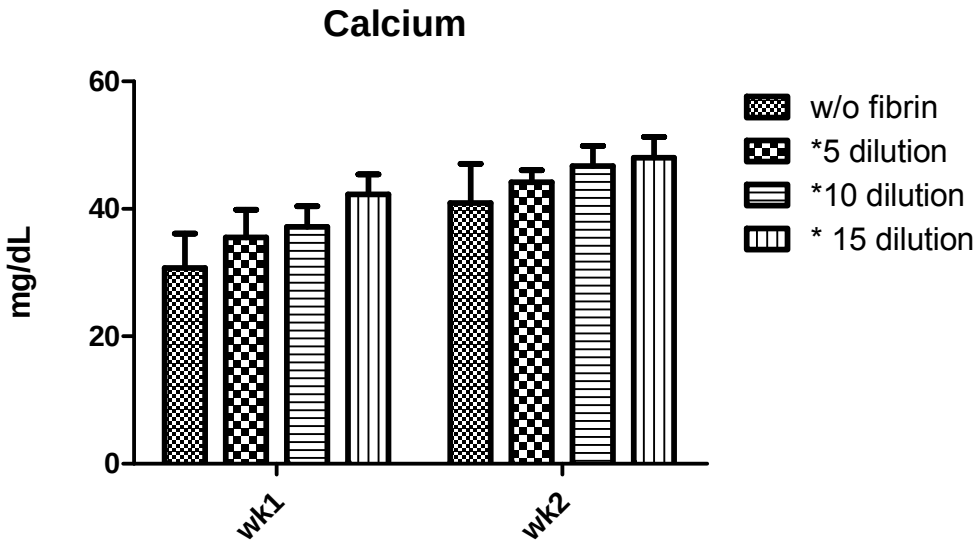


Fig.4.2. (C) Calcium concentrations of different dilutions

From the different concentrations of fibrin Fig.4.2, it can be concluded that 5X and 10X dilutions were more effective than without fibrin and also more effective than 15X dilution group. In the results of Pico green, 5X dilution got the highest level compared with other groups. From the ALP activity results, it also showed that 5X dilution fibrin should be the suitable concentration. While from the calcium concentration result, the calcium was the highest at week 1 with 15 X dilutions and at week 2, 15X still got the highest level.

This means that compared with other groups, 5* dilution of fibrin can be a premium choice in terms of the ds DNA of cells, especially at week 2. While 10* dilution and 15* dilution even leaded to lower levels of ds DNA. At week 1, the difference is not so obvious.

From microscope images (Fig.4.3), it could be shown that scaffolds without fibrin did not proliferate homogenously and there were some pores without cells. In contrast, the scaffolds with fibrin could have the homogenous cell proliferation. The scaffolds

with gel could retain more cells and less unoccupied pores, and cell adhesion was more homogenous compared to those without fibrin and retained high cellular viabilities in the core of the scaffolds

Overall, the 5X dilution of fibrin should be the proper one to continue the further experiments in bioreactor systems instead of only in static conditions.

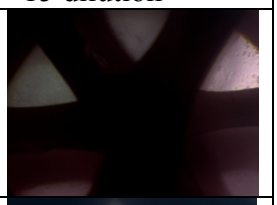
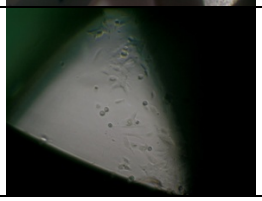
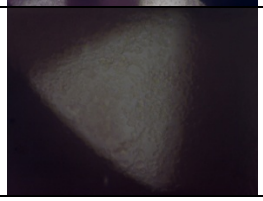
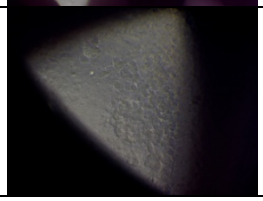
	w/o fibrin	*5 dilution	*10 dilution	*15 dilution
40X				
100X				

Fig.4.3. Microscope images of different fibrin dilutions

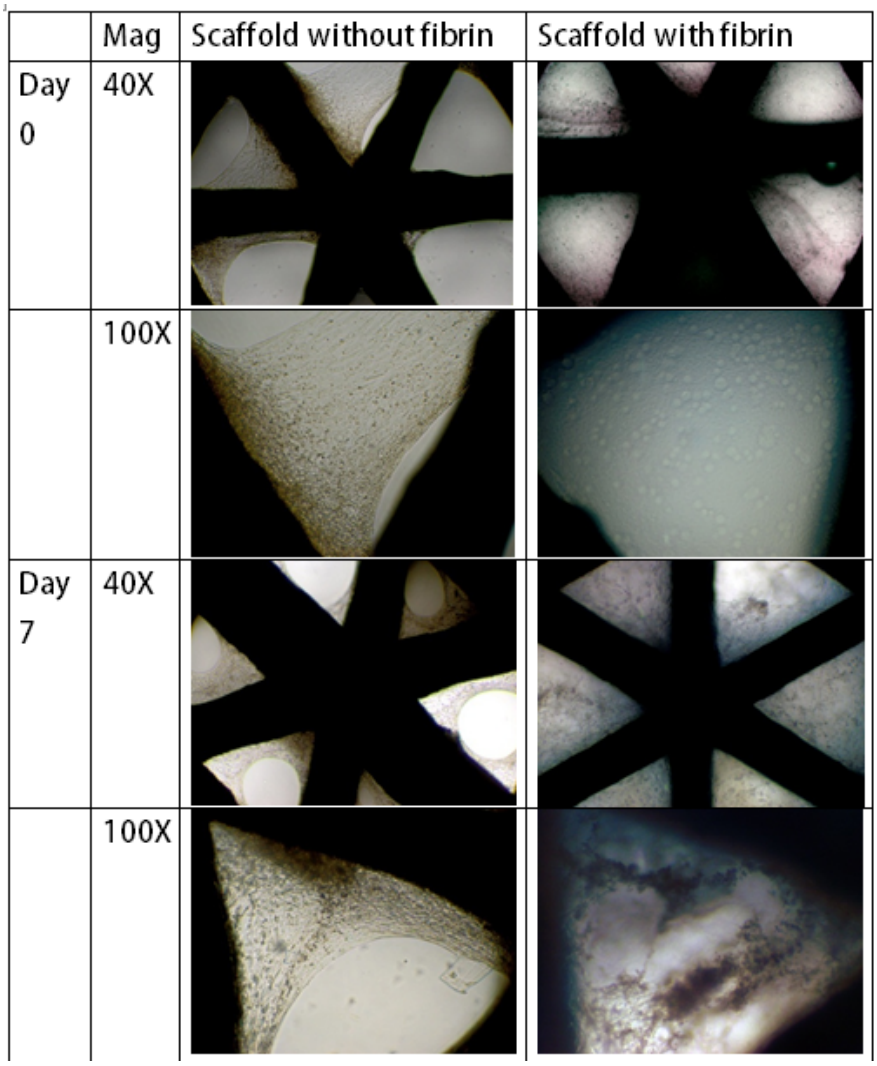


Fig. 4.4 (A) microscope images of scaffolds cultured in a biaxial bioreactor

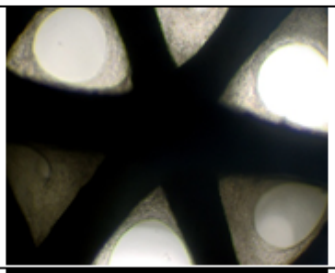
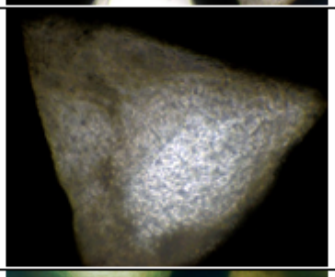
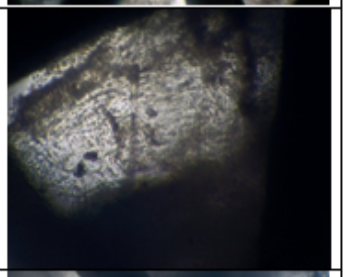
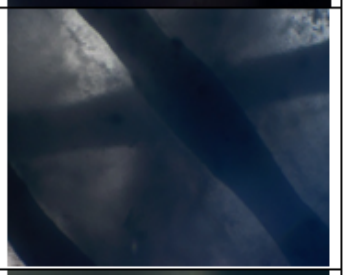
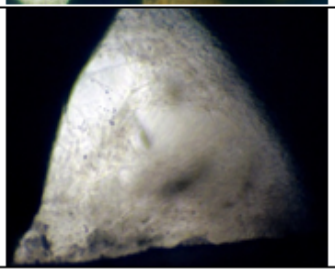
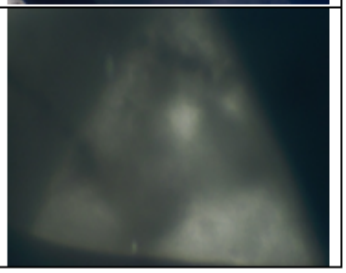
	Mag	Scaffold without fibrin	Scaffold with fibrin
Day 14	40X		
	100X		
Day 28	40X		
	100X		

Fig. 4.4 (B) microscope images of scaffolds cultured in a biaxial bioreactor

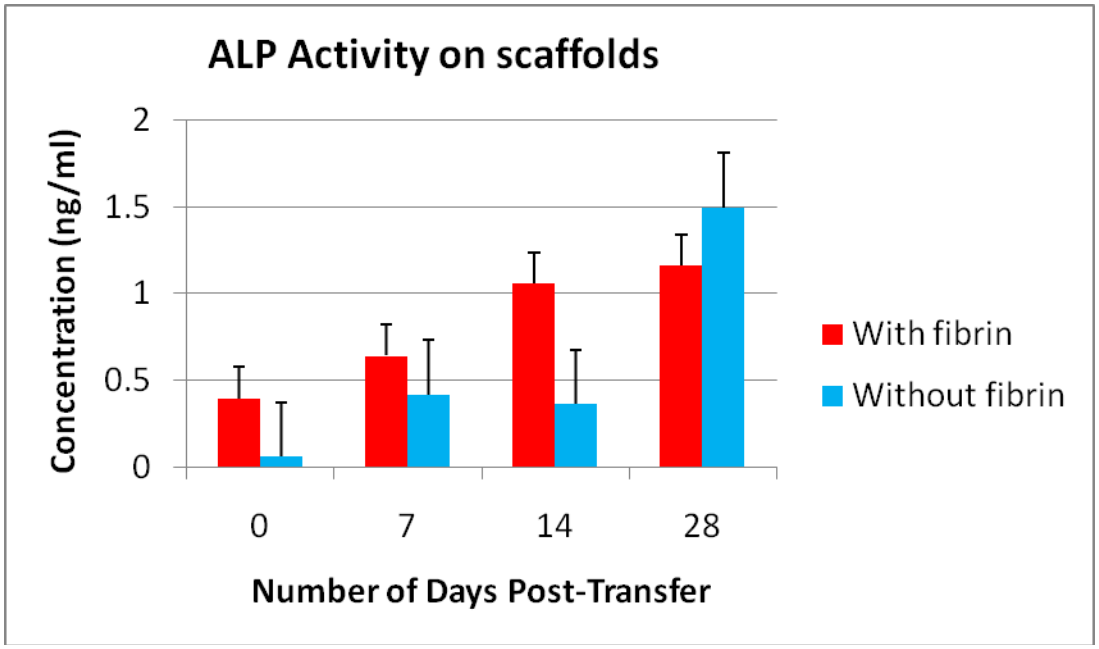


Fig. 4.5. ALP activity on scaffolds

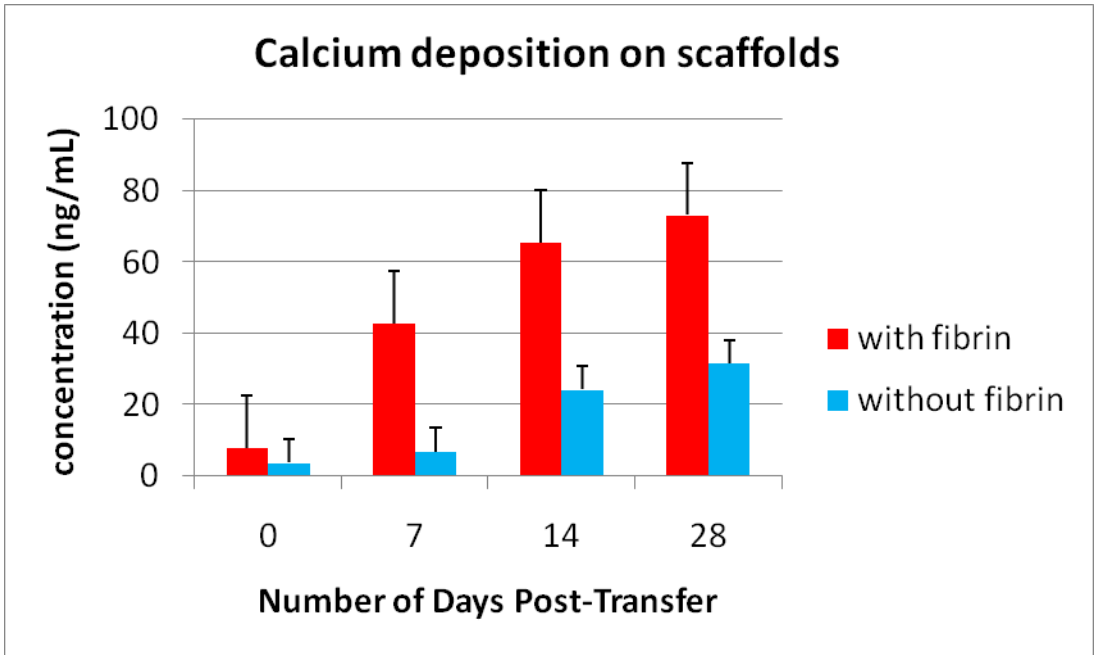


Fig.4.6. Calcium deposition on scaffolds

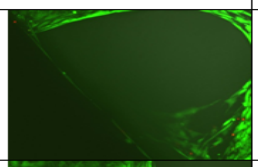
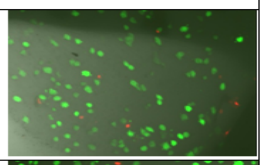
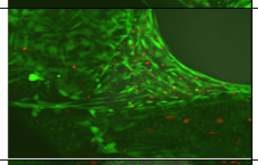
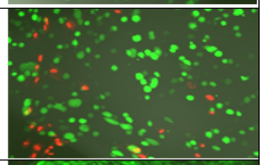
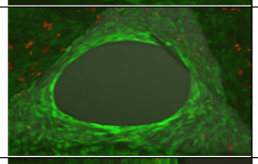
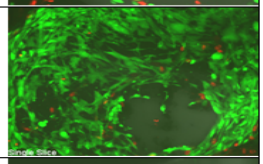
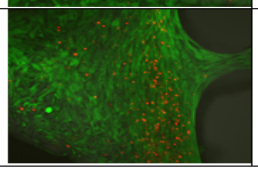
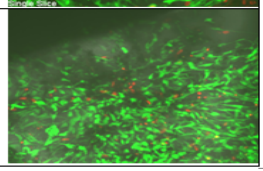
	View	Scaffold without fibrin	Scaffold with fibrin
Day 0	Planar		
	Core		
Day 7	Planar		
	Core		

Fig.4.7 (A) Confocal images

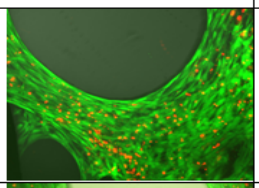
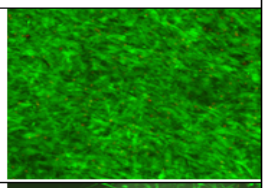
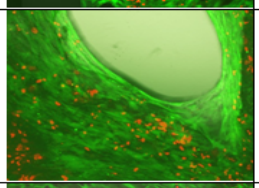
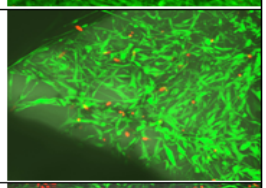
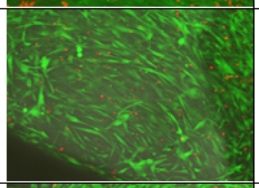
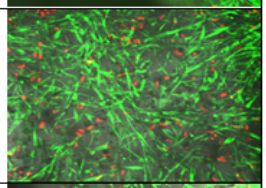
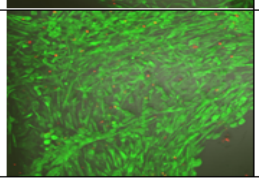
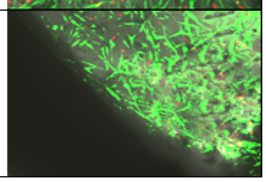
	View	Scaffold without fibrin	Scaffold with fibrin
Day 14	Planar		
	Core		
Day 28	Planar		
	Core		

Fig.4.7 (B) Confocal images

The scaffolds with Tisseel gel could retain more cells and less unoccupied pores, and cell adhesion was more homogenous compared to those without fibrin and retained high cellular viabilities in the core of the scaffolds (Fig.4.4 and Fig.4.7). The calcium concentration essay showed the increase in calcium concentration level in the experiment group with fibrin (Fig.4.5 and Fig.4.6). At week 4, calcium concentration remained 3 times higher than that of control group without fibrin. Meanwhile, the SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec USA) and Picogreen dsSNA assay also showed the enhanced osteogenic differentiation in the fibrin group.

In this study, we demonstrated that fibrin cell seeding method matured hfMSC/PCL-TCP scaffolds resulted in higher cellular proliferation, homogeneous distribution and enhanced osteogenic differentiation than control group without fibrin.

4.4 Conclusion

The scaffolds with Tisseel gel could retain more cells and less unoccupied pores, and cell adhesion was more homogenous compared to those without fibrin and retained high cellular viabilities in the core of the scaffolds The ALP and calcium concentration essay showed the increase in calcium concentration level in the experiment group with fibrin. At week 4, calcium concentraion remained 3 times higher than that of control gourp without fibrin.

In this study, we demonstrated that fibrin cell seeding method with hfMSC/PCL-TCP scaffolds resulted in higher cellular proliferation, homogeneous distribution and enhanced osteogenic differentiation than control group without fibrin.

The use of fibrin aided cell seeding method could improve cellular distribution, seeding efficiency and more osteogenic induction, demonstrating great potential in

coupling with bioreactor for clinical application. The efficiency of bioreactor system can be enhanced not only for in vitro but in vivo experiments for the further study.

Chapter 5: Chemical effect on hfMSCs culture

5.1 Introduction

Zinc is an essential factor for both humans and animals [30]. Being a nutritional element for bone growth retardation, zinc has been demonstrated that it can play a physiologic role to stimulate bone protein synthesis, promoting the regulation for bone formation both *in vivo* and *in vitro* [28]. For animal experiments, the insufficient dietary intake of zinc for rats can directly result in the reduce of the number of chondrocytes and osteoblasts in bones [44] and the study in monkeys have shown that zinc deprivation is associated with the delay of skeletal maturation and mineralization [45]. *In vivo* study, zinc can increase the number of osteoblasts like cells due to its ability of inhibitory on bone resorption and stimulatory effect [46]. However, there is a paucity of literature examining the effect of zinc sulfate on 3D scaffolds, not alone in bioreactor systems.

According to our existing biaxial bioreactor culture progress, the whole period of the hfMSCs differentiation and mineralization requires almost 1 month which is still a considerable amount of time required. Optimizing the bioreactor system by shortening the culture time and improving the osteogenic differentiation ability of hfMSCs can be an interesting issue for our experiment.

Our main aim is to: certify the impact of zinc on the 2D hfMSCs culture first, while finding the proper concentration of zinc for the cells growth; apply this suited concentration of zinc medium to the 3D scaffolds and obtain the comparison results with the control group; test the difference between common bone medium and zinc medium.

With regard to physiological significance, we hypothesized that zinc sulfate can enhance the proliferation and mineralization of hfMSCs in the static condition. If

proven to be true, we can further apply this chemical to our biaxial bioreactor system. With the addition of zinc, the origin culture period in the chamber can be shortened and the efficiency can be promoted, which will to some extent optimize the bioreactor's function.

5.2 Materials and methods

5.2.1 Preparation of zinc medium

The different concentrations of medium could be prepared by zinc sulfate chemical (Sigma company, Singapore). At first, the zinc–free medium, bone medium was prepared as a base medium. 2.9 mg zinc sulfate was dissolved in 1ml ddH_2O in a 5ml tube and then the concentration was 10^{-2} M. Next, 1ml 10^{-2} M zinc sulfate medium was diluted by adding 99ml bone medium. This medium was 100 μM zinc medium. Similar, if the total volume was 200 ml, the total mass of zinc sulfate should be 5.8 mg.

According to the experiments design, the zinc medium can be diluted for different concentrations with bone medium: 0 μM, 10 μM, 25 μM, 50 μM.

5.2.2 Cell culture and treatment

Culture the hfMSC of passage 3 in T-75 culture flask at 37 ℃ in a humidified atmosphere of 5％ CO_2 in incubator. Under the microscope, when the cells had around 80% confluence, they can be re-suspended and subsequently and seeded onto 5mm*5mm* 5mm cubic TCP-PCL (n=3). The density of hfMSCs in every scaffold is 0.5 million in a dropwise manner. Before adding the bone medium, the scaffolds with cells should be stored in the incubator for 3h in 12-well plates. After 3h, the additional 1.5ml of bone medium could be added to each wellwith bone medium (0.005% ascorbic acid, 0.3% β-glycerophosphate and 10nM dexamethasone), the

cellular scaffolds were incubated in at 37 C° and 5% CO_2 for week and change medium for 3 times. During this period, the cells can adhere on scaffolds much better.

Before the experiments, the concentration of 25 μM was tested first as a preliminary experiment to confirm whether this could have the effect on it. We hypothesized that if the effect could be tested, then the proper concentration for the zinc sulfate experiments would be determined.

5.2.3 Assay methods

As described before in Chapter 3 and Chapter 4.

5.3 Results and discussion

From the quantitative experiment (Fig.5.1, Fig.5.2 and Fig.5.3), the results showed that under the condition of zinc concentration of 25 μM, the dsDNA level can be enhanced because hfMSC proliferated rapidly. Pico green demonstrated the content of total double-stranded DNA. In contrast, zinc free cultured scaffolds only got the lower level of dsDNA ($p<0.05$).

We also observed the deposition of extracellular calcium crystals were enhanced in the zinc medium and scanning electron microscopy (SEM) revealed higher levels of crystal-like extracellular matrix (ECM) deposition in zinc cultured scaffolds (Fig.5.4).

Supporting this observation, we found that cells expressed significantly higher levels of ALP activity than the bone medium without zinc sulfate.

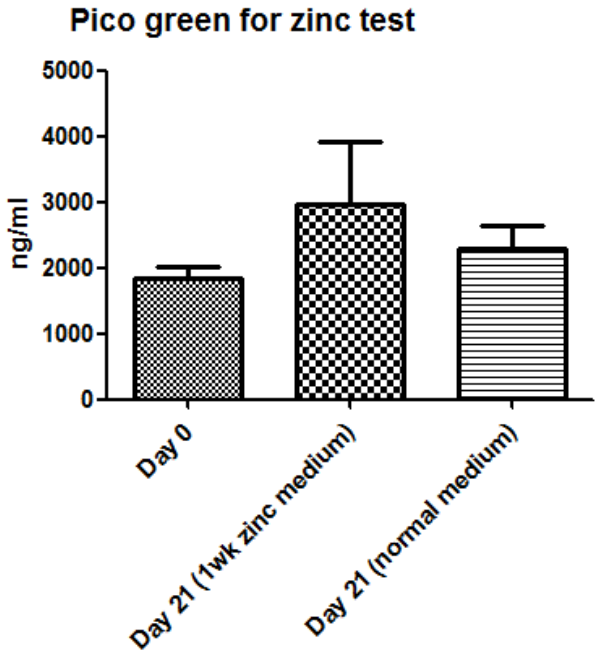


Fig. 5.1 ds DNA concentration comparisons with 25 μM

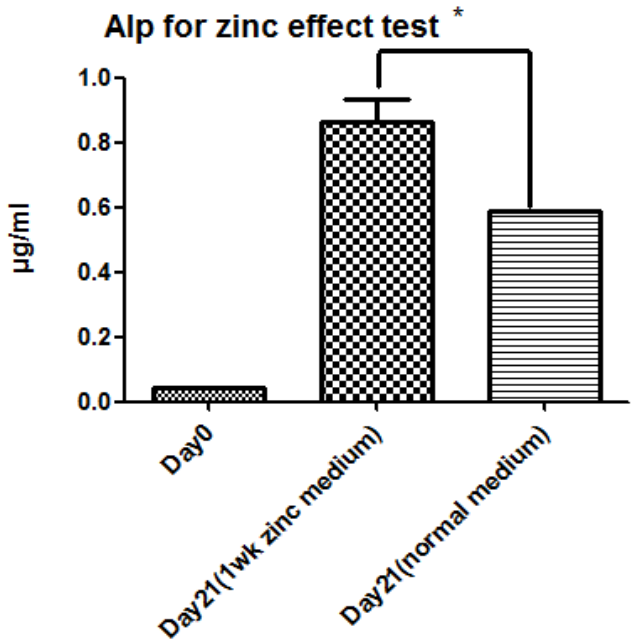


Fig. 5.2 ALP activity comparisons with 25 μM

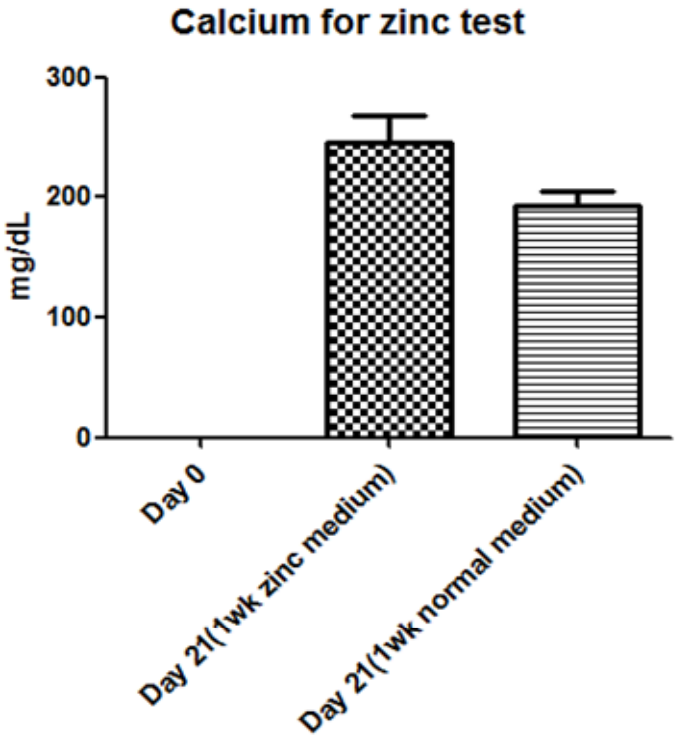


Fig.5.3 Caciulm comparisons with 25 μM

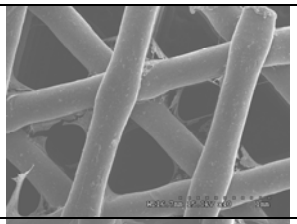
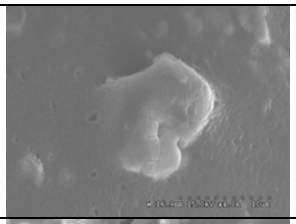
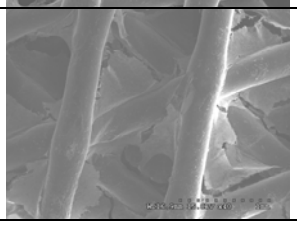
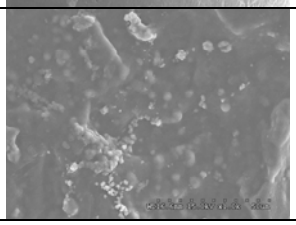
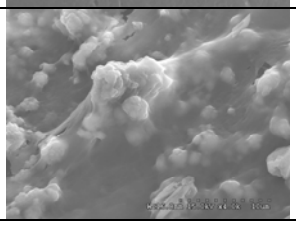
	40X	1K X	4K X
w/o zinc			
With zinc			

Fig.5.4. SEM images of two groups

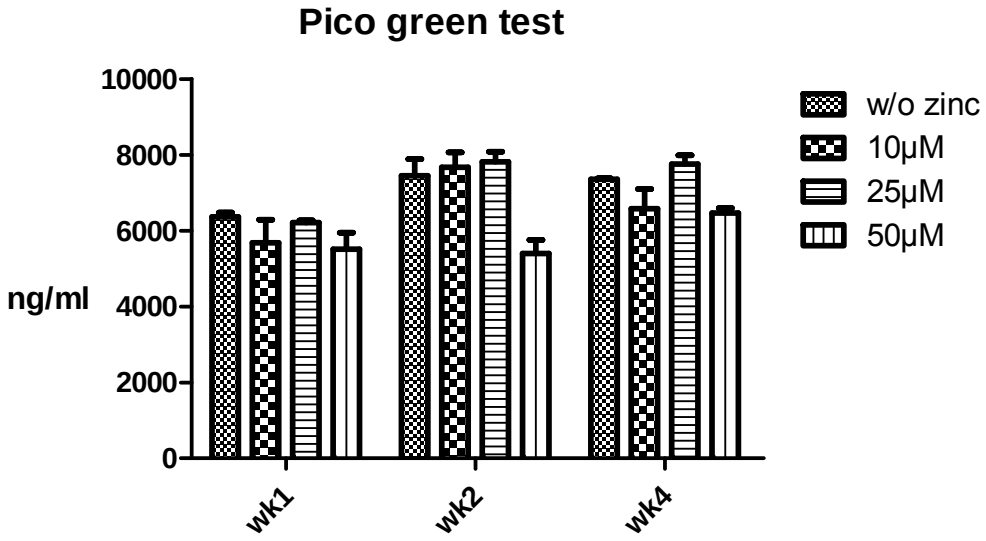


Fig.5.5. ds DNA concentrations from different concentrations of zinc sulfate

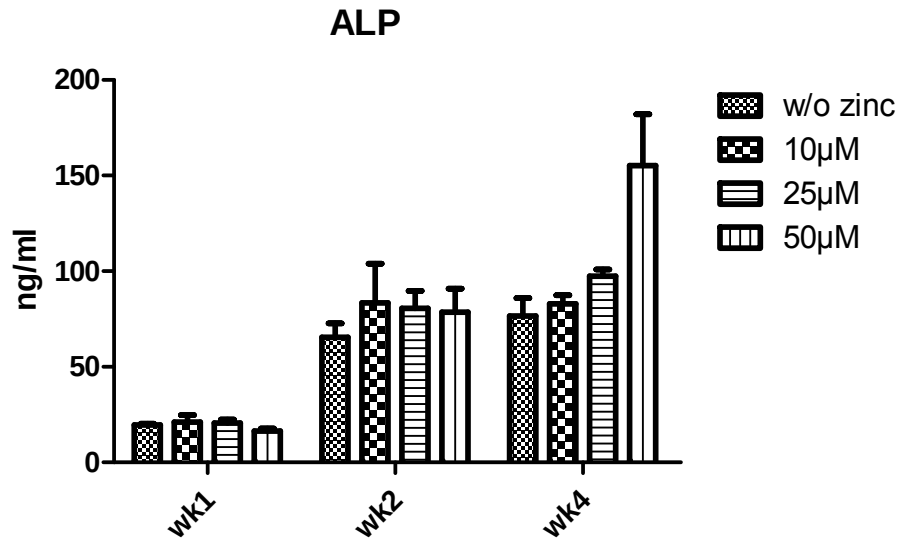


Fig.5.6. ALP activity from different concentrations of zinc sulfate

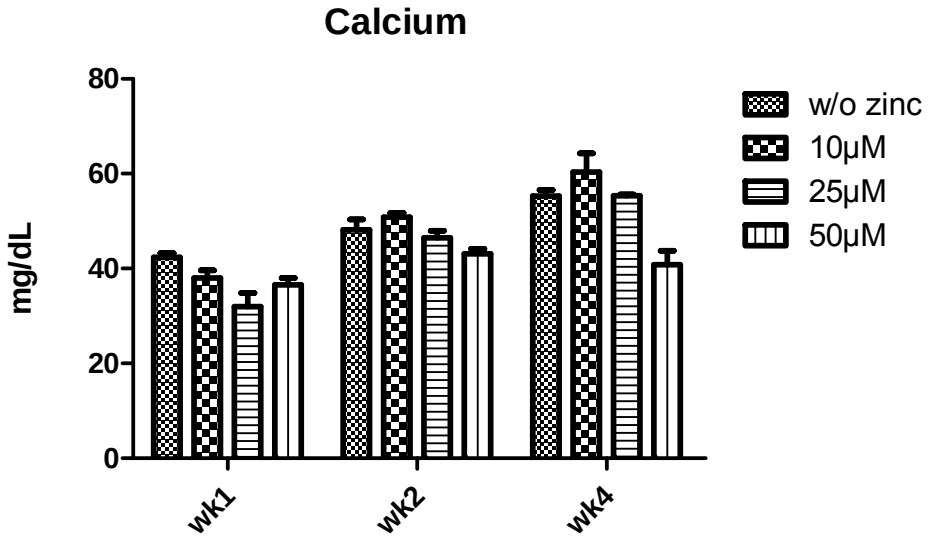


Fig.5.7. Calcium concentrations from different concentrations of zinc sulfate

The levels of dsDNA, ALP and calcium increased after a period of time. ALP activity increased during the proliferation and maturation steps, but deceased when mineralization was well progressed. From the different concentrations of Zinc sulfate, we can observe that 10 μM and 25 μM could be the suitable concentrations (Fig.5.5 Fig.5.6 and Fig.5.7). This is because zinc can suppress osteoclast differentiation and promoted osteoblast mineralization and can act as a potent NF-jB activation antagonist in osteoclast and osteoblast precursors [47].

5.4 Conclusion

The zinc sulfate experiments have showed the direct effect of zinc on the hfMSCs for the bone formation and can enhance the cell proliferation and differentiation. This optimization and improvement can help to make the bone graft with enhanced efficiency and can shorten the culture period time. Moreover, because of the zinc effect *in vivo*, zinc can also help to prevent osteoporosis and can also help osteoblasts for synthesizing, organizing and mineralizing. Inadequate intakes of zinc can be an important risk factor for the development of osteoporosis [48-50].

Chapter 6: Conclusion

6.1 Findings

To sum up, in the first part of project, mechanical stimulation have proven that it can to some extent enhance the osteogenic ability, although the research only focused on the continuous force, it still can be expanded to different kinds of mechanical force due to the physiological mimicking.

The individual requirements from each patient suggest that the current method of mechanical loading is inefficient. In the event where multiple functions can be used at simultaneously maximum labour and efficiency can be minimized with the shortest time. Besides, quantization can let us get more accurate results.

From the second part, fibrin as a tissue gel can effectively improve the cell loading efficiency as with the mixture of thrombin and fibrin; the cell attachment can be more homogenous and can also mimic ECM in grafts. From the result, it showed that a proper cell seeding method should be the first step of the whole research, especially for in vivo tests, efficiency of cell seeding can directly influence the periods.

The third part about the chemical stimulation demonstrated that with the additional of zinc, the bone medium can cultural more cells with higher ALP activity and calcium level. Zinc, for bone tissue engineering application, it can also prevent osteoporosis and to strengthen bones.

6.2 Limitations

In the optimization research, there are several limitations, for the continuous compressive force, the pins number was only 6 so the time point of essay was only one. If the positions for scaffolds can be increased, more essays can be done to get

more overall results. The external loading was applied by torque wrench, then we can try to use motor or frequency meter to induce this force.

From the Tisseel gel test, fibrin can play a critical role during the culture due to the high cost of Tisseel gel, the wide use of this method may have some limitations. The storage of the gel is also an important issue. Therefore, it still needs some further development.

Bone tissue bioreactors can provide the clinical application a much better process by taking into account different scales of materials and cell types during the cultivation. Moreover, they can provide the means to perform studies and research aimed at understanding the effects of biological, chemical and physical fields. They are able to provide a safe and convenient condition or environment. Furthermore, it can extend a wide way to optimization, such as mechanical stimulation, the method of cell seeding and for the once used disposable bioreactors.

For the future development of bioreactors, the bioreactor system can be an advantageous method in terms of low contamination risk, ease of scalability. Due to the complex progress of the whole bioreactor's design and fabrication, collaboration between biologists and engineers can lead to the understanding of issues. The advances will aid in fulfilling the expectations for revolutionizing medical care.

Chapter 7: Future recommendation

7.1 Mechanical stimulation

During humans' daily activities, the human skeletal system may sustain different kinds of forces and majority of them should be multiaxial and cyclical. This is important for improving cell proliferation and osteoblastic differentiation.

A. continuous compressive force

The continuous force on the scaffolds can be an initial step for the whole outline of our mechanical design. This basic foundation research is a test for the properties of our TCP-PCL materials. Compared with the origin chamber without this compressive load, this continuous force can to some extent optimize the culture condition in vitro.

B. cyclic compressive force

Cyclic stress is quite common during our daily life. Regarding to the example of walk, the mechanical force is cyclic as every step is extremely similar to the one before. Meanwhile it is also multiaxial because joints and muscles are pushing and pulling the bone in different directions.

In order to realize the function of this cyclic compressive force chamber, the usage of motor to control the frequency and the quantity of stress is important. We plan that with the addition of the cyclic force, the period of the exposure time under this is strictly accurate according to the different clinical applications and bone biomechanics.

C. enhanced shear stress

The fluid shear can exert effect on osteoblastic differentiation, which means the osteogenic signal expression of mesenchymal stem cells can be influenced. Because bone cells, in particular osteocytes, are quite sensitive to shear stress, which can be a

common phenomenon related to mechanical adaptation of bone. Meanwhile, the osteocytes can play a role of mechanosensors and transmit the mechanical information to adjacent cells through the intercellular communication network. Therefore, via this optimization, cells in vitro can be more effective and more adapted to the patients' requirements.

Based on our initial biaxial bioreactor, the combination of the pins and the holder of the bioreactor system can keep pins rotating. Because the origin bioreactor's chamber will rotate along X axis, when the pins now are fastened to the base, the motion of the chamber can let the scaffolds have a relative movement in the medium.

7. 2 Disposable bioreactor

In traditional design, bioreactors are always made of glass or stainless steel, but for disposal bioreactors, most of them can be made of plastic, such as polyethylene, polystyrene, polytetrafluorethylene, polypropylene (PP), or ethylene vinyl acetate. Actually, the only plastic chamber cannot satisfy the need of clinical application, because it still should have an external device to provide the premium condition for cell growth and even product formation. Therefore, for the bioreactors with 1L or larger culture volumes are usually equipped with disposable sensors, such as monitoring the pH, temperature and oxygen level (Fig.7.1).

The advantages of those disposable bioreactors are quite obvious: they have high flexibility, easy handling, saving time and lower incidence of cross contamination. For different patients and different requirements' standard, they can be fabricated according to the specific conditions. Meanwhile, the shortcomings of the disposable bioreactors cannot be ignored. The limitation of insufficient plastic material strength and single-use philosophy itself may also affect the widely use of this kind of bioreactors. The unstable property of PCL compared with glass chambers is also an

important negative factor. The reaction between the medium or the cell metabolism waste and PCL sometimes has an effect.

Overall, how to solve the problems of those disposable bioreactors and make them more available is a significant issue[51].

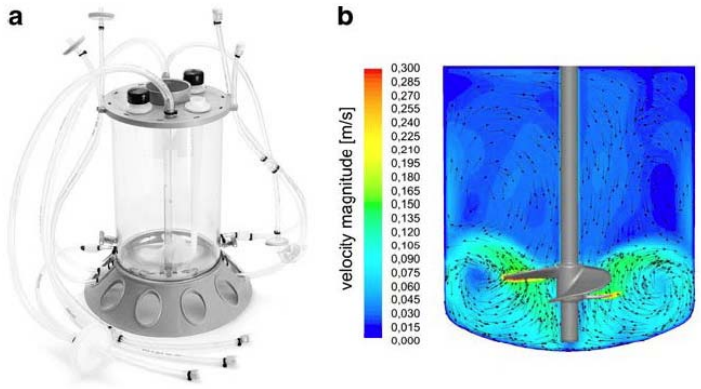


Fig.7 .1Mobius Cell Ready 3 L Bioreactor. a product picture. B velocity profile estimated with CFD [51].

7.3 Bioreactor modeling

Computational fluid dynamics (CFD) modeling is one of the effective techniques used in simulation flow fields. The models are validated by such as laser Dopper anemometry and particle-image velocimetry (PIV) [51], but they are always time consuming. CFD simulation method provides a powerful means for solving these limitations and enabling the characterization of 3D flow fields.(Fig.7.2) For instance, hydrostatic pressure can lead to the improved mass transfer of large and small molecules and can also induce a mechanical stimulation of embedded cells. Therefore, the simulation methods can support the experimental studies, CFD or finite-elment approach can be a good way to predict.

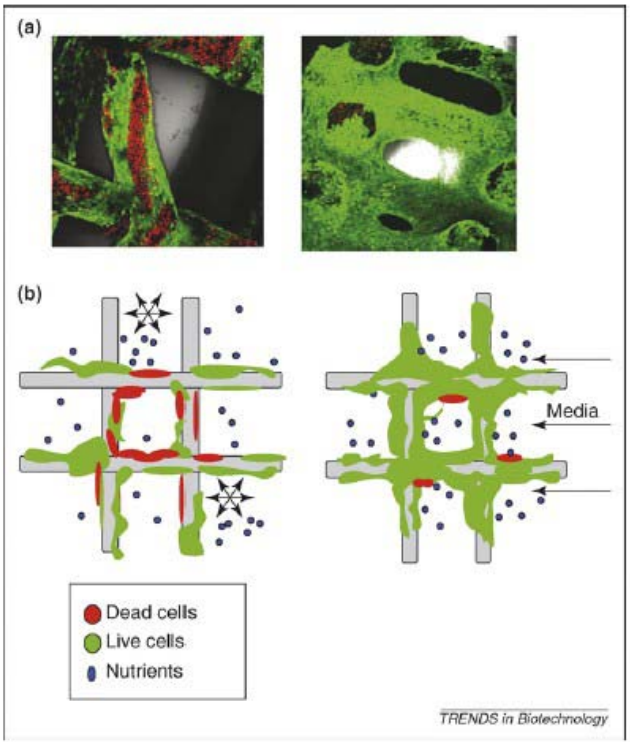


Fig .7.2. (a) Graphical illustration showing the effects of static(left) and dynamic(right) flows on nutrient diffusion and cell survival at the scaffold. (b) Confocal microscopy images of seeded scaffolds stained for live (green) and dead (red) cells reveal higher numbers of dead cells under static condions compared with the dynamically cultured scaffolds [51].

Besides, Omasa et al. applied another kind of method, analytic hierarchy process (AHP). In order to evaluate a bioreactor, five criteria should be concerned, safety, scalability, cell growth condition, mimicking native tissue's functions and handling.

Specifically, the parameters of bioreactors can be predicted and calculated before the fabrication, such as shear stress and the external loading, which can optimize the growth of cells. In our bone tissue engineering, for the porous scaffolds, the modeling of the dynamic effect is more useful. The Navier-Stoke equation in bio fluid region in the porous medium is numerically solved. For the porous-fluid interface, the Whitaker's and Ochoa-Tapia stress can be used to show the effect on flow and mass

transfer. The kinetic reaction is based on Michaelis-Menten type. The simulation results are related to parameters for the purpose of generalized results. This in the design analysis can be an application.

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Appendices
I

SensoLyte™ *p*NPP Alkaline Phosphatase Assay Kit

KIT COMPONENTS

Component A: *p*NPP, colorimetric alkaline phosphatase substrate (1 vial)
Component B: 10X Assay buffer (50 mL)
Component C: Stop solution (25 mL)
Component D: Triton X-100 (500 µL)
Component G: Alkaline Phosphatase Standard, Calf Intestine (10 µg/mL, 50 µL, sterile)

PROTOCOL

1. Prepare *p*NPP stock solution

• Reconstitute by adding 300 µL of ddH_2O into the ***p*NPP vial(component A)**. Mix well. Aliquot in 30ul per vial, and freezed in -80°C. (The stock solution will be good for 3-4 weeks if stored at -20°C).

2. Prepare *p*NPP reaction solution

• Dilute the *p*NPP stock solution 1:100 with **2X assay buffer (component B)**. Prepare fresh reaction mixture for each experiment.

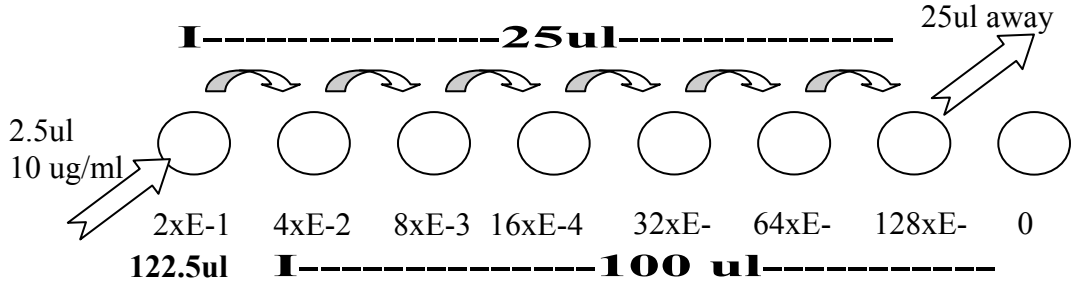
Prepare pNPP reaction solution				
total test	total volume of pNPP reaction mixture(ul)	to prepare(ul)	stock of pNpp(ul)	2X assay buffer
96	4900	5100	51	5100

3. Alkaline phosphatase standard.

• Prepare ddH2O with 2 mg/mL **bovine serum albumin**
• Prepare alkaline phosphate **dilution buffer:** Dilute **10X lysis buffer (component D)** to 1X with ddH_2O (1.11mg/mL **BSA)**.

prepare 1x dilution buffer			
1x dilution buffer(ul)	10x buffer(ul)	ddH2O	2mg/ml BSA(ul)
850	85	340	425

• Dilute **alkaline phosphatase standard (10 µg/mL component G)** to 0.2 µg/mL (1:50) in dilution buffer. Then make five-fold serial dilutions to get the concentration of 0.04, 0.008, 0.0016, 0.00032, 0.000064, 0.0000128, and 0 µg/mL of alkaline phosphatase solution. In 96 well plate, dilute as follows
Note: Unused portion of diluted alkaline phosphatase solution should be discarded.



Conc.	2xE-1	4xE-	8xE-	16xE-	32xE-	64xE-	128xE-7	0

(ug/ml)		2	3	4	5	6		
1x dilution buffer (ul)	**122.5**	100	100	100	100	100	100	100
From previous	**2.5 from 10 ug/ml**	25	25	25	25	25	**Remove 25**	0

4. Detect alkaline phosphatase activity

• Add 50 µL/well (96-well plate) of biological samples containing alkaline phosphatase.

• Add 50 µL/well (96-well plate) of *p*NPP reaction mixture. Mix the reagents by gently shaking the plate for 30 sec.

• Measure absorbance:

Incubate reaction at the desired temperature for 20 min. Add 50 µL/well (96-well plate) of stop solution (component C). Shake the plate on a plate shaker for 1 min before the reading. Measure absorbance at 405 nm.

II

Bone differentiation
----monolayer

Bone differentiation medium

Dexamethasone: **10^{-8} M D2915 – 100mg 69 GBP**
Ascorbic Acid: **0.2 mM A4544 – 100 g 19.80**
b-Glycerophosphate: **10 mM G9891 – 25 gm 24.90**

DEXAMETHASONE (100X; Stock conc: 10^{-6} M)

1. Put 3 sterile 1.5 ml eppendorf tubes in a rack
   Tube 1: 1 ml ddH20
   Tube 2: 1 ml 10% FBS (10% FBS-DMEM)
   Tube 3: 4500 µl 10% FBS
2. Weigh 6 mg Dexamethasone
3. Dissolve it in 1 ml ddH2O in tube 1 (10^{-3} M)
4. Aliquot 10 µl of 10^{-3}M into tube 2 (100x dil) (10^{-5} M)
5. Aliquot 500 µl of 10^{-5} M into tube 3 (10x dil) (10^{-6} M)
6. Freeze in -20 degree.

ASCORBIC ACID(100X; Stock conc: 0.02 M)

1. Put 2 sterile 1.5 ml eppendorf tubes in a rack
   Tube 1: 1 ml ddH20
   Tube 2: 4667 µl 10% FBS
2. Weigh 50 mg Ascorbic Acid
3. Dissolve it in 1 ml ddH2O in tube 1 (0.3 M)
4. Aliquot 333 µl of 0.3 M into tube 2 (0.2 x 10^{-1} M) 15 X Dilution
5. Freeze in -20 degree.

Beta-GLYCEROPHOSPHATE(Stock conc: 0.9 M)

1. Weigh 200 mg beta-glycerophosphate
2. Dissolve it in 1 ml ddH2O (0.9 M)
3. Freeze in -20 degree.

For 10 ml of Osteogenic medium:

1. In a 15 ml tube add

   - 9,689 µl 10% FBS in DMEM
   - 100 µl 10^{-6} MDexamethasone (100 x dil; 10^{-8} M)
   - 100 µl 0.2 x 10^{-1} M Ascorbic Acid (100x dil; 0.2 x 10^{-3}M)
   - 111 µl beta-Glycerophosphate 0.9 M (90x dil; 10 mM)

2. Filter (0.2 µ) the medium after addition of the substances.

Differentiation:

1. Seed the cells at 2 x10^4 MSC/cm^2 in regular growth medium:
2. Let the cells adhere over night.
3. On the second day, take picture and change to bone differentiation media.
4. Change media every 3-4th day for 2-3 weeks and take picture before change medium. Note: In the end of the differentiation, change the medium **very gently**!
5. Cells change shape and produce minerals. Calcium salts is detected with Alizarin Red staining and phosphates are detected with Von Kossa staining.
6. The osteogenic gene expression is determined by Real time RT PCR, cells are frozen in -80 degree before the extraction of mRNA.