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IGF-I RELEASING PLGA SCAFFOLDS FOR GROWTH PLATE REGENERATION

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ABSTRACT OF THESIS

IGF-I RELEASING PLGA SCAFFOLDS FOR GROWTH PLATE REGENERATION

Growth plate is a highly organized cartilaginous tissue found at the end of long bones and is responsible for longitudinal growth of the bones. Growth plate fracture leads to retarded growth and unequal limb length, which might have a lifelong effect on a person's physical stature. This research is a tissue engineering approach for the treatment of growth plate injury. Insulin-like growth factor I (IGF-I), which can stimulate cartilage formation, was encapsulated within PLGA microspheres that were then used to form porous scaffolds. The release profile of the IGF-I from the PLGA scaffold showed a biphasic release pattern. In vitro studies were done by seeding rat bone marrow cells (BMCs) on the top of IGF-I encapsulated PLGA scaffolds, and the results showed an increase in cell multiplication and glycosaminoglycan content. The final in vivo studies were conducted by creating growth plate injury and implanting scaffolds in the tibiae of the New-Zealand white rabbits. Histological analysis of tissue sections showed regeneration of cartilage, albeit with disorganized structure, at the site of implantation of IGF-I encapsulated scaffolds. This work will be a significant step towards tissue engineering of growth plate cartilage.

KEYWORDS: PLGA, Tissue engineering, growth plate, drug delivery and IGF-I

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THESIS

Sharath kumar Chinnakavanam Sundararaj

The Graduate School

University of Kentucky

2010

IGF-I RELEASING PLGA SCAFFOLDS FOR GROWTH PLATE REGENERATION

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biomedical Engineering
in the Graduate school at the University of Kentucky

By
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2010

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1. INTRODUCTION

Growth plate is an area of developing cartilaginous tissue found in the end of the bones. The chondrocytes present in the growth plate undergo division by mitosis along with matrix synthesis aid in the elongation of the bone. The growth plate ossifies or hardens after a certain age and determines the length and shape of the mature bone.

Growth plate injury is a type of injury or fracture that occurs at the physis region of the bone where the growth plate is present. Growth plate injuries are seen more commonly in boys and girls at the end of their growth period and young adulthood, whose growth plate has not matured and ossified in to bone. Damage in the growth plate results in retarded growth and unequal limb length.

Usually the damaged cartilage in the body is replaced by fibrous cartilage, which does not possess the same properties of the hyaline cartilage and will not be able to perform the necessary functions. If an injury occurs at the growth plate region, the cartilage does not get regenerated or replaced by fibrous cartilage as observed in the usual case but it is replaced by bone. The process of formation of a bone bar to replace the damaged cartilage makes the condition worse and increases the immediate need for a better treatment option. There is no current treatment that regenerates the injured growth plate and its functions completely.

Tissue engineering of articular cartilage is a treatment option that is growing in popularity. Promising preclinical animal models and clinical pilot studies have encouraged further research in this field. Most of the tissue engineering approaches involve the harvesting of cells from the donor and replenishing them in the damaged or the injured site for healing and regeneration. One of the major drawbacks with this method is the removal of cells from a healthy site of the donor's body. Some growth factors are also required for the in vitro culture of these cells. The expanded culture is associated with a scaffold for the delivery of the cells to the injured site. The other method of tissue engineering involves the direct loading of growth factors in the scaffolds for delivery in to the treatment site for tissue regeneration. This method avoids the removal of healthy tissues or cells from the patient and eliminates the need for more than one surgery. A

biodegradable scaffold is preferred for this delivery purpose in both the cases because of its ability to be easily removed from the treatment site as the tissue starts to regenerate.

This research focused on the tissue engineering of the growth plate by the delivering the growth factor insulin-like growth factor I (IGF-I). The biodegradable poly(lactic-co-glycolic acid) (PLGA) polymer served as the vehicle for the delivery of growth factors and also provided support to the fractured portion of the bone during the initial stages of recovery. In this case the IGF-I was microencapsulated in PLGA and made in the form of porous scaffold for delivery of the growth factor (IGF-I). PLGA is one of the most widely used polymers for drug delivery and tissue engineering. PLGA is biocompatible in nature and it is easier to achieve desirable release kinetics. These factors make PLGA a practical choice for the current purpose.

Tissue engineering of cartilage is gaining heavy importance in the field of tissue engineering. But most of the research till now is mainly focused on the regeneration of articular cartilage and very little progress has been made in tissue engineering of growth plate. This work was aimed at providing a significant contribution towards tissue engineering of growth plate. We hypothesized that microencapsulation of IGF-I in PLGA would be an appropriate growth factor delivery system for regeneration of growth plate cartilage both in vitro and in vivo.

2. BACKGROUND AND SIGNIFICANCE

2.1 Growth plate injury and treatments

The growth plate is the hyaline cartilage present in the metaphysis at the end of the long bones. Growth plate cartilage consists of chondrocytes and the extracellular matrix which the chondrocyte produces and reside in [1]. Growth plate can be further represented as a highly organized structure mainly consisting of chondrocytes at different stages of proliferation and differentiation in a scaffold of oriented extracellular matrix components consisting of collagen proteoglycans and proteoglycans aggregates [2]. Based on the differentiation of the chondrocytes the growth plate is divided in to three distinct zones, reserve, proliferative and hypertropic zones [3]. In some literature the proliferative zone and hypertropic zones are further subdivided in to upper and lower parts to form five zones [4]. The longitudinal growth of the long bones and vertebrae occurs in the growth plate. Growth plate is the most fragile region of the long bone and is susceptible to injuries. Injury or damage to the growth plate is called as the epiphyseal fracture or growth plate fracture [5, 6] . The growth plate fractures account for approximately 15 – 20 % of the long bone fractures in children. Growth plate does not have the ability to regenerate on its own after an injury. The damage to the growth plate cartilage usually leads to the formation of the osseous bridge at the site of injury. Partial damage to the growth plate results in the shortening and progressive angular deformation of the bone, while more severe damage to the growth plate results in complete arrest of the longitudinal bone growth [6-8].

There have been several attempts made to prevent the partial growth plate closure and induce the regeneration of growth plate. To prevent the closure of the growth plate, the osseous bridge is surgically removed and replaced with an inert material. A variety of natural and synthetic materials have been tried for this purpose, some of them include bone wax, beeswax, methyl methacrylate, siliconized Dacron, silicone rubber, grafts of fat and cartilage. All of these materials were able to provide only limited protection against bone re-growth, while fat and cartilage were found to be relatively better options [7]. For regeneration of growth plate the transplantation of the cartilage from other sites of the body have been tried. These methods involving the transplantation have resulted in only temporary graft survival. The treatment methods which are being practiced now have relatively small number of satisfactory results [7].

Research involving tissue engineering and gene therapy for the regeneration of the growth plate cartilage is gaining rapid importance. Use of growth factors capable of signaling molecules that control mitogenic behavior and cell differentiation is also being widely studied [8].

2.2 Insulin-like growth factor I (IGF-I)

IGF-I is a polypeptide endocrine hormone with the ability to stimulate cartilage growth. IGF-I has a molecular weight of 7600 Daltons and it is synthesized by the cartilage and stored within the ECM of the cartilage [9],[10]. IGFs can be found in the organic skeletal matrix and circulation, with serum containing significant concentrations of both IGF-I and IGF-II [11]. There are several evidences in the literature supporting the role of IGF-I in skeletal growth and development through classic endocrine and autocrine or paracrine mechanisms [12]. It has been proved that IGF-I has the ability to stimulate mitotic activity and matrix synthesis in growth plate chondrocytes in primary monolayer cultures, which proves the general anabolic role of IGF-I in growth plate development [8, 12]. The IGF-I receptors present in the growth plate chondrocytes are stimulated by IGF-I with respect to mitosis and expression of differentiation properties of the chondrocytes [12]. In a study by Tripple et al., it was proved that IGF-I purified from human serum showed the ability to bind to IGF-I receptors on bovine growth plate chondrocytes [13].

IGF-I stimulates growth plate activity by inducing the process of cell proliferation, matrix production and hypertrophy [14]. Along with general enhancement of mitotic activity of chondrocytes, IGF-I has the capability of stimulating chondrocytes at all stages of differentiation [8, 12]. Despite the ability of IGF-I to stimulate chondrocytes at all stages of differentiation, the degree of its effect varies with respect to the cell activity [14]. Proliferative zone cells of the growth plate show a better response to IGF-I when compared to the cells present in the reserve zone, due to the greater IGF-I binding capacity of the proliferative zone cells than resting zone cells. These studies further confirm that the effect of IGF-I on cell population of the growth plate is dependent on the cell population's stage of maturational development [15].

IGF-I not only has an effect on chondrocytes at various developmental states, it also shows positive effect on the stem cells by influencing chondrocyte differentiation. Hunziker et al. have shown the ability of IGF-I to stimulate stem cells towards chondrogenic differentiation [14].

Ability of IGF-I to stimulate mitotic activity and differentiation of growth plate chondrocytes have been proved in vitro along with its ability to enhance longitudinal growth in vivo [12].

These effects, such as mitosis and differentiation of the growth plate chondrocytes, were generated by nanomolar concentration of IGF-I in vitro, a range that is consistent with a physiological role in vivo [15]. IGF-I not only plays a regulatory role in cartilage and bone formation, but is also important in regulating bone fracture repair [6]. There are many other research studies from the literature showing the importance of IGF-I in cartilage development. Despite these findings, systemic administration IGF-I has shown only marginal effects on the development of cartilage [9]. The main reason for the failure in the use of IGF-I systemically is due to the inability of these peptides to reach the target area in a fully bioactive and functional state. IGF-I is a short lived cytokine which requires a proper delivery system to transport it to the desired anatomical site [8]. The fate of the tissue is mainly determined by the local IGF-I activity [11], which creates the need for the local delivery of the IGF-I for desired effect.

2.3 PLGA for drug delivery and tissue engineering

Properties such as good biocompatibility, biodegradability, desirable mechanical strength along with the ability to act as carriers for various classes of drugs, such as vaccines, peptides and proteins, has generated immense interest in PLGA for the purpose of drug delivery and tissue engineering. The drug carried by PLGA is released by diffusion through water filled network of pores or by erosion of the polymer material or by combination of both [16, 17]. The PLGA undergoes hydrolytic degradation (both in vivo and in vitro) by cleavage of its backbone ester linkages. PLGA polymer biodegrades into its monomers lactic acid and glycolic acid. These byproducts are biocompatible, toxicologically safe and are eliminated by normal metabolic pathways. Lactic acid produced enters the tricarboxylic acid cycle and is eliminated as carbon dioxide and water. Glycolic acid is excreted unchanged in the kidney or it also enters the tricarboxylic acid cycle and eliminated as carbon dioxide and water [16].

The properties of the PLGA used to encapsulate the drug play an important role in determining the rate of degradation of the polymer, which ultimately decides the release kinetics. Some of the factors on which the rate of degradation of the PLGA polymer depends include copolymer

composition (ratio of lactic acid and glycolic acid), polymer chemistry (PLGA with acid end group or aliphatic end group) and crystallinity of the polymer. All these factors control the amount of water that is accessible to the ester linkages and thus control the rate of degradation [16, 17]. Tracy et al. showed that degradation of the PLGA was more dependent on the chemistry of the polymer end group than on the molecular weight [18]. The acid-ended PLGA polymer degrades faster both in vivo (3-4 fold faster) and in vitro (2-3 fold faster) when compared to the aliphatic ended PLGA polymer. The faster degradation of acid-ended PLGA is due to its ability to take up water at a faster rate. The acidic end groups released by degradation decreases the pH further catalyzing the hydrolysis of ester bonds resulting in the autocatalytic cycle. Studies done by Tracy et al. also showed that encapsulation of a protein with PLGA microspheres did not have any significant effect on the rate of degradation [18].

The rate of degradation of PLGA is also determined by the medium in which the polymer dissolves. Faisant et al. showed that the rate of PLGA polymer degradation depends on the pH and temperature of the medium in which it degrades [19]. The rate of degradation increases with the decrease in the pH. Thus the increase in the degradation products (higher content of carboxylic acid end groups) creates acidic conditions (decrease in pH) and further accelerates degradation in an autocatalytic manner. This is also the reason behind the faster degradation of larger PLGA devices when compared to the smaller devices. In larger PLGA devices the degradation takes place heterogeneously and faster in the center. This is due to the accumulation of degradation byproducts, which decreases the pH at the center of the device when compared to the surface. PLGA polymer also shows increased degradation rate at pH of 9. The pH range from 3 to neutral shows the slowest degradation rate [20], this may be because of the increased degradation products saturating the medium slowing down further degradation. Studies conducted by Tracy et al. showed that the PLGA dissolved 1.7-2.6 times faster in vivo when compared to in vitro. They also proved that irrespective of the molecular weight or the end group, degradation of PLGA occurred faster in vivo. The reason for the faster degradation in vivo might be because of the foreign body response and accumulation of cells, like macrophages, or because of the lipids or other biological compounds present in the in vivo condition that act as plasticizers aiding the uptake of water in to the polymer [18, 21]. Other factors which might affect the rate of degradation include the size, shape and porosity of the PLGA device [18] .

Studies done by Klose et al. showed that the change in the shape of the PLGA device with microparticles had a significant effect on the relative release rate of the drug. Their studies also confirmed that the type of drug did not significantly affect the degradation kinetics of the polymer [22].

In this research, acid-ended PLGA polymer containing 50:50 ratio of lactic acid and glycolic acid was used. The glass transition temperature for this PLGA copolymer is 42ºC which makes it glassy in nature. The glassy nature gives the polymer a fairly rigid chain structure, making it mechanically strong enough to be used as drug delivery devices [16]. The physiochemical and biological properties of the PLGA polymer are also suitable for fabricating sustained drug delivery system capable drug release from days to months [23]. PLGA is also Food and Drug Administration (FDA) approved and is being used for various clinical purposes [16]. Some of the PLGA devices used for protein release available in the market are Lupron Depot®, Sandostatin LAR® Depot, Nutropine Depot® and Zoladex® [24].

2.4 Microencapsulation and controlled release of proteins

Literature shows that a variety of proteins have been encapsulated in polymeric materials for drug delivery purposes. PLGA is one of the most commonly used biodegradable polymer for encapsulating proteins for drug delivery [16]. These various proteins encapsulated in PLGA possess different physiochemical properties. The proteins with different molecular weight, solubility, distribution coefficient and therapeutic functions interact in a different way with the PLGA polymer [17]. So according to the type of protein their stability and bioactivity differs when encapsulated with the PLGA polymer. The microencapsulation technique selected is based on the type of drug, properties of the polymer, intended use and the duration of the therapy. The technique used to microencapsulate a drug with the polymer must be able to protect the stability and bioactivity of the drug, must yield microspheres of required size range, produce microspheres of reproducible quality and the drug release profile and produce drug loaded microspheres in a free that does not aggregate or adhere to each other [16].

The other important factor that must be taken into consideration for designing a drug delivery devise is the release kinetics of the drug. Drug release kinetics are mainly governed by the

polymer properties, fabrication method and the percentage of drug loaded [25]. The double emulsion method (water/oil/water method) is considered as the most suitable method for microencapsulation of water soluble drugs like proteins, peptides and vaccines [16]. In the water/oil/water method (w/o/w), aqueous solution of protein or drug is mixed with a organic phase containing PLGA dissolved in dichloromethane to form the w/o emulsion. The emulsion formed is again mixed with water containing polyvinyl alcohol (PVA) to form the w/o/w emulsion. The whole solution is stirred in atmospheric pressure to remove the dichloromethane by evaporation. The drug loaded microspheres obtained by this process are washed, lyophilized and sieved [16]. Cohen et al. showed that the exposure of the proteins to the dichloromethane during the process of microencapsulation or the process of freeze drying did not have any significant effect on the enzymatic activity or the solubility of the protein. Their research also showed that the encapsulation of the protein with PLGA in the form of microspheres enabled it to retain activity for a prolonged period of time. The protein not encapsulated in the microspheres lost 80% of its activity in a solution at 37 ºC when compared to the ability of the protein to retain more than 55% of its activity when encapsulated in PLGA and incubated at 37 ºC for 21 days [25].

The processing conditions employed during the preparation of microspheres have a direct effect on the microsphere properties such as the size, morphology, porosity, encapsulation efficiency and drug distribution. These microsphere properties ultimately decide the release kinetics of the protein loaded in the polymer [17]. Studies show that the changes made in the procedure during microsphere preparation such as the mixing rate or the volume of the inner water phase or the organic phase will have an effect on the release profile of the drug encapsulated within the polymer microsphere [25]. IGF-I has been successfully encapsulated with PLGA for therapeutic purposes. Microencapsulation of IGF-I in PLGA not only maintains the bioactivity and stability of IGF-I, it also mediates controlled release of the protein [26].

2.5 Significance

Currently there is no treatment available for growth plate injury, which leads to complete healing or regeneration of the damaged growth plate. Growth plate injury in children leads to retarded growth or complete arrest of the growth in long bones. The growth plate injury, if not treated properly, might have a lifelong effect on a person's physical stature. The treatment methods that are employed at present are mostly aimed to replace the growth plate cartilage with an inert material. These methods only avoid the formation of bone bridge, but they do not help in regeneration of growth plate cartilage and its functions.

The PLGA used for microencapsulation of IGF-I is FDA approved and has been used for drug delivery purposes. Studies have also proved that there is no adverse effect on the bioactivity of IGF-I during the process (double emulsion method) of microencapsulation with PLGA. The ease in processing of these IGF-I encapsulated microspheres also makes this method an appropriate choice for this growth factor delivering device. The PLGA scaffold which carries the IGF-I also provides mechanical support to the damaged part of the bone after removal of bone bridge. IGF-I has been proved to have positive effect on growth plate chondrocytes and growth plate development as a whole. The practical difficulty in the use of IGF-I is due to its short biological half life. Encapsulation of IGF-I with PLGA protects the bioactivity and stability of IGF-I. Thus this drug encapsulation method with PLGA can aid in the regeneration of the growth plate cartilage and will also be an alternative to the materials being currently used to block and prevent the formation of osseous bridge.

This research is aimed to achieve site-specific and controlled delivery of growth factor (IGF-I) using porous PLGA scaffolds for regeneration of growth plate cartilage.

3. MATERIALS AND METHODS

3.1. IGF-I

IGF-I is the growth factor that was used in this research for the regeneration of the growth plate. It was purchased from Peprotech inc. (Rocky Hill, NJ). The protein was available in 1 mg vials in lyophilized form.

3.1.1. Fluorescent labeling of IGF-I

IGF-I was labeled using Alexa 488 fluorescent dye (Invitrogen Eugene, OR). IGF-I was dissolved with 0.1 M sodium bicarbonate buffer (pH 8.3) to obtain the protein solution of concentration 3 mg/ml. 1 mg of the Alexa 488 dye was dissolved with 100 µl of dimethylsulfoxide (DMSO). The dye solution obtained was mixed with the protein solution and stirred at room temperature (25ºC) for one hour. The solution of protein and dye was transferred to centrifugal filter tubes (Amicon Bioseparation. Bedford,MA) with a molecular weight cut off (MWCO) of 3000 Daltons. The protein dye mixture was centrifuged for 100 minutes at 14,000g and phosphate-buffed saline (PBS) was added to the centrifugal filters for further centrifugation to wash out the excess unbound dye. The BCA Protein Assay (Pierce Chemical Co.. Rockford, IL) was performed to determine the concentration of the labeled protein. Depending on the concentration, the labeled protein was diluted to obtain a desired concentration of 0.5 mg/ml. Calibration curve was obtained with the labeled protein at various concentration, which was used as the standard for measuring the amount of protein released during the release studies.

3.2. PLGA

PLGA was used to encapsulate the IGF-I for the preparation of the biodegradable scaffolds. PLGA obtained from Durect Corp. (Pelham, AL) was used in this research is a copolymer of lactic acid and glycolic acid with the ratio of 50:50 and carboxylic acid group at the end of the chain. The inherent viscosity of the polymer ranges from 0.15 - 0.25 with the corresponding molecular weight range of 5000 – 6700 Daltons.

3.2.1. Microencapsulation of IGF-I with PLGA microspheres

IGF-I was microencapsulated into hydrophilic PLGA by double emulsion method. 24 gm of polyvinyl alcohol (PVA) (Sigma-Aldrich, St. Louis, MO) was dissolved in 2.4 liters of deionized

(DI) water by mixing it at 600 rpm (rotations per minute). 3.2 gm of PLGA was dissolved in 24.48ml of dichloromethane (Sigma-Aldrich, St. Louis, MO) and 2.72 ml of labeled IGF-I (unlabelled IGF-I for cell studies) of concentration 0.5 gm/ml was added to it to obtain the PLGA-IGF-I solution. PLGA-IGF-I solution was sonicated at 25 watts for ten seconds and added to the PVA solution and homogenized at 3500 rpm for two to three minutes. After homogenization the solution was stirred at 400 rpm for 12 hours, and the microspheres were allowed to settle for three to four hours. The microspheres encapsulated with IGF-I was washed with DI water by centrifugation for three times at 1500 rpm for 10 minutes. The washed microspheres were stored at -80ºC and lyophilized. The microspheres were sieved to obtain uniformity in size. Microspheres with the size range of 50-150 microns were used for this research.

3.2.2. Fabrication of scaffolds

The IGF-I encapsulated microspheres were mixed with NaCl (sodium chloride) in the mass ratio of 60(NaCl):40(microspheres). For this research 45 mg of NaCl was mixed with 30 mg of microspheres to make scaffolds with the thickness of 0.2 cm and diameter of 0.6 cm. The mixture was transferred to a die and was compressed using a Carver press by applying a load of two and half tons for two minutes. The discs were "sintered" just above the glass transition temperature of 43ºC for 48 hours to fuse the microspheres and were subjected to overnight leaching of salt at room temperature in DI water to create porous scaffolds. The porous scaffolds were lyophilized or air dried to remove the moisture.

3.2.3. Adsorption of protein on scaffolds

Blank scaffolds were adsorbed with protein by immersing them in lysozyme (0.5 mg/ml) for varying time periods (one hour and 24 hours) at room temperature.

3.3. In vitro studies

3.3.1. Release studies

To study the release profile of the fluorescently labeled protein from the PLGA scaffolds, the scaffolds were degraded in PBS (2ml) with a pH of 7.4. The samples were kept in a shaker rotating at 86 rotations per minute (rpm), incubated at 37 ºC. The supernatant from the immersed

disc was collected on consecutive days for first one week followed by every three days for the rest of the study (50 day time period). Fluorescence reader (SPECTRA max GEMINI XS; Molecular Devices, Sunnyvale, CA) was used to determine the amount of protein released from the scaffolds. The release studies were first performed with lysozyme as a pilot protein and repeated with the protein of interest, IGF-I. Fluorescent labeling of both IGF-I and lysozyme were done in the same way. To examine the effect of encapsulation of labeled protein in PLGA on the release profile, the release profile of the protein encapsulated scaffold was compared with the release profile of scaffold with labeled protein adsorbed on its surface. The same type of release study was also performed with the protein encapsulated scaffolds degraded in cell culture medium (alpha MEM and DMEMHyclone. Logan, Utah) with 10% fetal bovine serum (FBS Hyclone. Logan, Utah). 0.02 % sodium azaide was added to the cell culture medium to prevent contamination during the drug release and mass loss studies. The third type of the release study involved the scaffolds degraded in the cell culture medium (DMEM) along with macrophages seeded on the top of the scaffold. The macrophages (RAW 264.7) were seeded at the density of 2000 cells/scaffold on the top of the sterilized scaffold. The release study was conducted by removing the cell culture medium and replacing it with fresh medium for every 48 hours. When the scaffolds were degraded in the cell culture medium, they were stored in a 5% CO_2 incubator without shaking.

3.3.2. Mass loss studies

To study the degradation properties of the porous PLGA scaffolds, mass loss studies were conducted by incubating the scaffolds in PBS (2ml) with a pH of 7.4. The samples were kept in a shaker and incubated at 37 ºC. 1 ml of the PBS was replaced with fresh PBS every day for first one week and every three days from day 8 to day 36. Every six days three scaffolds were removed from PBS and lyophilized to determine the mass. Mass of the scaffolds was measured at day 6, 12, 18, 24, 30 and 36. Similar mass loss study was also performed for the discs degraded in cell culture medium incubated in a 5% CO_2 incubator without shaking.

3.3.3. Seeding of cells on the scaffolds

Bone marrow cells (BMCs) were harvested from the femora of Sprague Dawley rats (3 month old male rats) and were seeded on the PLGA scaffolds. The bone marrow cells were seeded at a

seeding density of 20,000,000 cells/ml on top the IGF-1(unlabeled) encapsulated scaffolds. The control groups included BMCs seeded on blank PLGA scaffolds and BMCs seeded on blank scaffolds with the addition of soluble IGF-1 (250 ng/ml). To prevent the cells from flowing out of the scaffolds and into the medium, 7.5µl of cells were mixed with of 7.5µl fibrinogen (20 mg/ml in 0.9% saline) (Sigma-Aldrich, St. Louis, MO), and 15µl of the cell-fibrinogen mixture was seeded on top of the scaffold. After 30 minutes, 15µl of thrombin (50 U of thrombin/ 1ml of 40 mM $CaCl_2$) (Sigma-Aldrich,) were added to form a clot of the cells on/within the scaffold. The cell culture medium was replaced every two days, with the replenishment of IGF-1 in the soluble growth factor controls.

3.3.4 Confocal imaging cell seeded scaffolds

Leica TSP SP5 laser scanning Confocal microscope was used to obtain the images of the cells at various depths of the scaffold. Three weeks after seeding the BMC's on top of the scaffold, the cells were labeled with a fluorescent intracellular probe (Cell Tracker™ Green CMFDA (5-chloromethyl fluorescein diacetate)Molecular Probes™. Eugene ,OR). 20 images were obtained at various depths of each blank scaffold, blank scaffold with IGF-I added externally and IGF-I encapsulated scaffold.

3.3.5 GAG assay and DNA assay of cells seeded on scaffolds

The GAG and DNA assays were performed at 3 week and 4 week time periods after the seeding of the BMCs on the scaffolds. Scaffolds were transferred to centrifuge tubes, and 1 ml of cold sterile PBS was added to them. The scaffolds were then subjected to freezing and thawing cycles for three times. The scaffolds were sonicated for 30-40 seconds at 25 watts after the completion of three freezing and thawing cycles. The GAG assay was performed using a commercial GAG assay kit (Blyscan, Sulfated Glycosaminoglycan assay Bicolor Ltd. N.Ireland) to determine the total amount of GAG content produced by the cells present in the scaffolds. Chondroidin sulphate was used to obtain the standard curve for the GAG assays. The Pico Green DNA Assay (Invitrogen) was performed to obtain the amount of DNA from the cells present in the scaffold. The DNA content from each scaffold was used to standardize the amount of GAG produced in each scaffold.

3.4. In vivo studies

3.4.1. Animal surgery for scaffold implantation

For in vivo studies, animal surgeries were performed on New Zealand white rabbits which were six to eight weeks old. The porous scaffolds used for animal implantation were of different dimension when compared to those of the porous scaffolds used for the in vitro studies. For in vivo studies, 180 mg of NaCl was mixed with 120 mg of microspheres to make scaffolds with the thickness of 0.1 cm and diameter of 1.3 cm. The mixture was transferred to a die and was compressed by applying a load of seven tons for two minutes. The rest of the procedure for scaffold fabrication was same as the procedure followed for the scaffolds used in release studies. Before the surgery the animals were anesthetized and radiographic images of their hind limbs were obtained. Incision was made in the anterior side of the tibia and the medial tibia was exposed with an intact growth plate (Figure 3.4.1.1). A 1 mm burr (Stryker Medical Kalamazoo, MI) was used to remove the medial half of the tibial growth plate (Figure 3.4.1.2). The place of injury was irrigated well before the incision was closed. The animals were allowed to recover for three weeks before the implantation of the porous hydrophilic PLGA scaffolds. Before the implantation of the scaffolds, radiographic images of the hind legs were obtained for confirmation of the growth plate injury and formation of the bone bar. The burr was used to remove the bone bar, and it was replaced with blank or IGF-I loaded scaffold (Figure 3.4.1.3). In the case of the control legs, the bone bar was removed and it was left without any replacement with a scaffold. The animals were allowed to heal for eight to sixteen weeks, after which they were euthanized. The hind limbs were harvested for further studies, after obtaining the radiographic images.

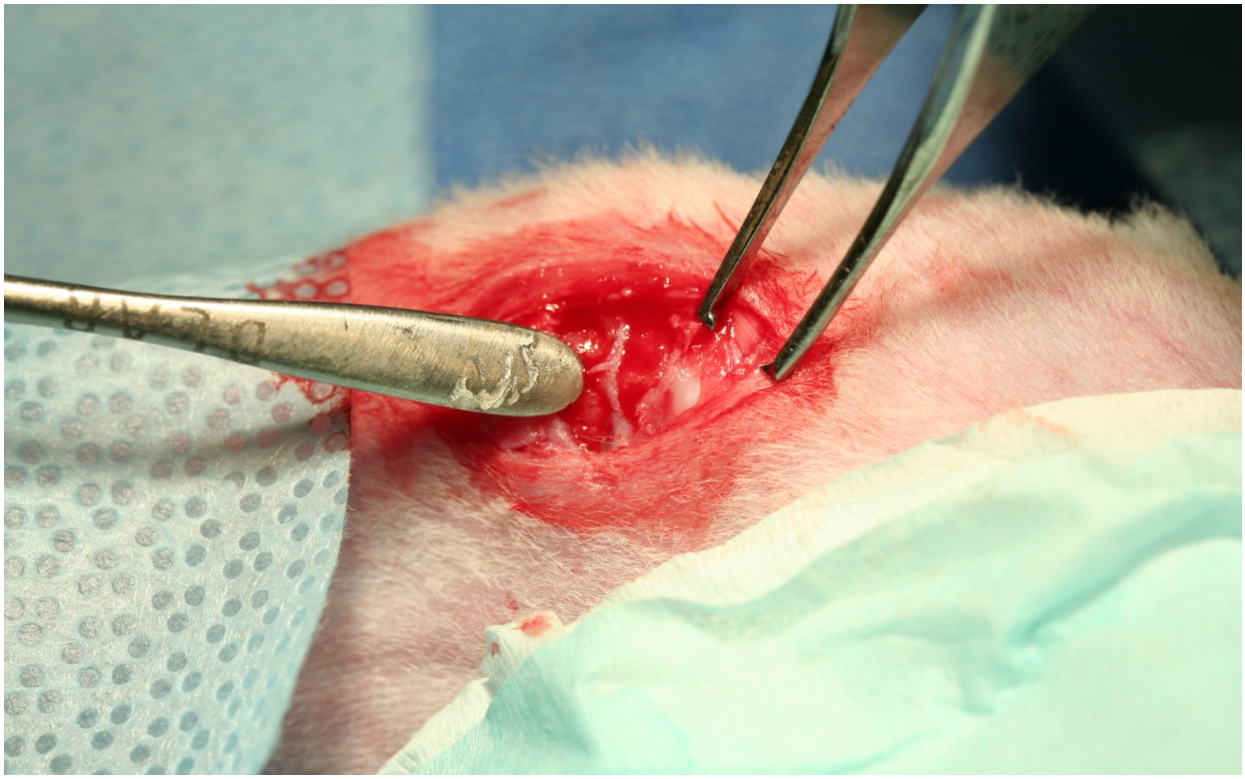


Figure 3.4.1.1: Incision made in the anterior side of the tibia to expose the intact growth plate.

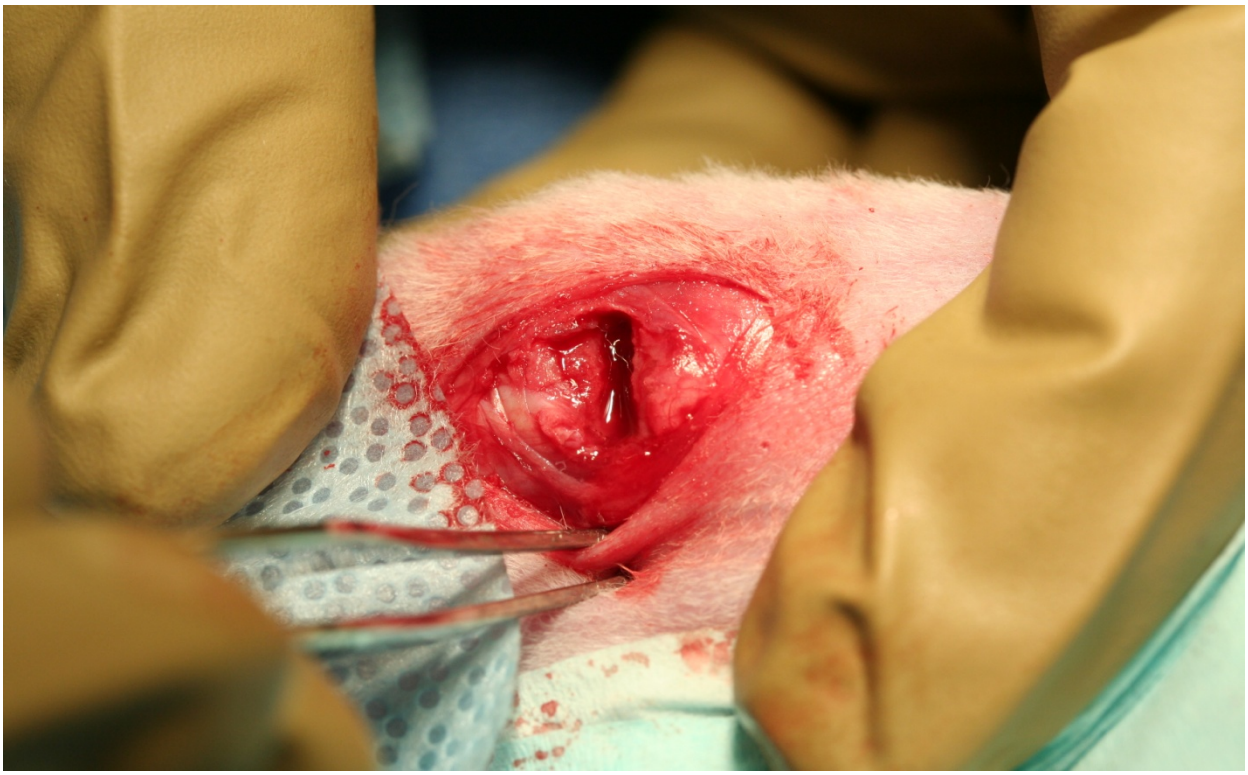


Figure 3.4.1.2: Removal of the medial half of the tibial growth plate.

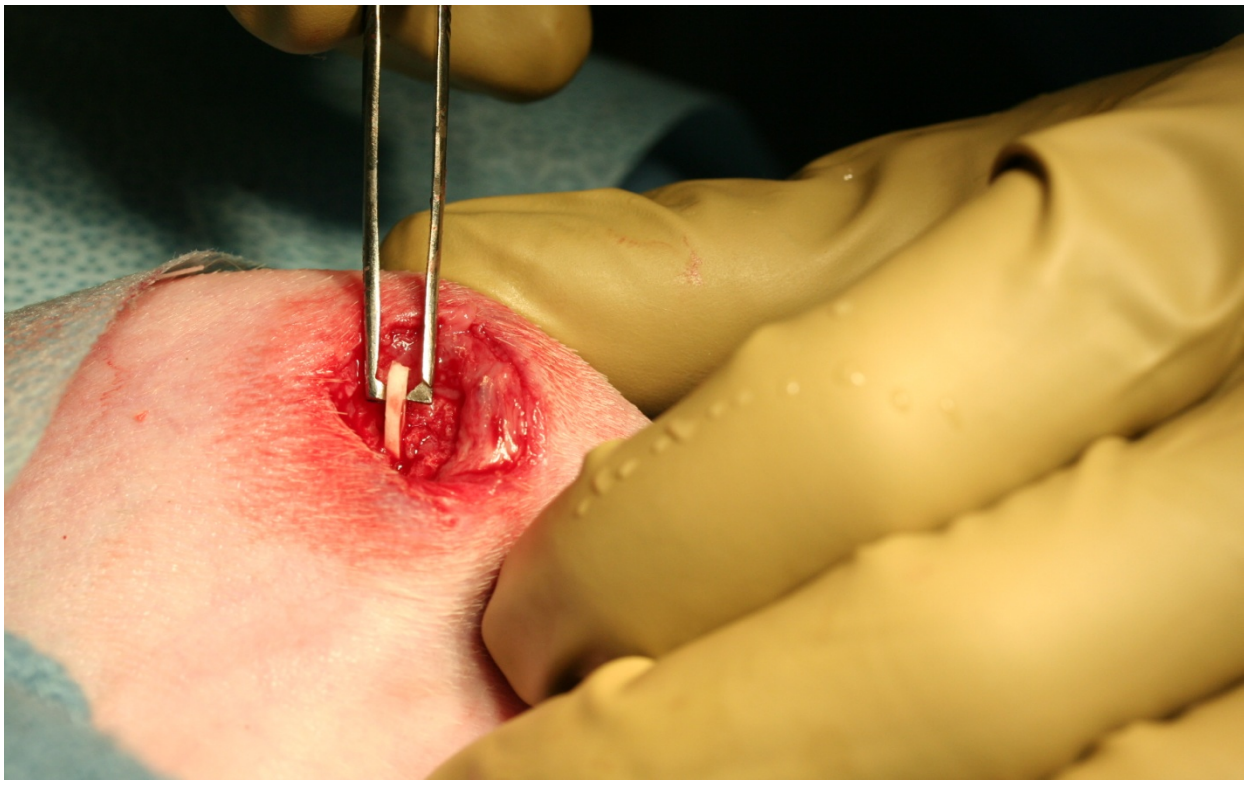


Figure 3.4.1.3: Implantation of the scaffold after removal of bone bar.

3.4.2. Radiographic imaging for bone angle measurement

Radiographic images of the animals' legs were obtained at three time points . The first images were obtained at the start of the study, before causing the growth plate injury. The second set of the radiographic images were obtained after the formation of the bony bar. The final images were obtained eight or 16 weeks after the implantation of the scaffold, before euthanizing the animal. The lateral distal femoral angle (LDFA) and the medial proximal tibial angle (MPTA) were measured in all the cases (Figure 3.4.2.1). The LDFA is the angle made by th distal part of the femur on the lateral side of the knee region. MPTA denotes angle made by the proximal part of tibia on the medial side of the knee region. The LDAF and MPTA measured were compared during various stages of the surgery, treatment and recovery.

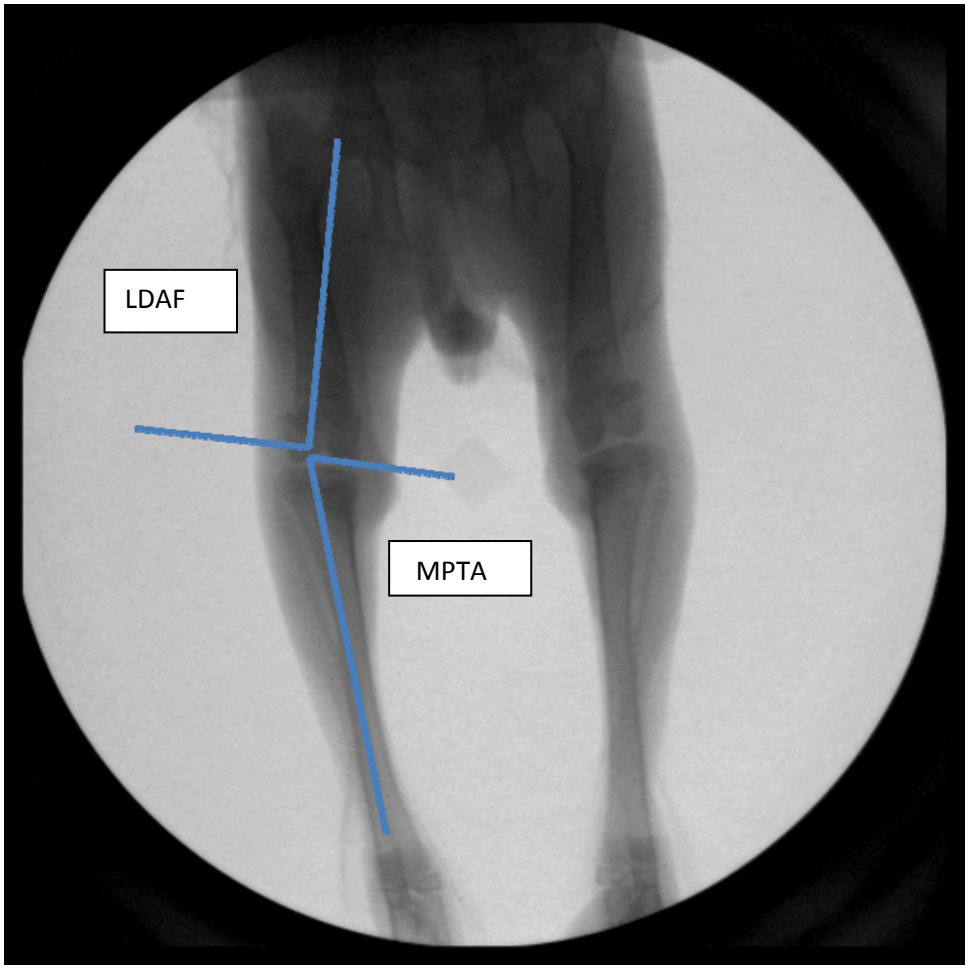


Figure 3.4.2.1: Angle measurement from radiographic images.

3.5 Histology

3.5.1 Decalcification and sectioning of bone

The harvested bones were decalcified using 10% 10 % nitric acid or EDTA. The decalcification solution was changed every five days to perform decalcification test. Decalcification test was performed by mixing 5 ml of decalcification solution with 10 ml of 5% ammonium hydroxide/5% ammonium oxalate (50:50) working solution. The test solution was left to stand for an overnight period to check the formation of precipitate. The complete decalcification of the bone was confirmed when no precipitate was observed on two consecutive days of testing. The decalcified bone was cut in half through the coronal plane, and the anterior and posterior parts obtained were again treated with decalcification solution to make sure that the inner parts of the bone were completely decalcified. The anterior and posterior parts of the bones were embedded separately in paraffin and sectioned using a microtome (Finesse ME+ Waltham, MA). Sections with a thickness of 10 µm were obtained and heat-fixed at 42ºC onto Superfrost slides.

3.5.2 Staining of tissues

The heat-fixed sections were deparaffinized using xylene (Fisher Scientific, Fair Lawn, NJ) followed by hydration using graded ethanol (Fisher Scientific) series. Two staining techniques were used: 1) hematoxlin and eosin (H&E) for visualization of the overall tissue organization and 2) safranin O for identification of GAG-containing tissue. For H&E, the deparaffinized and hydrated sections were immersed in Gills 3 hematoxylin (Richard Allan Scientific; Kalamazoo, MI) for 2 minutes and eosin (Schmid & Co.; Washington, D.C.) for 30 seconds. For safranin O, other deparaffinized and hydrated sections were immersed in acid fast green (Fisher Scientific) for 5 minutes and safranin O (Sigma). After staining, all the sections were dehydrated using graded alcohol series and xylene, and thenthe slides were coverslipped using Permount SP15-500 (Fisher Scientific). The stained sections were observed using a microscope (Nikon Eclipse E600) and the images were obtained using the attached Nikon DN 100 digital camera.

4. RESULTS

4.1 Release profiles

4.1.1 Lysozyme-encapsulated PLGA scaffolds

The release profile of lysozyme from the PLGA scaffolds obtained as a pilot study is shown in Figure 4.1.1.a. There was an initial burst (a very high peak in the release seen in the first 24 hours) in the release of lysozyme from the scaffolds which was followed by a gradual reduction in the amount of protein released. The cumulative release of lysozyme from the scaffolds degraded in PBS is shown in Figure 4.1.1b. At the end of day 10, 84% of the encapsulated protein had been released.

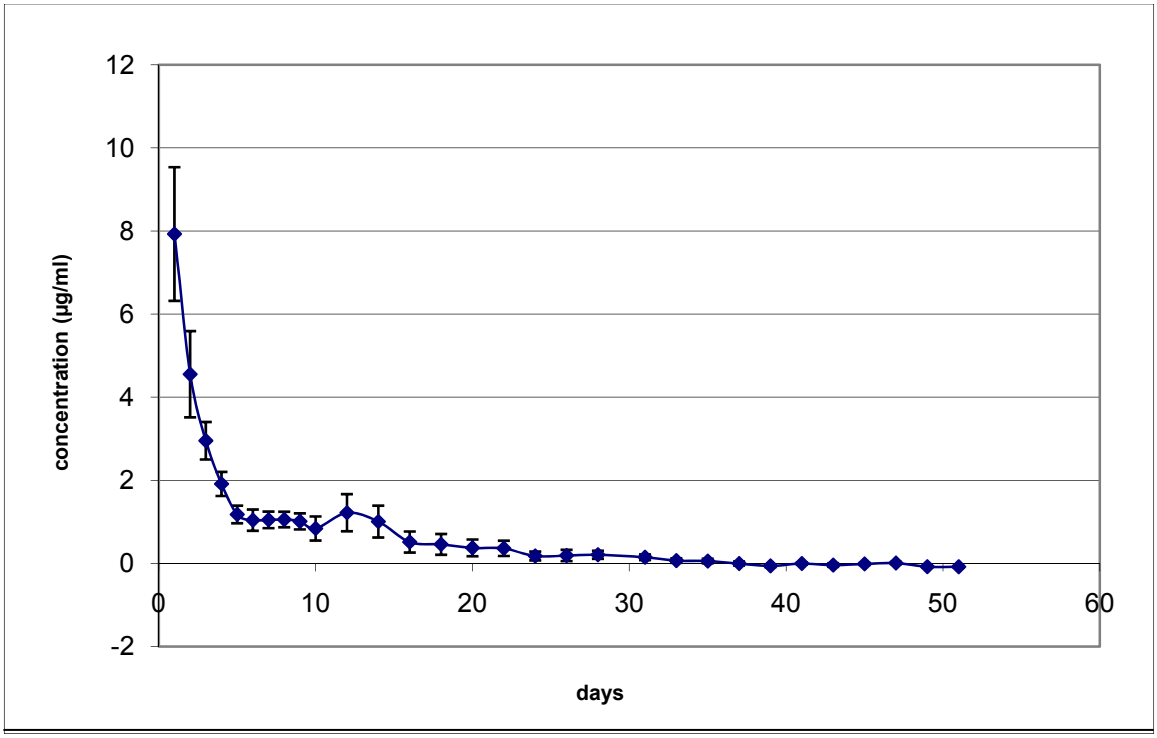


Figure 4.1.1a: Release profile of lysozyme encapsulated PLGA scaffolds degraded in PBS

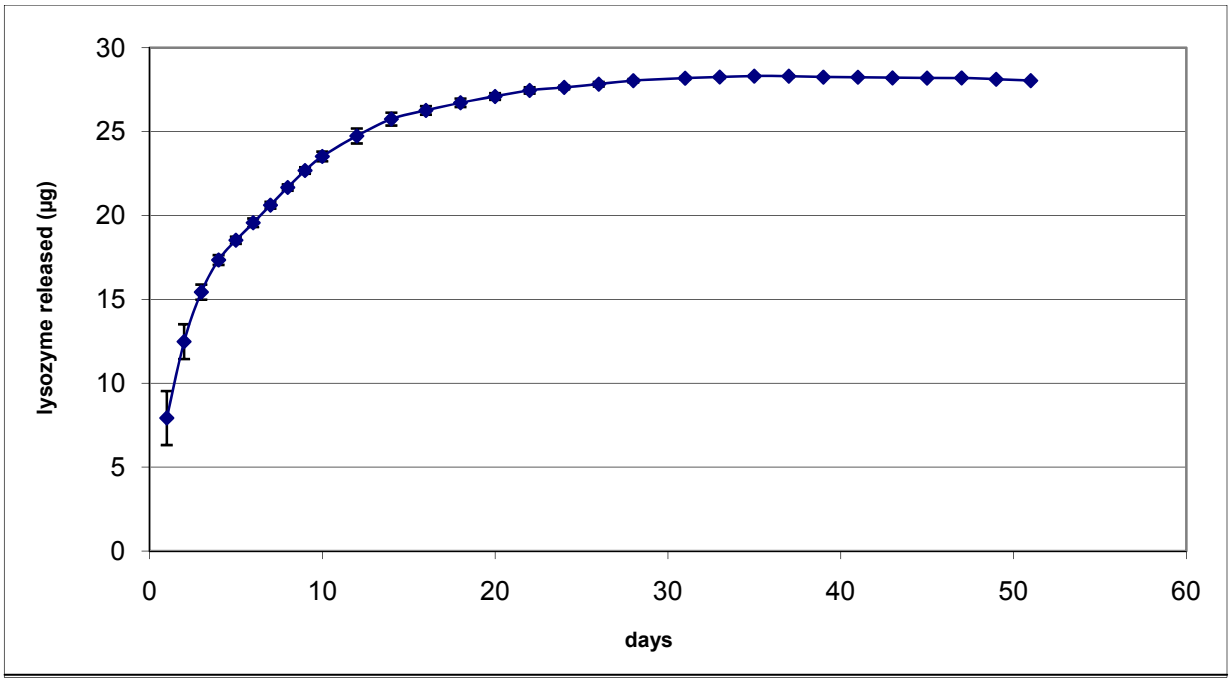
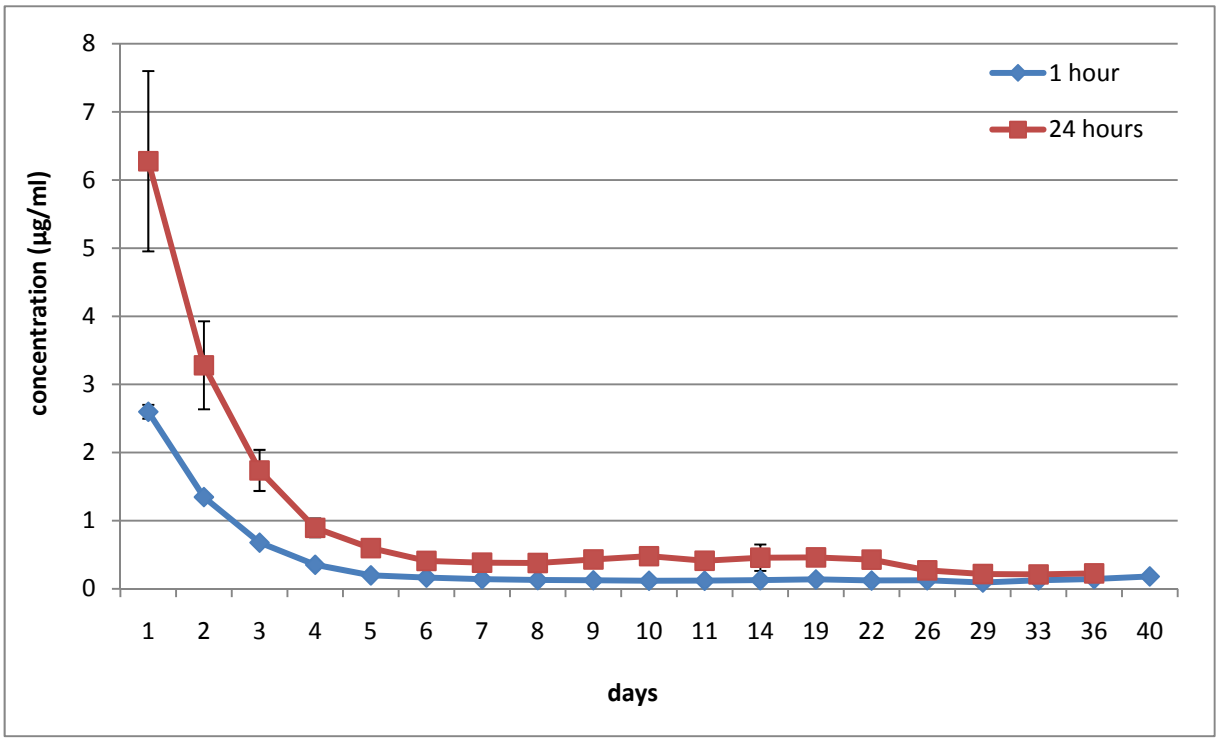


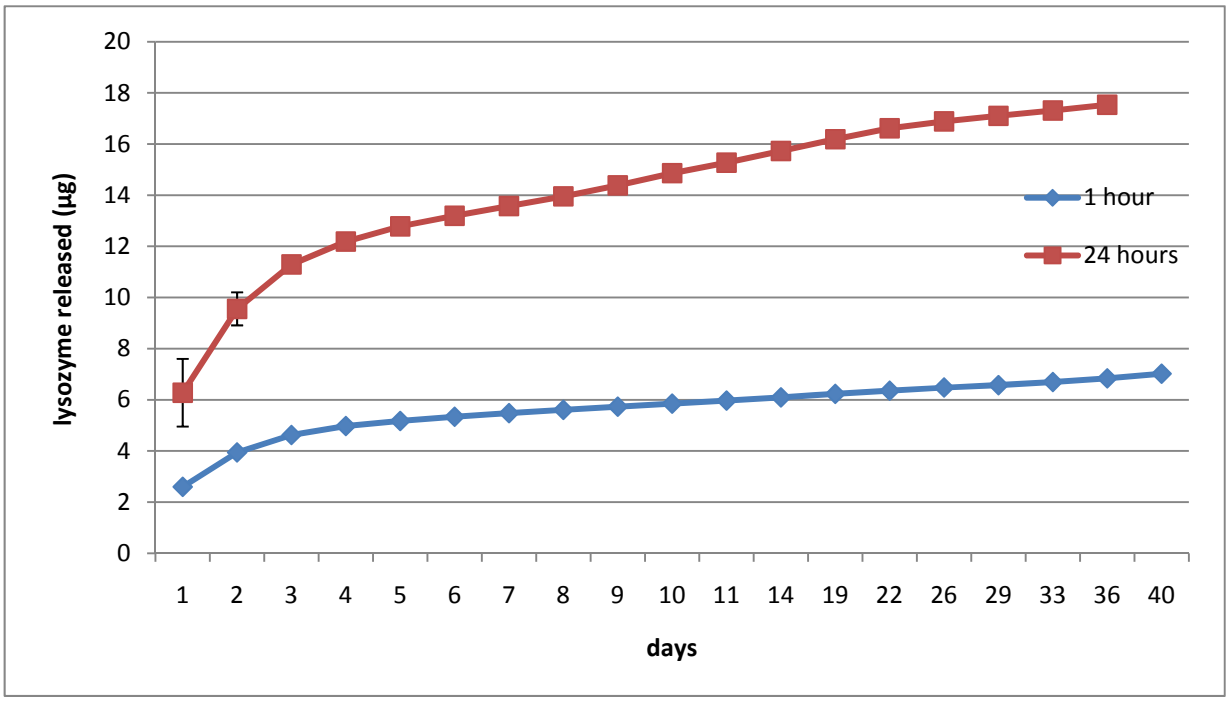
Figure 4.1.1b: Cumulative lysozyme release from PLGA scaffolds degraded in PBS

4.1.2 Lysozyme-adsorbed PLGA scaffolds

Release profiles of blank scaffolds adsorbed with lysozyme for one hour and the blank scaffolds adsorbed with lysozyme for 24 hours are shown in Figures 4.1.2a, and their cumulative release is shown in Figure 4.1.2b. In both the cases the initial release was very high during the first 2 days and the release was not significant after the first week. The blank discs that were immersed in the lysozyme for 24 hours had higher amount of protein adsorbed and released when compared to the blank disc immersed in the lysozyme for one hour. The amount of lysozyme adsorbed and released from the scaffolds adsorbed with lysozyme for 24 hours was found to be 60% higher than the scaffolds adsorbed with lysozyme for one hour.



Figures 4.1.2a: Comparison of release profiles from scaffolds adsorbed with lysozyme for 1 hour and 24 hours and degraded in PBS



Figures 4.1.2b: Comparison of cumulative releases from scaffolds adsorbed with lysozyme for 1 hour and 24 hours and degraded in PBS

To showcase the effect of encapsulation of protein within the scaffold, the release profile of the scaffolds encapsulated with lysozyme was compared with the release profile of the scaffolds adsorbed with lysozyme for 24 hours on their surface. The comparison of the cumulative release from the lysozyme encapsulated and adsorbed (for 24 hours) scaffold is shown in the Figure 4.1.2d. In the adsorbed scaffolds 70% of the adsorbed protein was released by day four compared to the release of 61% of lysozyme by day four in the case of lysozyme encapsulated scaffold. The cumulative release also shows that the total amount of lysozyme released at the end of the study from the encapsulated scaffold (28.24µg) was 61% more than the total amount of lysozyme (17.54µg).

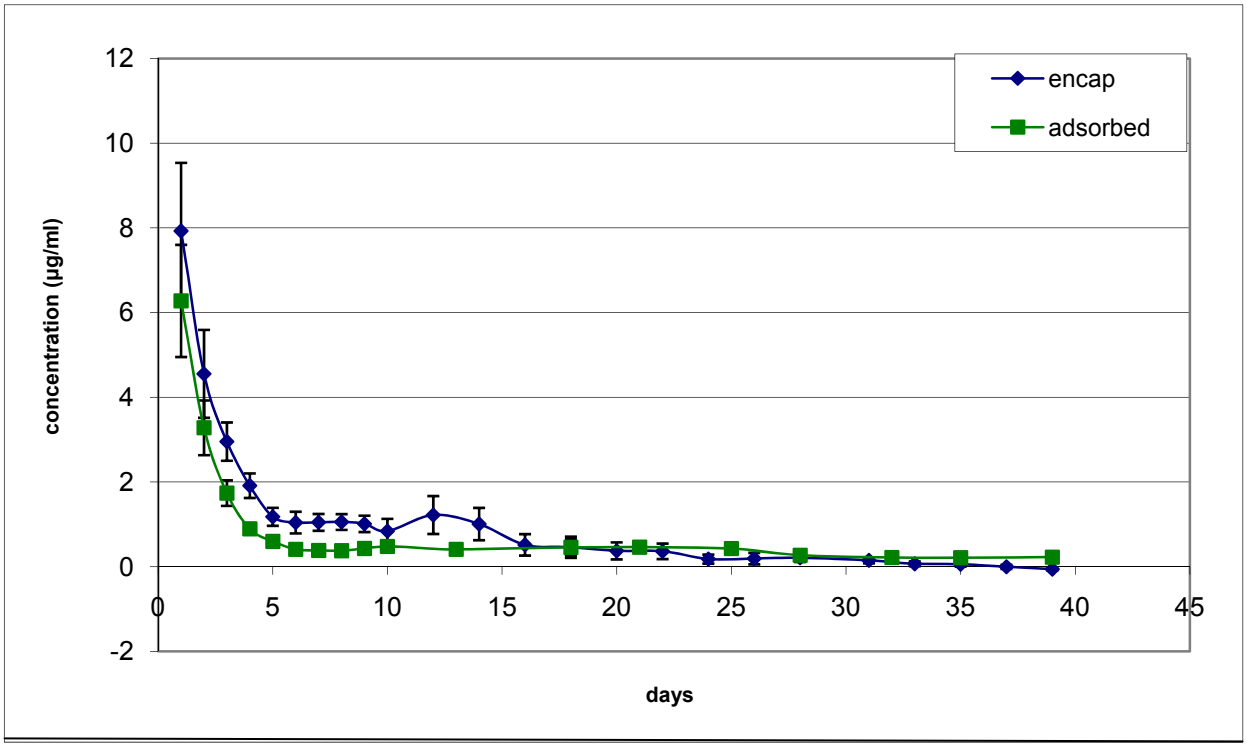


Figure 4.1.2c: Comparison of lysozyme released from encapsulated and adsorbed (for 24 hours) scaffolds

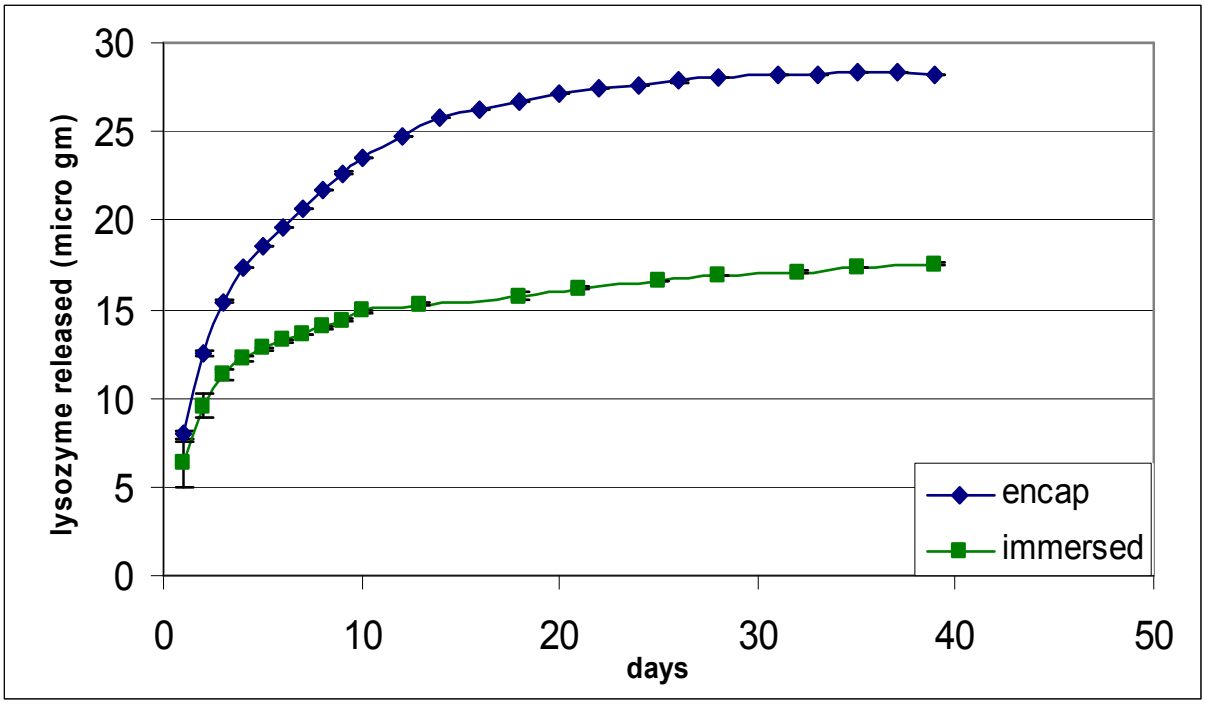


Figure 4.1.2d: Comparison of cumulative release of lysozyme from encapsulated and adsorbed scaffolds

4.1.3 IGF-I-encapsulated PLGA scaffolds

Following the pilot study using lysozyme, the release profile of the IGF-I from the IGFI-I encapsulated scaffolds was also obtained (Figure 4.1.3a). The release of IGF-I followed a similar pattern to that of the lysozyme. The release was characterized with an initial burst resulting in significant amount of protein released in the first week. There was relatively less protein released after one month, and it took around 50 days for the scaffolds to degrade completely. The cumulative release pattern of the IGF-I encapsulated scaffolds degraded in PBS is shown in the Figure 4.1.3b. Approximately 85% of protein was released within the first 10 days.

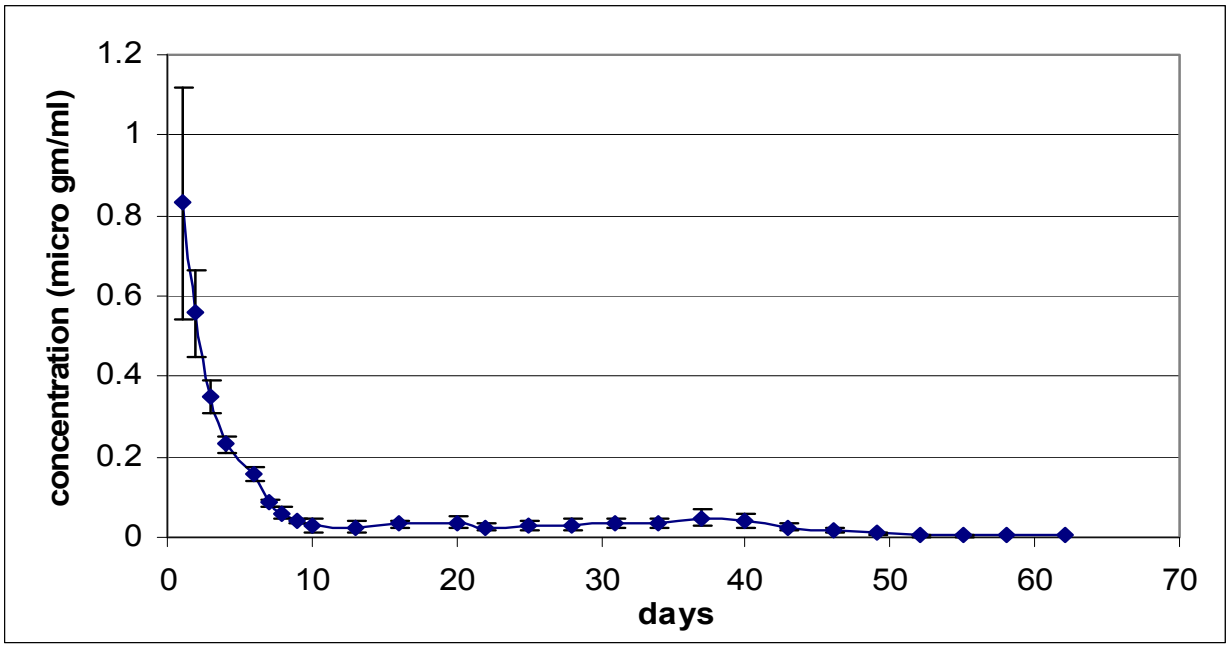


Figure 4.1.3a: Release profile of IGF-I encapsulated PLGA scaffolds.

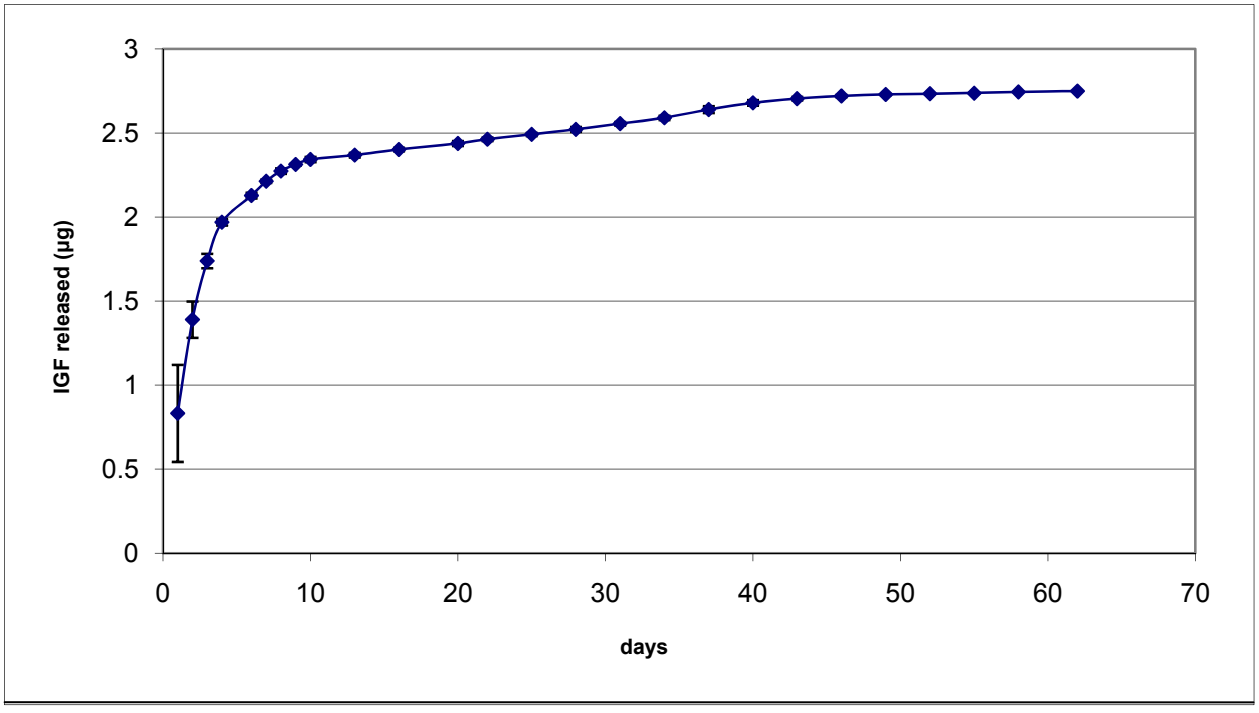


Figure 4.1.3b: Cumulative IGF-I release from PLGA scaffolds.

4.1.4. IGF-I-encapsulated PLGA scaffolds in cell culture medium

The study conducted by degrading the scaffolds in cell culture medium (DMEM) showed a different kind of a release profile compared to scaffolds degraded in PBS. The comparison between the release of IGF-I from IGF-I encapsulated scaffolds degraded in PBS and cell culture medium can be more clearly seen from Figure 4.1.4a.

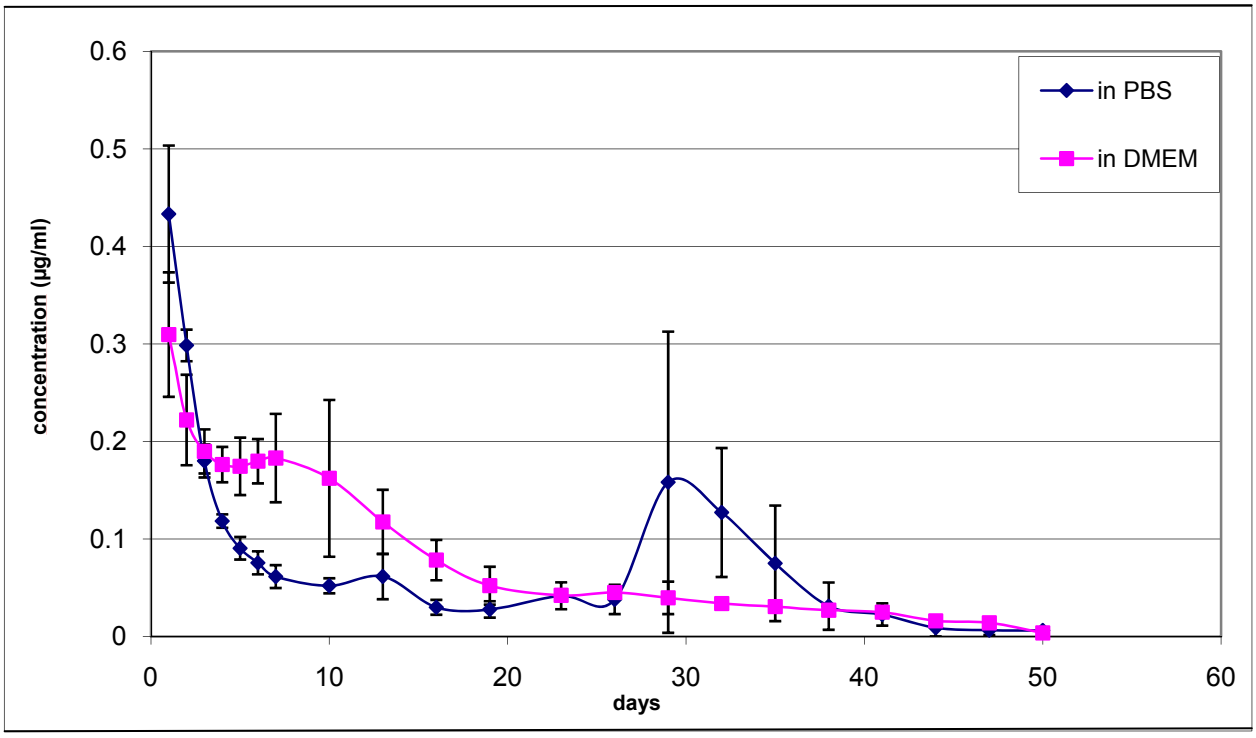


Figure 4.1.4a: Comparison between the release of IGF-I from IGF-I-encapsulated scaffolds degraded in PBS and cell culture medium.

The release of IGF-I from the IGF-I encapsulated scaffolds seeded with macrophages was also obtained. Figure 4.1.4b compares the release profile of IGF-I from the scaffolds degraded in PBS, cell culture medium and cell culture medium with macrophages. The statistical analysis showed that the slopes of the release curves of scaffolds degraded in PBS, cell culture medium and cell culture medium with macrophages were significantly different. Slopes of the release curves were calculated at two different regions of the curve, the first slope was calculated from the release curve by fitting a line between the days 1 to 10, and the second slope was calculated from the release curve by fitting a line between the days 12 to 25. Both these slopes from the

release profile of IGF-I in PBS were compared with the respective slopes from the release profiles of IGF-I in cell culture medium and cell culture medium with macrophages. The results were found to be significantly different for the slopes from day 1 to 10 (P=0.0002) and for the slopes from day 12 to 25 (P=0.0039). The area under the release curves was also calculated for all the three release curves, but it was not found to be significantly different (P=0.2179) in all the three release curves. The cumulative release of the IGF-I from the scaffolds degraded in PBS (1.9 µg), cell culture medium (2.1 µg) and cell culture medium with macrophages (2.3 µg) showed that the total amount of IGF-I released in all three cases were almost the same (Figure 4.1.4c).

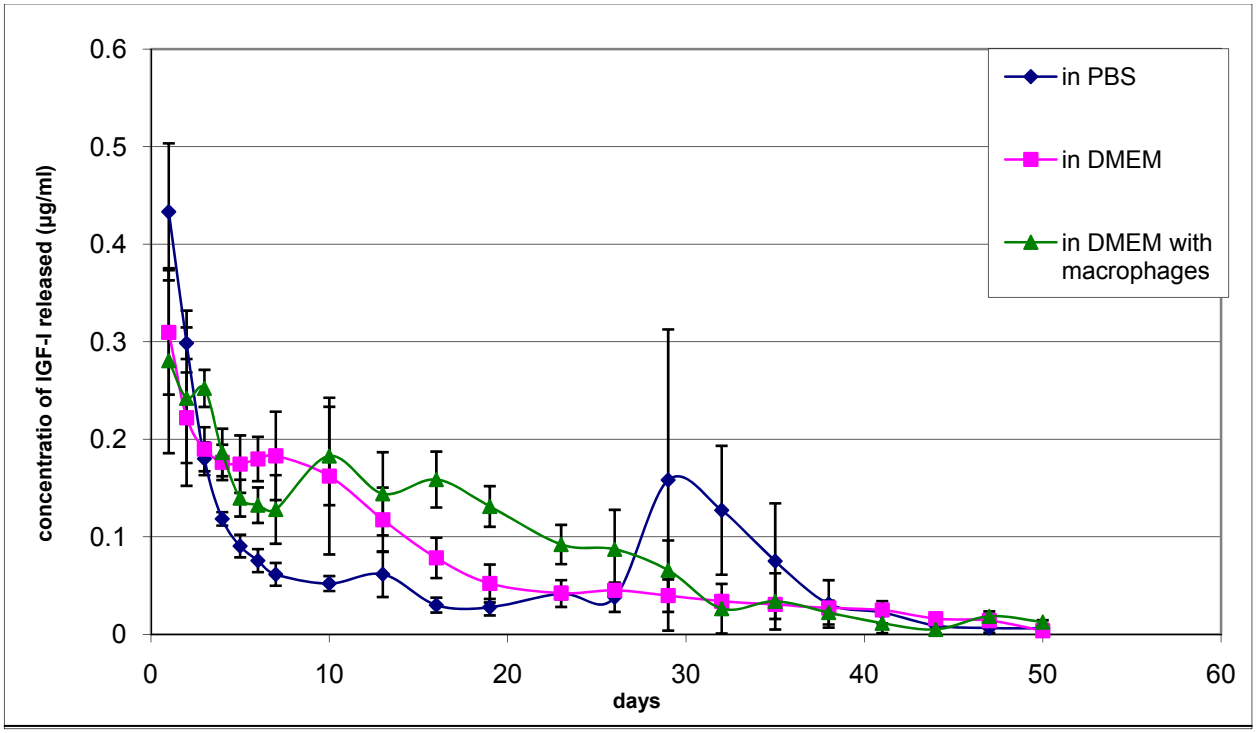


Figure 4.1.4b: Comparison of the release profile of IGF-I from the scaffolds degraded in PBS, cell culture medium and cell culture medium with macrophages.

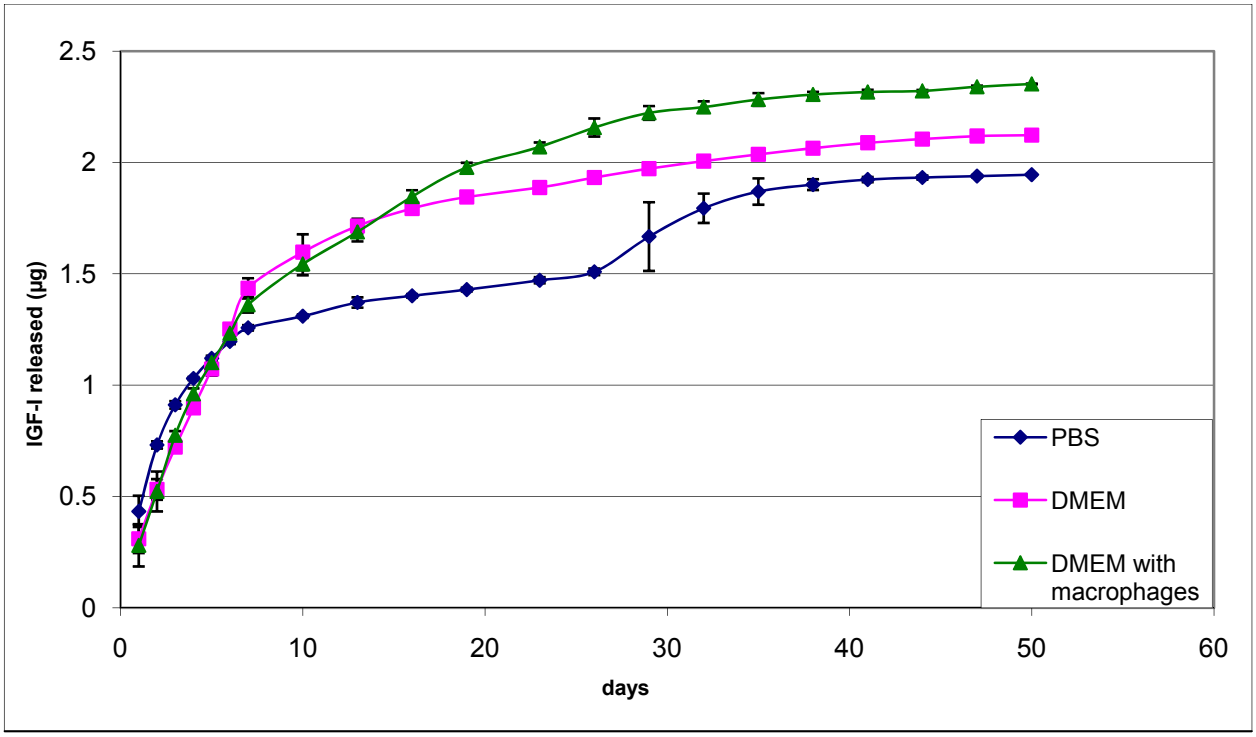


Figure 4.1.4c: Comparison of the cumulative release of IGF-I from the scaffolds degraded in PBS, cell culture medium and cell culture medium with macrophages.

Table 4.1.4.1. : Comparison of the release profile of IGF-I from the scaffolds degraded in PBS, cell culture medium and cell culture medium with macrophages with their p values.

Comparison of the release profile of IGF-I from the scaffolds degraded in PBS, cell culture medium and cell culture medium with macrophages.	**P value**
Slope of the line fitted on the release curves from day 1 to 10	**0.0002**<0.05
Slope of the line fitted on the release curves from day 12 to 25	**0.0039**<0.05
Area under the release curves	**0.2179**>0.05

4.2 Mass loss studies

4.2.1 Mass loss profile of PLGA scaffolds

The mass loss profile of the porous PLGA scaffolds degraded in PBS is shown in the Figure 4.2.1.1. This data was obtained by destructive mass loss studies. Mass loss profile followed a linear pattern with a gradual decrease in the mass of the scaffolds with the time. The mass loss study was also conducted in the cell culture medium instead of PBS. In this case the porous PLGA scaffolds were degraded in cell culture medium and incubated in a 5% CO_2 incubator. The scaffolds swelled up and increased in size as they started degrading. Figure 4.2.1.2 shows the changes in the size and shape of the scaffolds degraded in cell culture medium at various time points. The scaffolds increased to a maximum of 150% by day 12 when degraded in cell culture medium. The mass loss profile of scaffolds degraded in cell culture medium (Figure 4.2.1.3) was similar to that of the scaffolds degraded in the PBS.

Figure 4.2.1.1: Porous PLGA scaffolds degraded in cell culture medium at various time points

The comparison of the mass loss profile of the porous PLGA scaffold in PBS and cell culture medium is shown in the Figure 4.2.1.4. The mass loss profiles in both the cases are very similar in spite of the difference in the morphology of the scaffolds observed during the course of degradation. The Figure 4.2.1.5 shows the morphology of the scaffolds degraded in PBS, cell culture medium and cell culture medium with macrophages at day 20. The image clearly shows that the scaffolds degraded in the cell culture medium and in the cell culture medium with macrophages in a different way. The scaffolds swelled up more than their usual size when degraded in the cell culture medium. When the scaffolds were degraded in the cell culture medium with macrophages seeded on top of them, the scaffolds contracted unlike the scaffolds degraded in the cell culture medium.

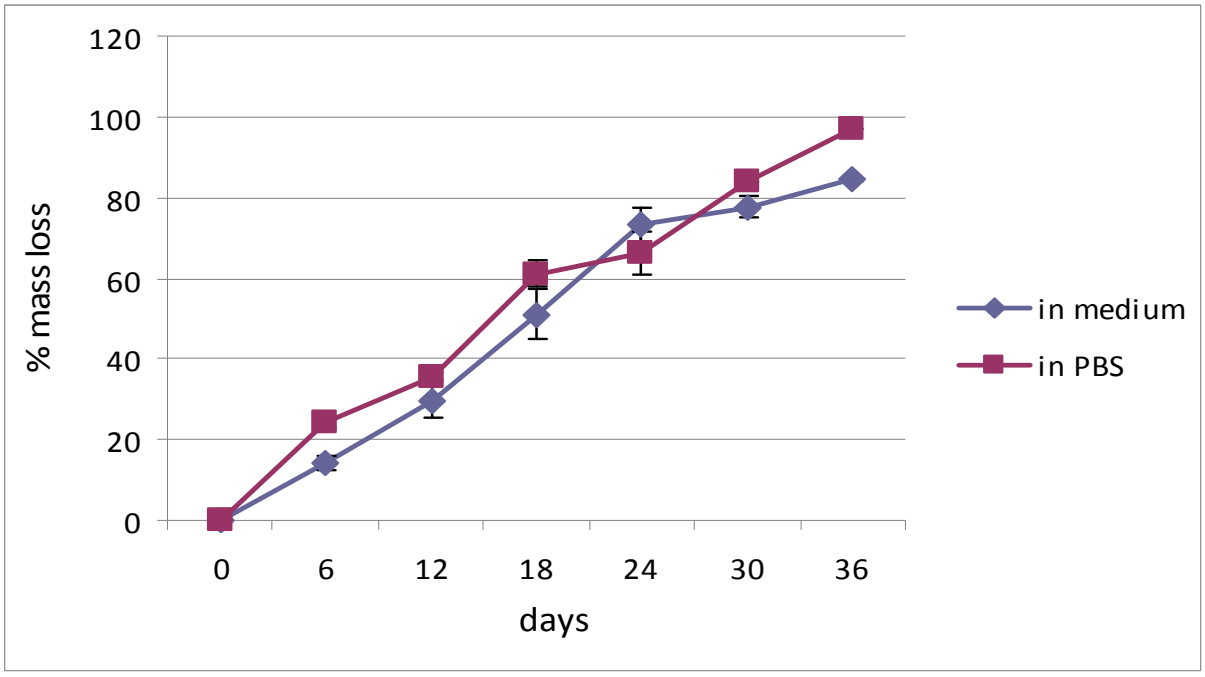


Figure 4.2.1.2.: The comparison of the mass loss profile of the porous PLGA scaffold in PBS and cell culture medium

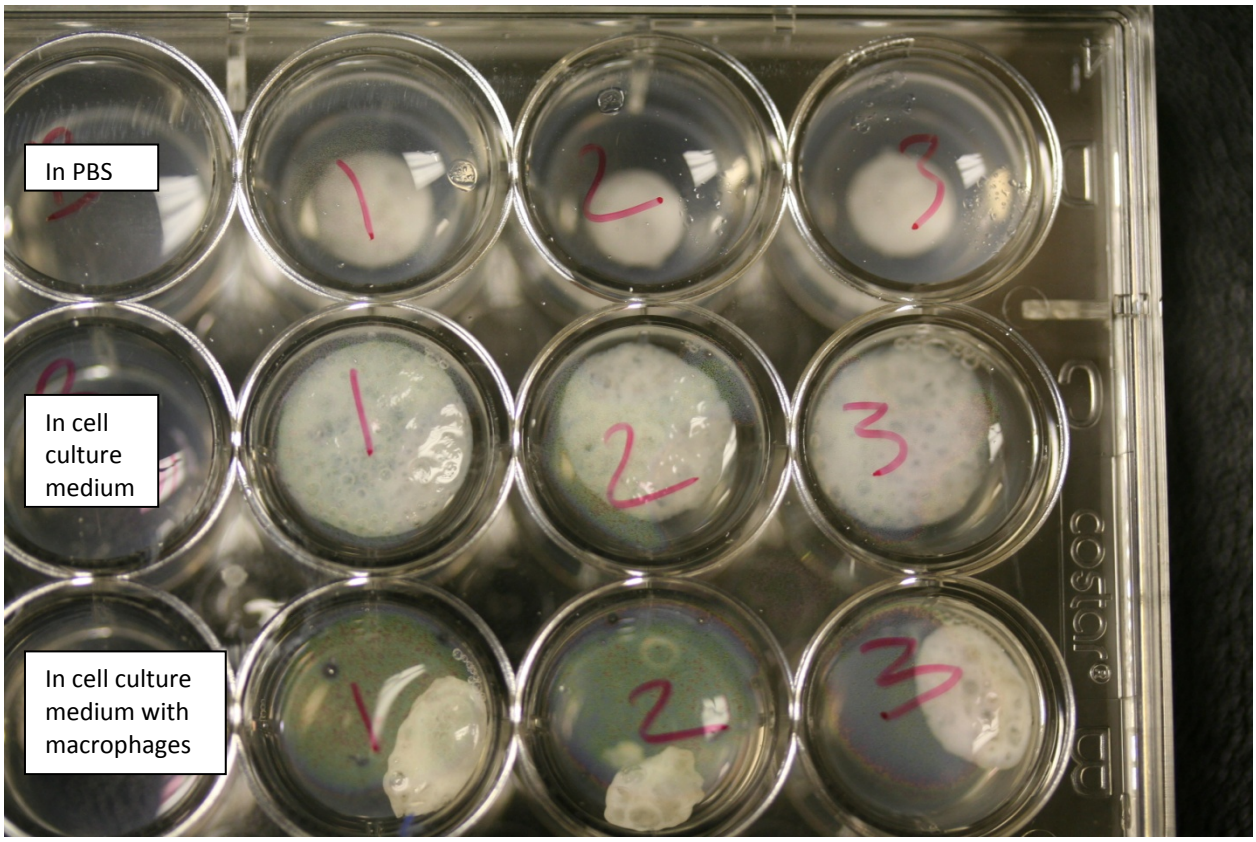


Figure 4.2.1.3: Scaffolds degraded in PBS, cell culture medium and cell culture medium with macrophages at day 20.

4.3 Cell seeding on IGF-I encapsulated scaffolds

4.3.1 Confocal images of the cells seeded on the scaffolds

Three weeks after seeding the bone marrow cells on top of the blank scaffolds and IGF-I encapsulated scaffolds, they were labeled with Cell Tracker and visualized using a confocal microscope. Figure 4.3.1.1 shows a representative image obtained from a blank scaffold. The image shows the cells (green spots) present in the porous scaffold. This image shows the cells that have penetrated the pores present in the scaffold three weeks after seeding. Similar confocal images obtained from the porous scaffolds seeded with BMCs and IGF-I added externally showed the presence of cells after three weeks. Figure 4.3.1.2 show the images obtained from the blank scaffold with IGF-I added externally. The confocal images from the IGF-I encapsulated scaffolds clearly showed the presence of the cells at the edges of the pores present in the scaffolds (marked by arrows). Confocal images confirmed the presence of labeled cells present within the scaffolds both in the case of the controls and the IGF-I encapsulated scaffolds.

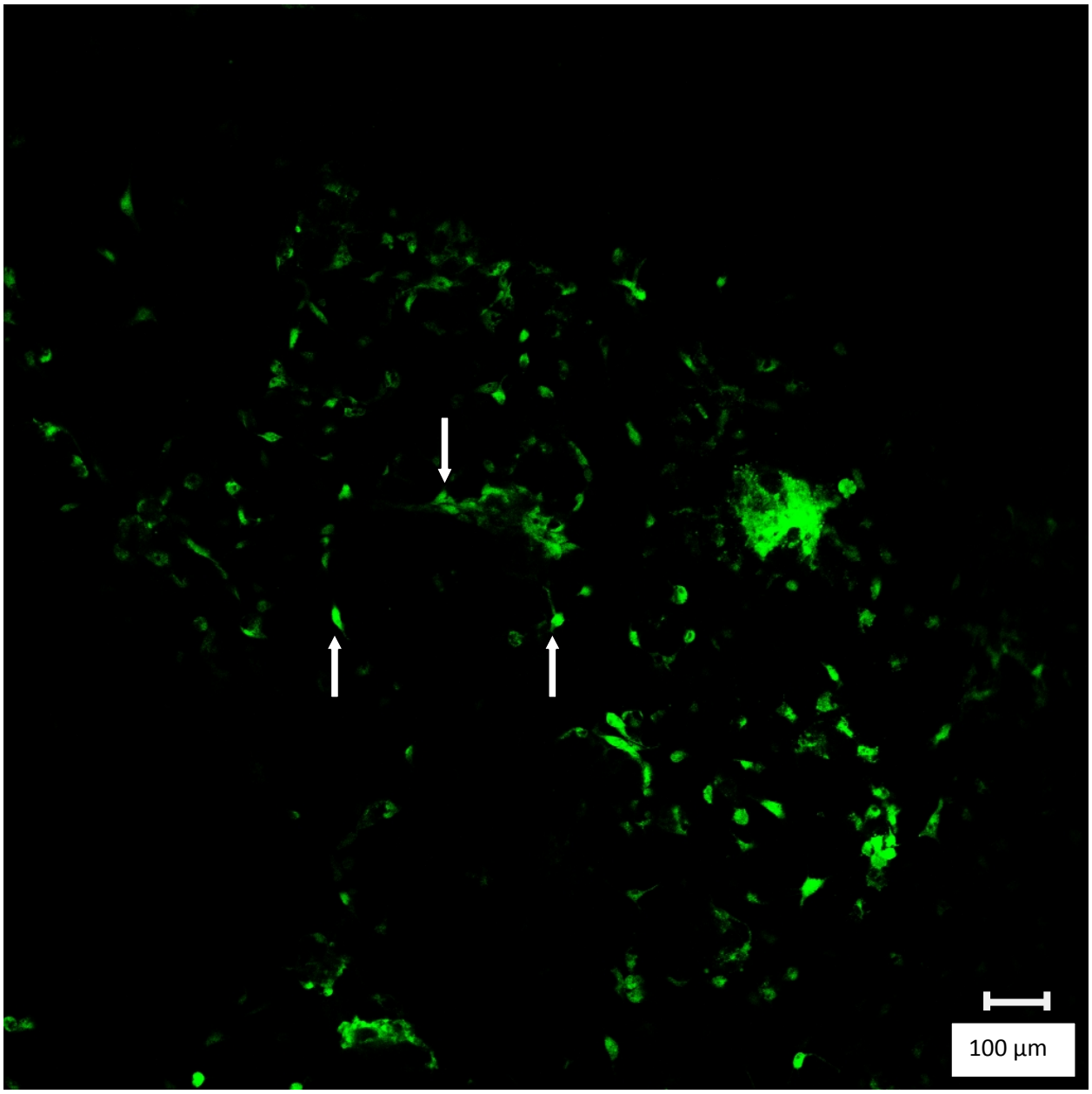


Figure 4.3.1.1: Confocal image of BMCs on a blank scaffold after three weeks.

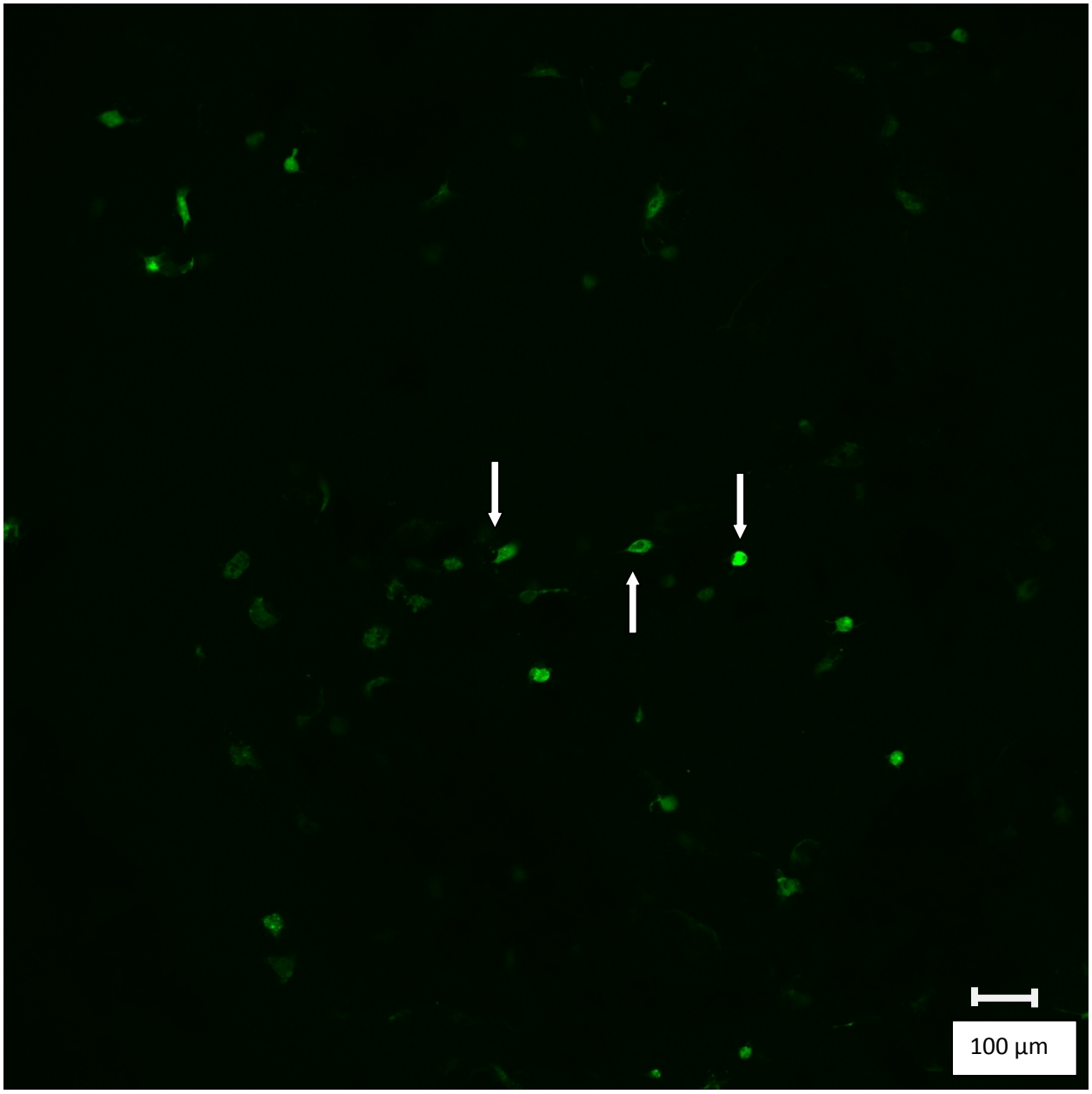


Figure 4.3.1.2: Confocal image of BMCs on a blank scaffold after three weeks of culture with IGF-I added externally.

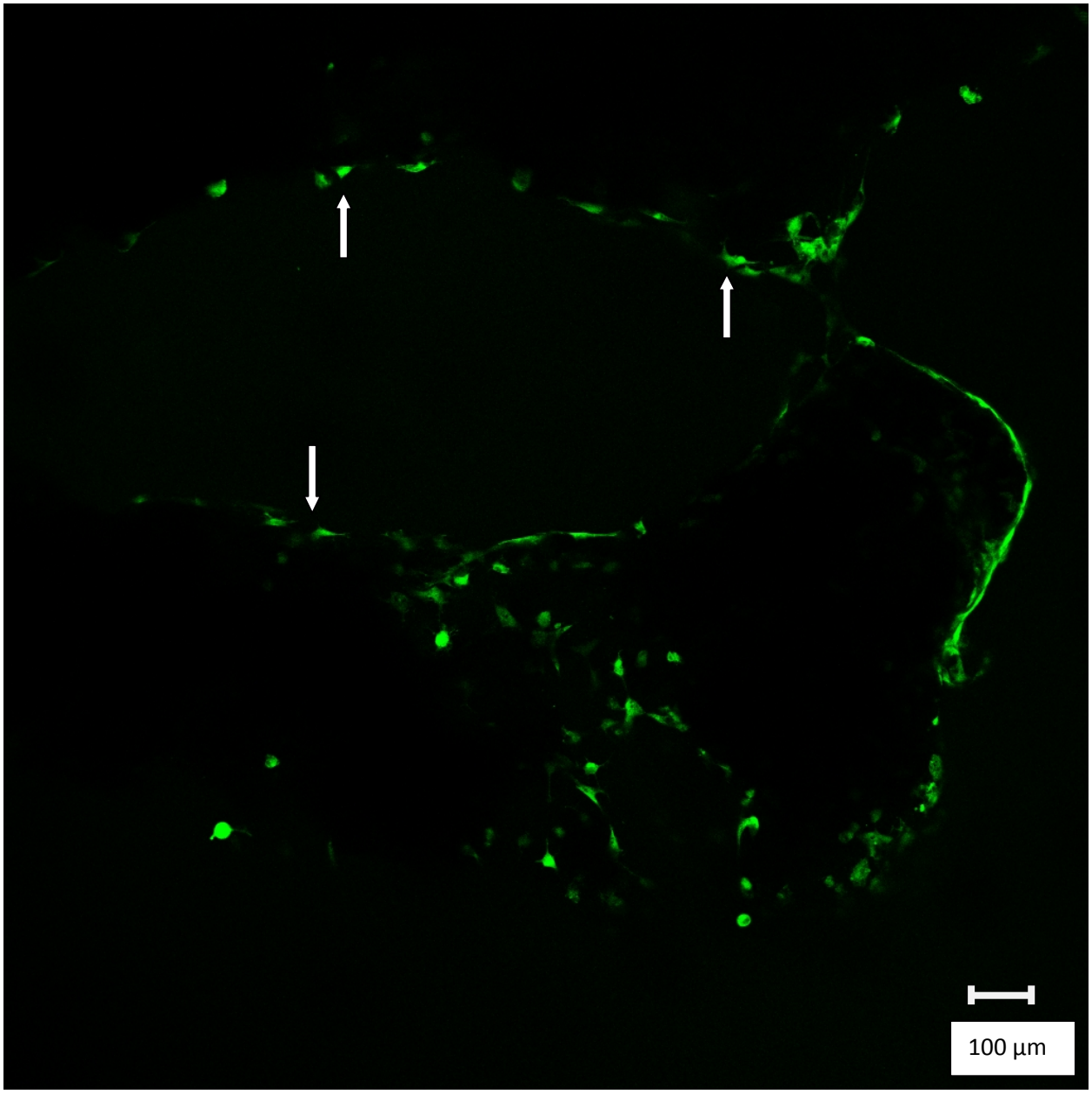


Figure 4.3.1.3: Confocal image of BMCs on an IGF-I encapsulated scaffold after three weeks.

4.3.2. GAG and DNA assay

The GAG assay was performed with the blank scaffolds and IGF-I encapsulated scaffolds seeded with bone marrow cells. At the end of the three week period, the results showed an increased amount of GAG production in the case of IGF-I added blank scaffolds and IGF-I encapsulated scaffolds compared to that of the blank scaffolds seeded with the bone marrow cells (Figure 4.3.2.1). The results after the four week time period showed that the amount of GAG produced was almost equal in the blank scaffolds seeded with bone marrow cells and blank scaffolds seeded with bone marrow cells and IGF-I added externally (Figure 4.3.2.2). Even after four week time period the IGF-I encapsulated scaffolds seeded with bone marrow cells showed more production of the GAG when compared to its control.

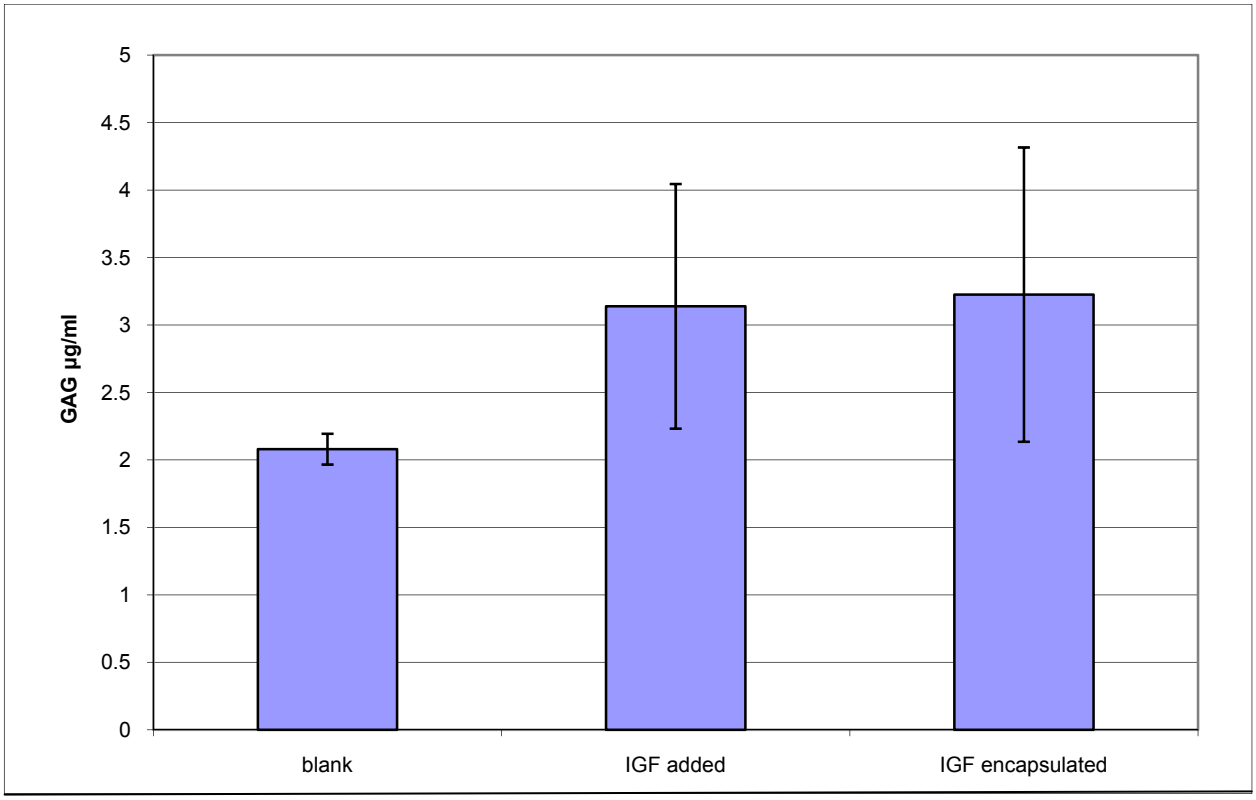


Figure 4.3.2.1: Concentration of the GAG, measured from BMCs cultured on PLGA scaffolds for three weeks.

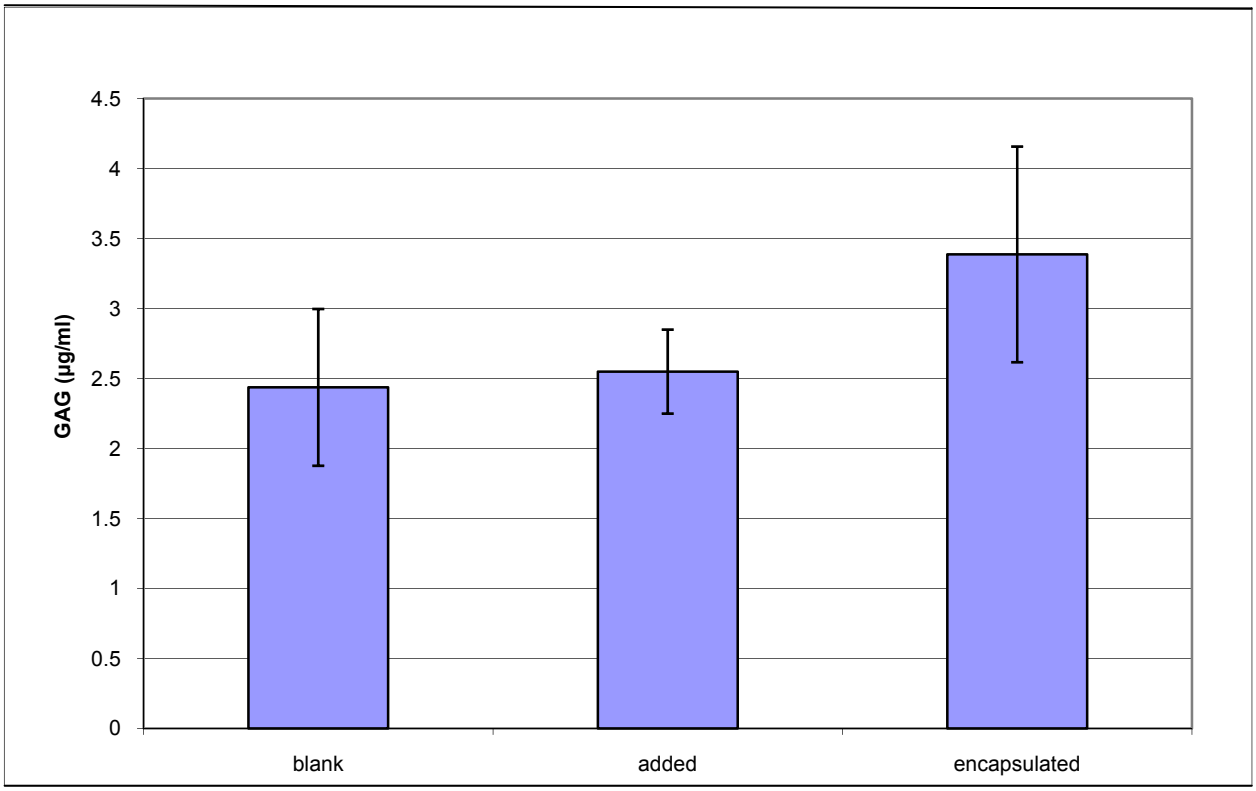


Figure 4.3.2.2: Concentration of the GAG, measured from BMCs cultured on PLGA scaffolds for four weeks.

The GAG assay results obtained were normalized by dividing the amount of GAG produced by the total amount of cells present in the scaffolds. The amount of cells present in each scaffold was determined by Pico green DNA assay. The amount of DNA was found to be more in the case of IGF-I added and encapsulated scaffolds compared to the blank scaffolds after three week and four week time periods, indicating the effect of IGF-I on cell multiplication (Figure 4.3.2.3 and Figure 4.3.2.4). Figure 4.3.2.5 shows the normalized data after three week time period. These results did not show any significant difference between the normalized GAG concentrations of the IGF-I encapsulated scaffolds and the controls. Similar results were obtained from four week time period data (Figure 4.3.2.6). There was no significant difference between the normalized GAG concentrations of the IGF-I encapsulated scaffolds and the controls even after four weeks.

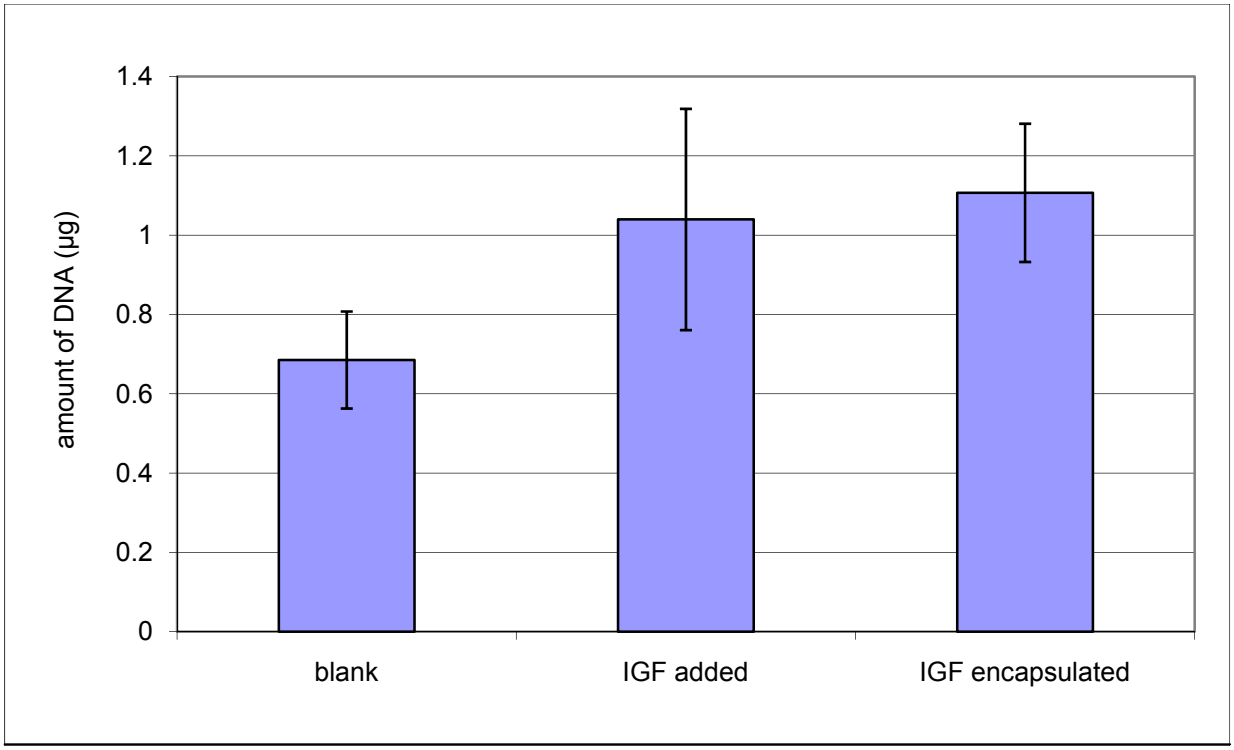


Figure 4.3.2.3: Amount of DNA from BMCs cultured on PLGA scaffolds for three weeks.

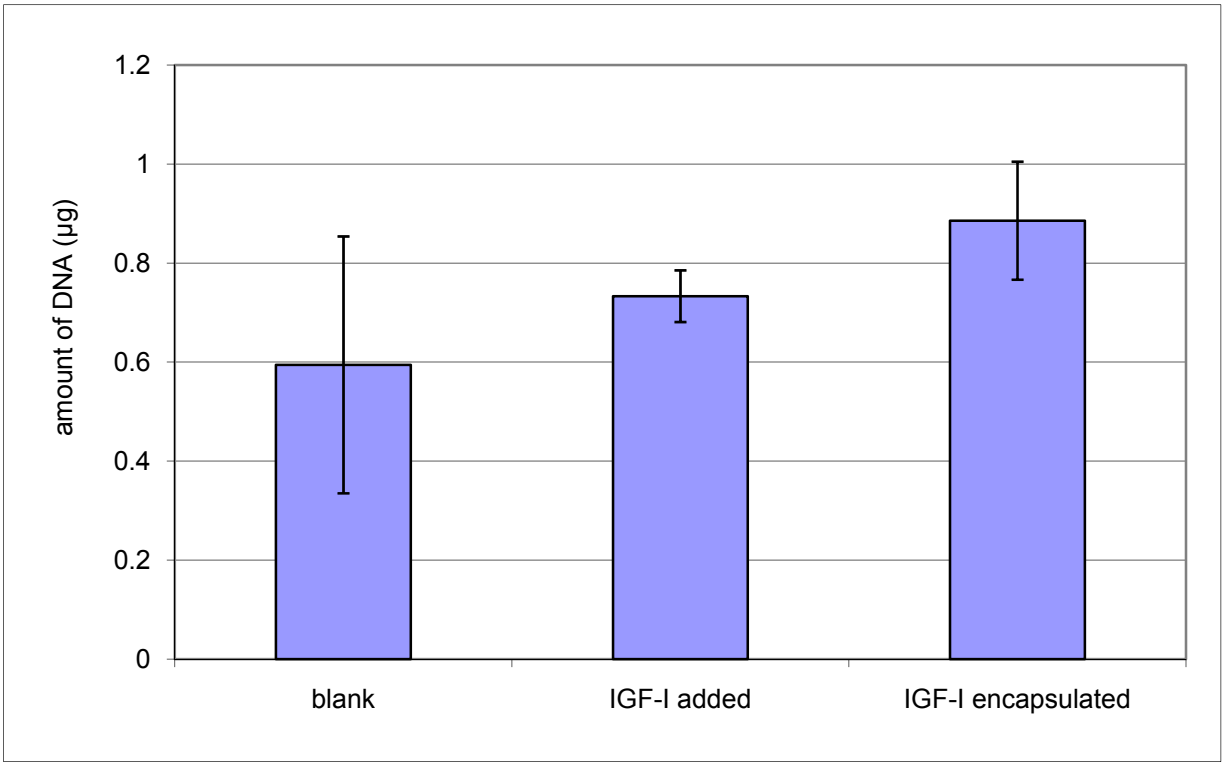


Figure 4.3.2.4: Amount of DNA from BMCs cultured on PLGA scaffolds for four weeks.

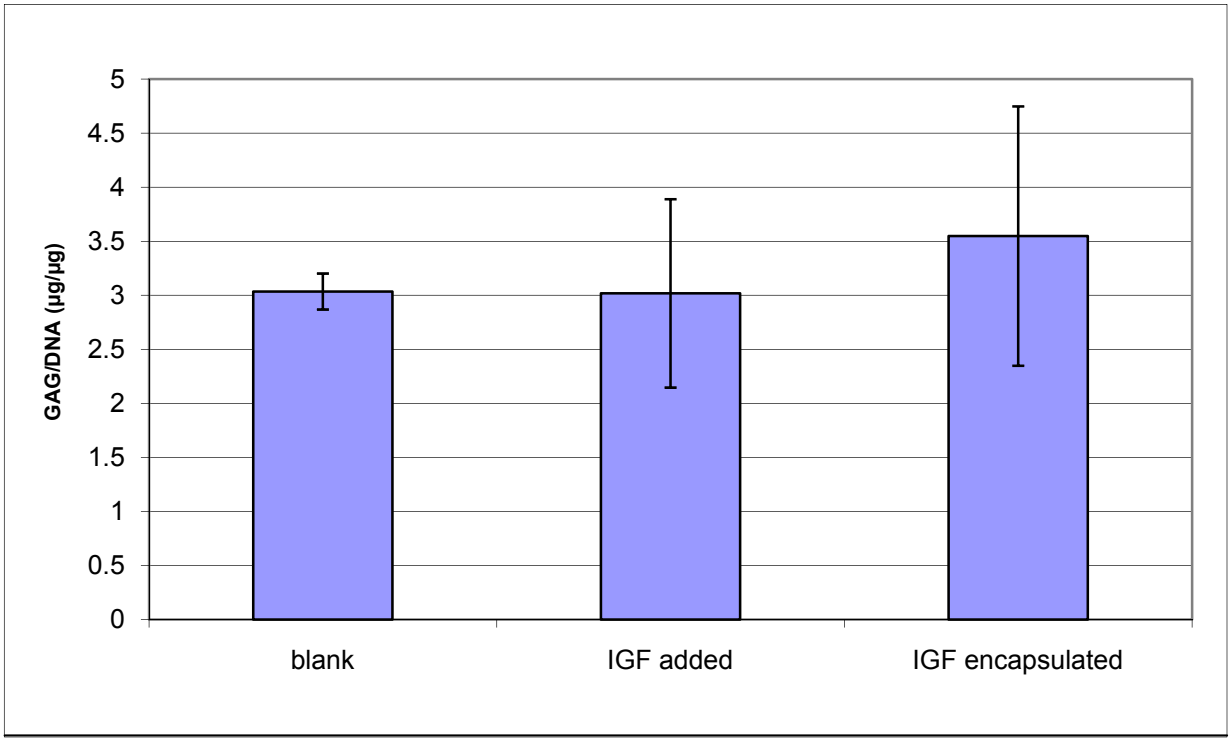


Figure 4.3.2.5: Normalized GAG content of BMCs cultured on PLGA scaffolds for three weeks.

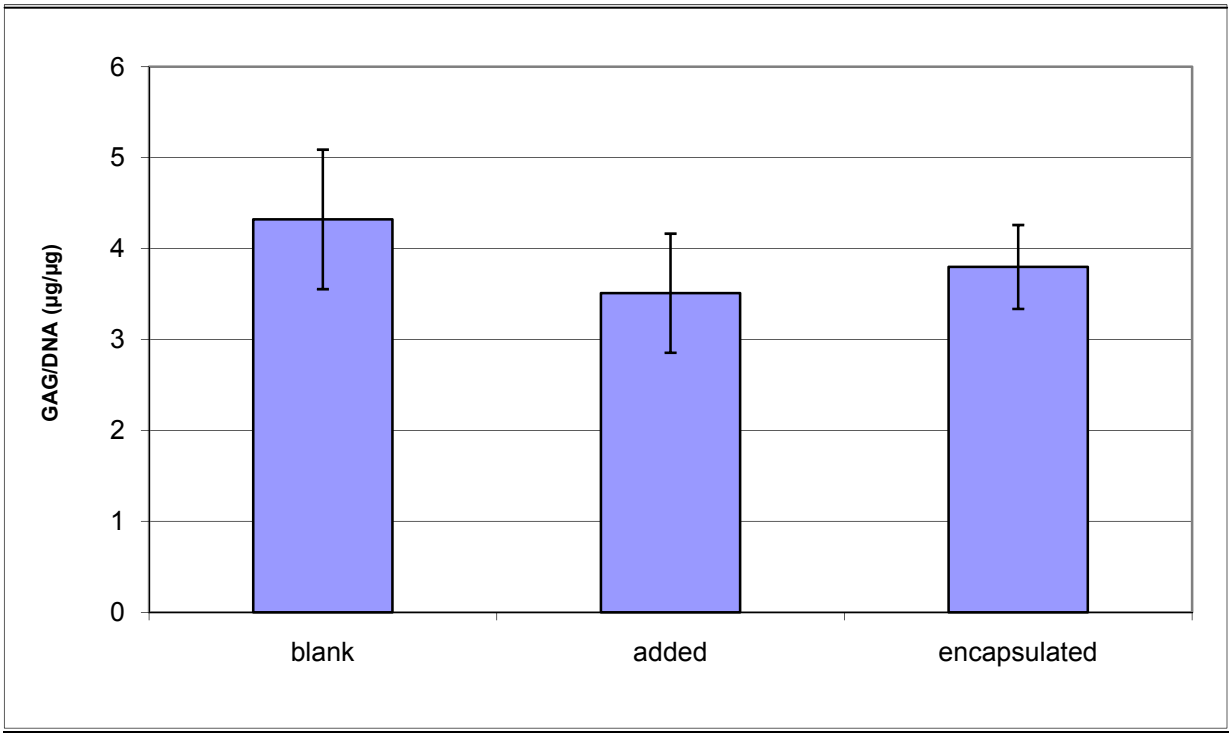


Figure 4.3.2.6: Normalized GAG content of BMCs cultured on PLGA scaffolds for four weeks.

4.4 Animal study

4.4.1 Angle measurements from the radiographic images

The measured LDFA and MPTA were compared between the animals which were implanted with blank scaffolds or IGF-I encapsulated scaffolds and animals without the scaffold (controls). Figure 4.5.2.1 shows the results of the comparison of the LDFA. During the first radiographic images the angle was around 90 degrees, but there was no significant change in the angles measured at the start of the study, after three weeks (after formation of bone bar) and after 11 weeks (at the end of the treatment). Figure 4.5.2.2 shows the MPTA of the animals at various stages of the study. There was no statistically significant change in the MPTA measured at the start of the study, after three weeks (after formation of bone bar) and after 11 weeks (at the end of the treatment).

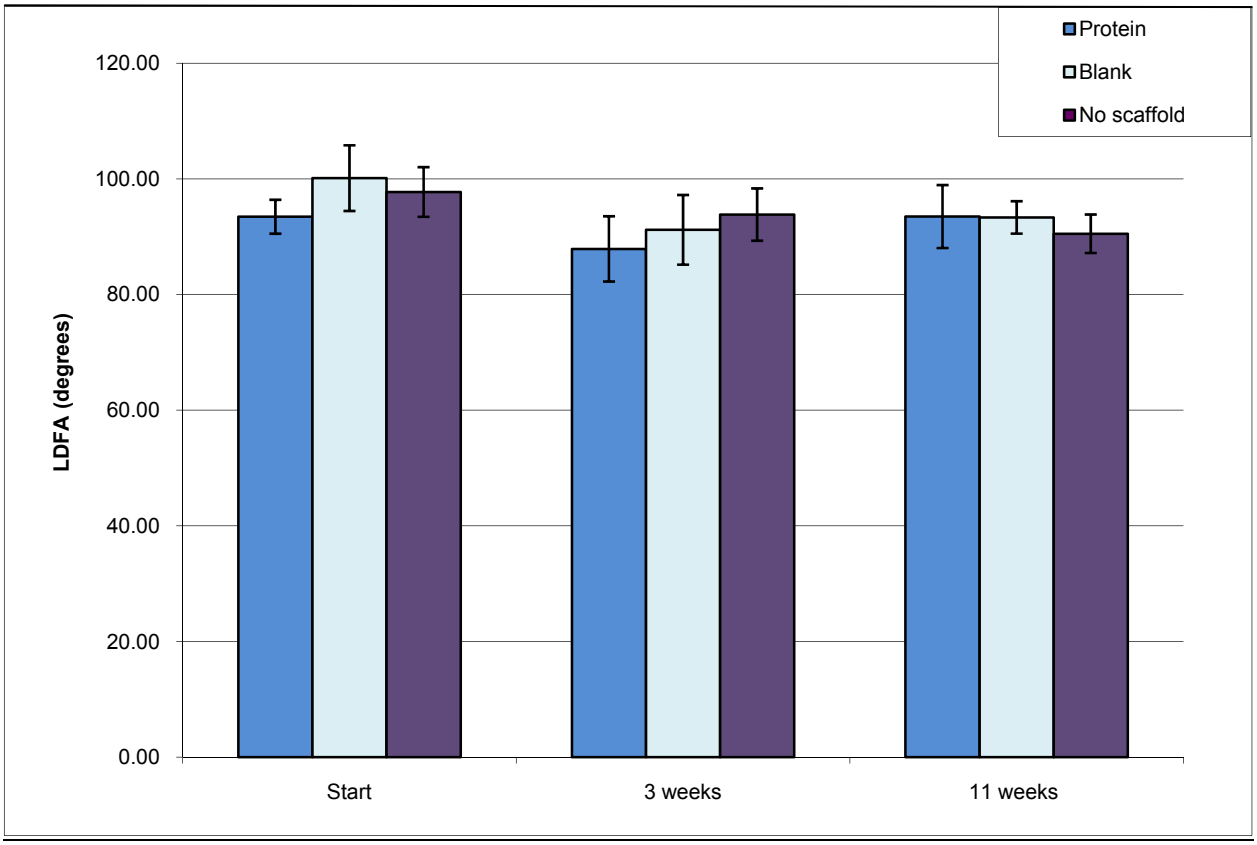


Figure 4.4.1.1: Comparison of LDFA during various stages of the study.

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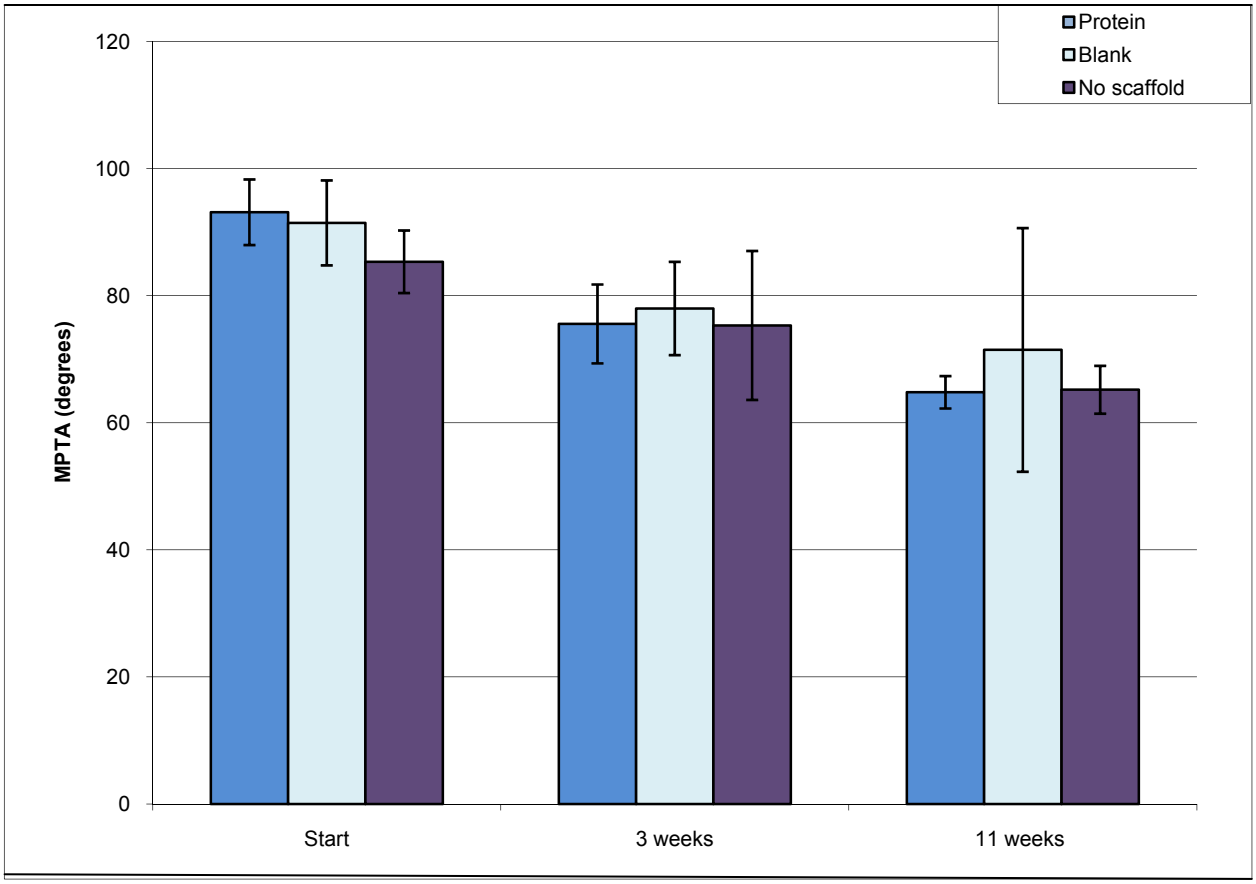

Figure 4.4.1.2: Comparison of MPTA during various stages of the study.

4.5 Histology

Tissue sections stained by H&E and safranin O staining were compared between the control, blank scaffold and drug loaded scaffold implanted bone sections. The images showed intact growth plate on the lateral part of the proximal tibia where the growth plate was not disturbed during the surgery. In the medial half of the proximal tibial growth plate where the injury was created and the scaffold was implanted showed regeneration of cartilage in the case of bones implanted with IGF-I encapsulated scaffold. The visual observation of the regenerated cartilage showed that unlike the native growth plate the chondrocytes were not stacked but scattered in the extracellular matrix.

4.5.1 Images from the H&E staining

H&E staining was performed on the tissue sections obtained from control (animals without the scaffold treatment), animals implanted with blank scaffold and IGF-I encapsulated

scaffold. The images from the H&E stained slides are shown below. Figure 4.6.1.1 shows the H&E stained image from coronal section of proximal tibia from the control rabbit. The coronal section shows both the medial half (M) and lateral half (L) of the proximal tibia. The implant site does not show any signs of growth plate regeneration. The same kind of observation was also made in the case of the bones implanted with blank scaffold (Figure 4.6.1.2). In the animals where the injured growth plate was treated with implantation of IGF-I encapsulated scaffold (Figure 4.6.1.3) cartilage regeneration was observed.

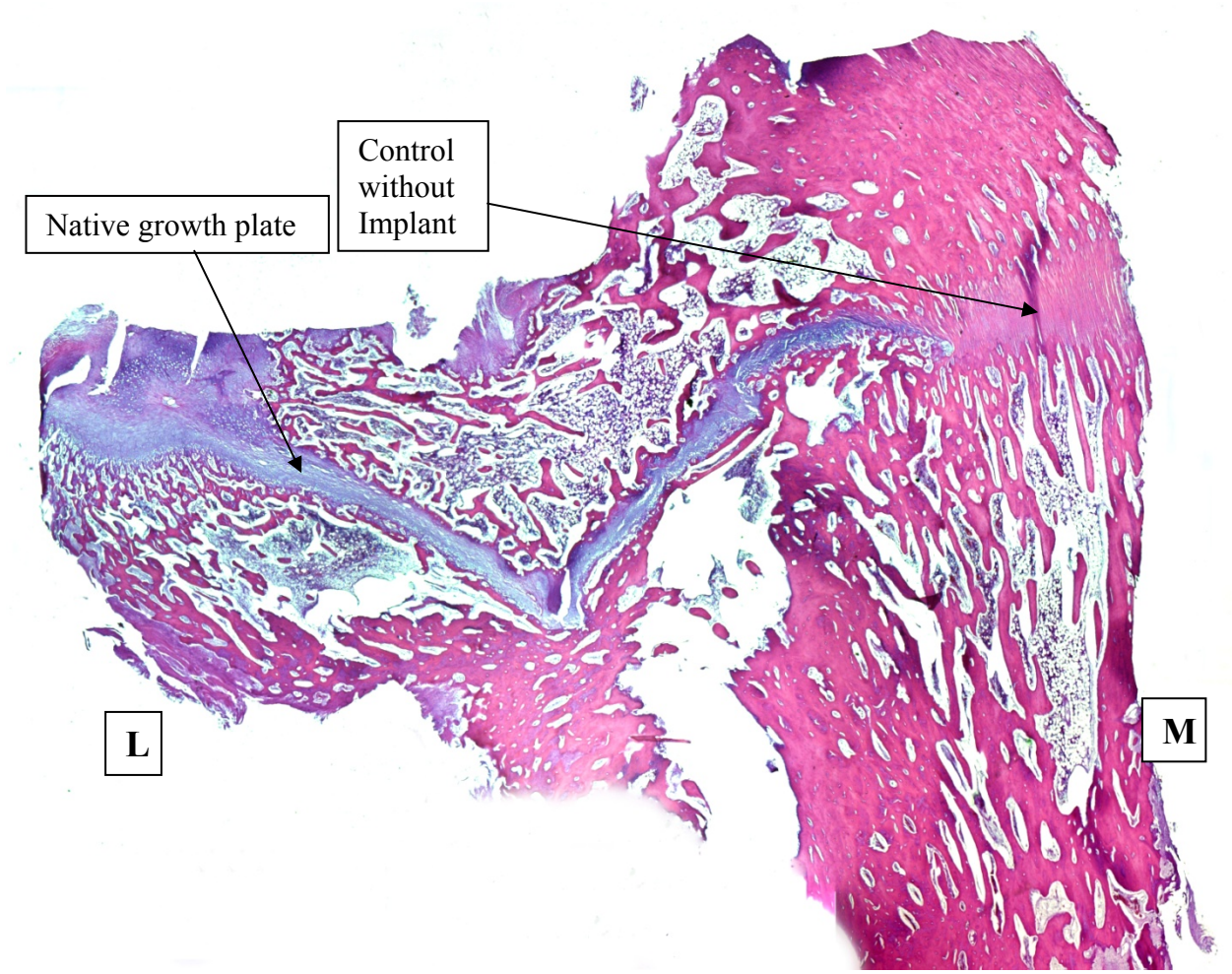

Figure 4.5.1.1: H&E stained coronal section of rabbit proximal tibia (control - without scaffold implantation) at the end of the study.

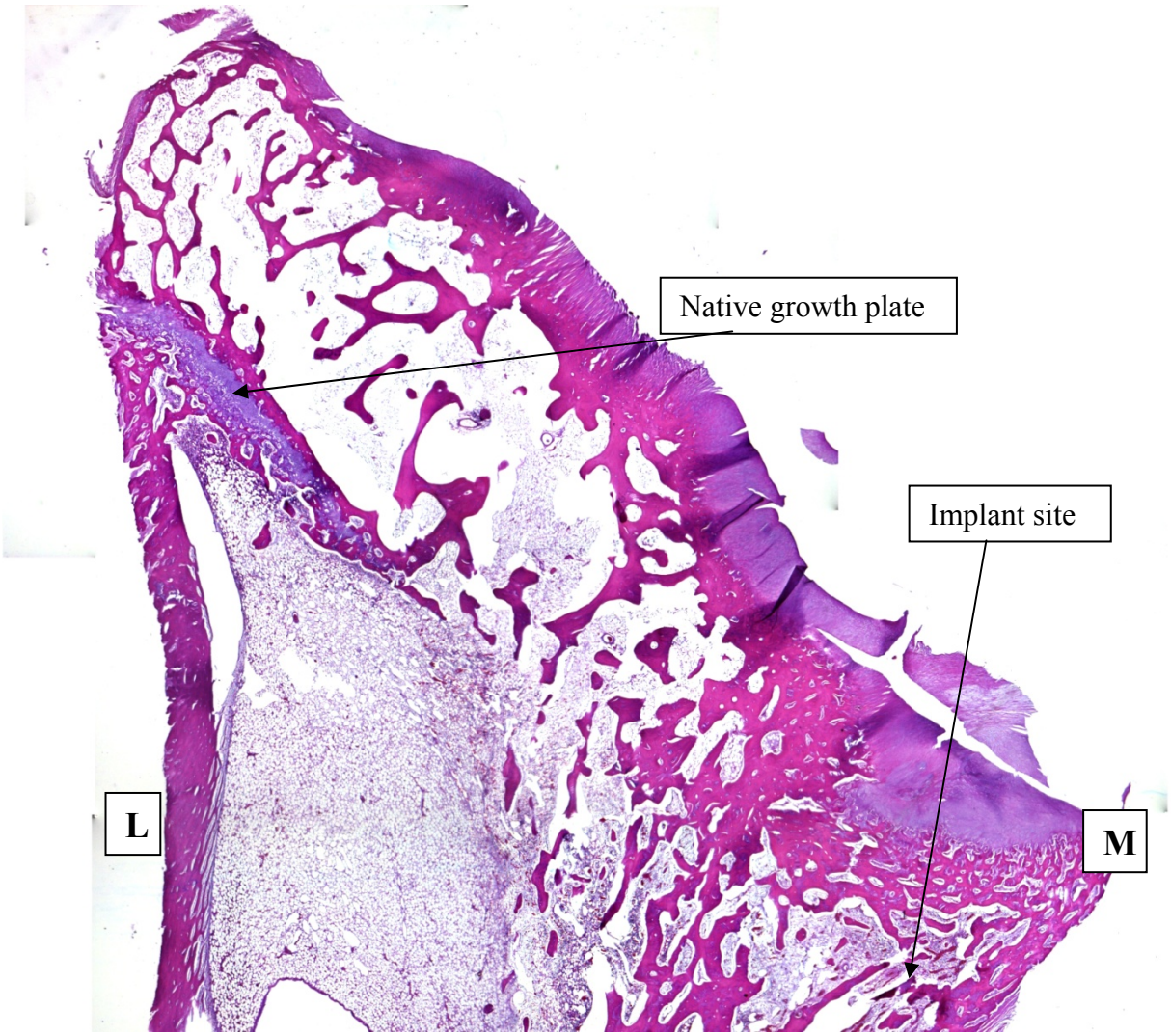

Figure 4.5.1.2: H&E stained coronal section of rabbit proximal tibia (with blank scaffold) at the end of the study.

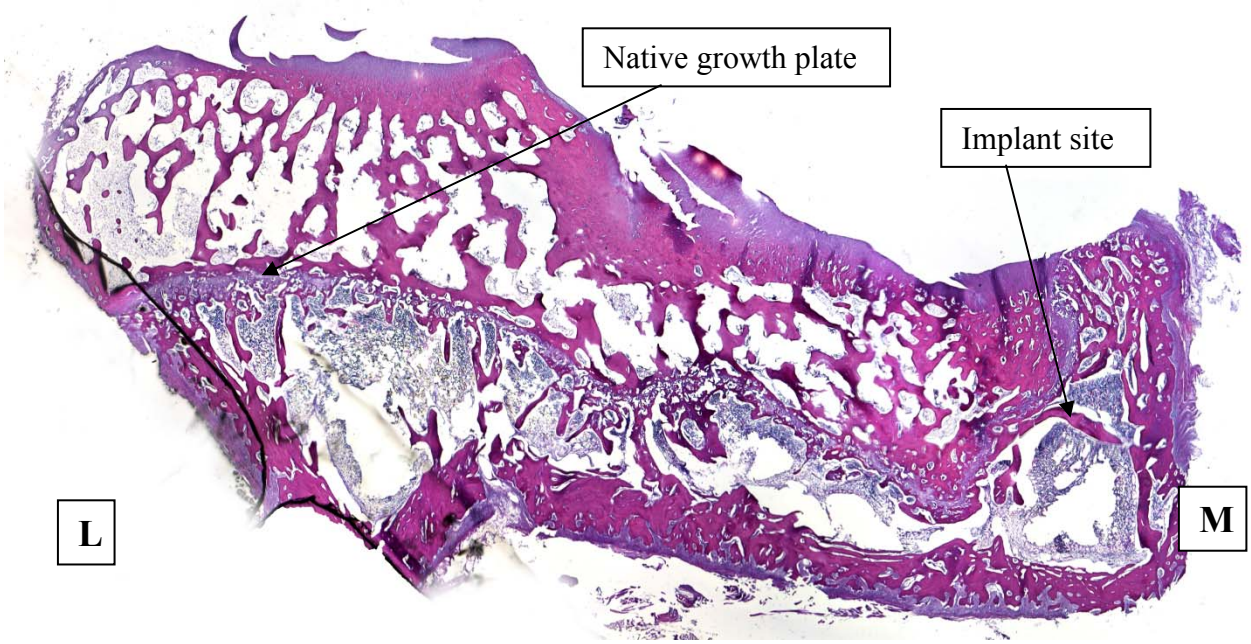

Figure 4.5.1.3: H&E stained coronal section of rabbit proximal tibia (with IGF-I encapsulated scaffold) at the end of the study.

Further magnified images of the native growth plate (Figure 4.6.1.4) and the regenerated growth plate Figure (4.6.1.5) were also obtained. Stacks of chondrocytes were observed in the case of the native growth plate. In the regenerated growth plate the chondrocytes were not found in the usual stacked manner. Single chondrocytes were distributed in the extra cellular matrix in the regenerated growth plate.

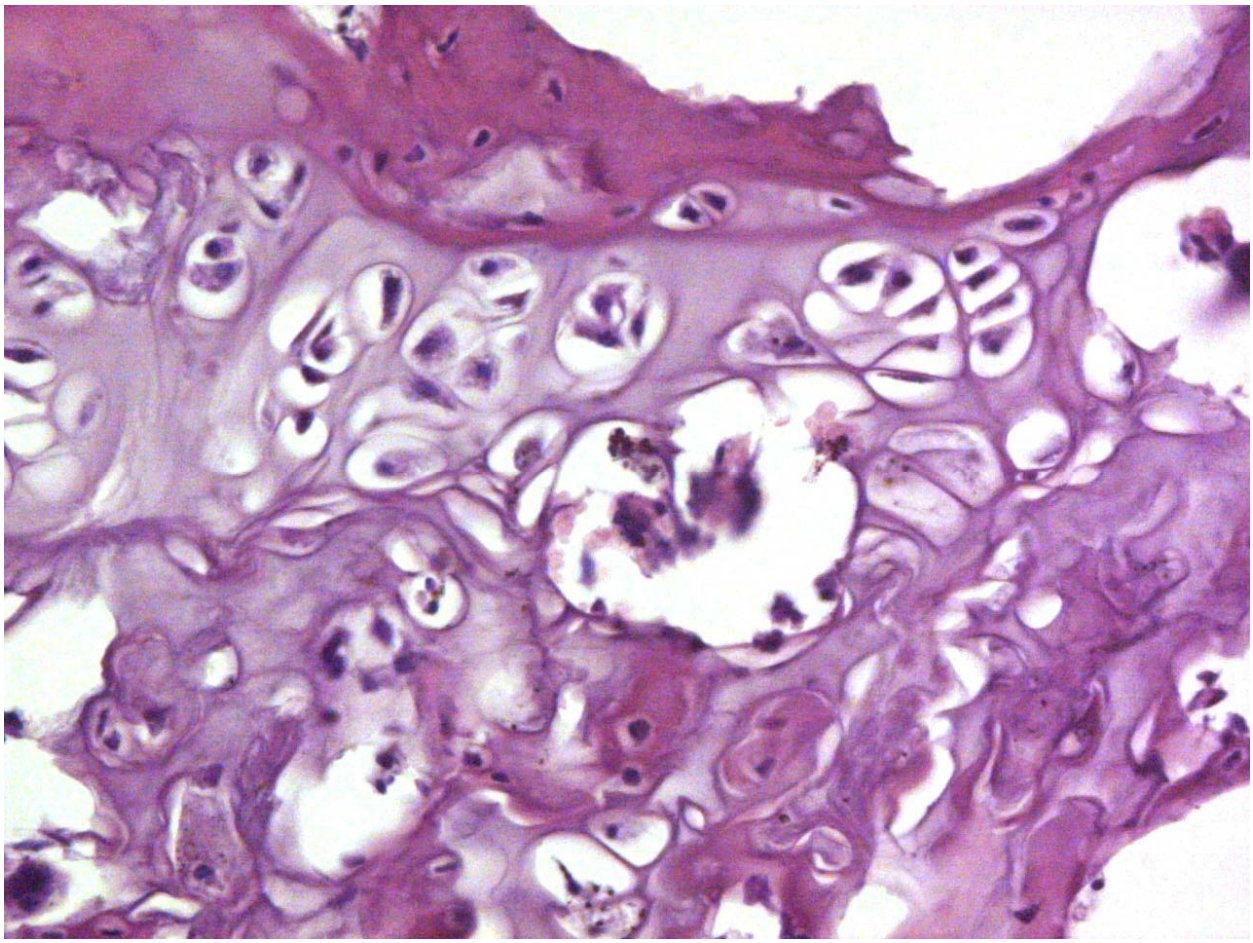

Figure 4.5.1.4 : Magnified image of the native growth plate with chondrocytes stacked.

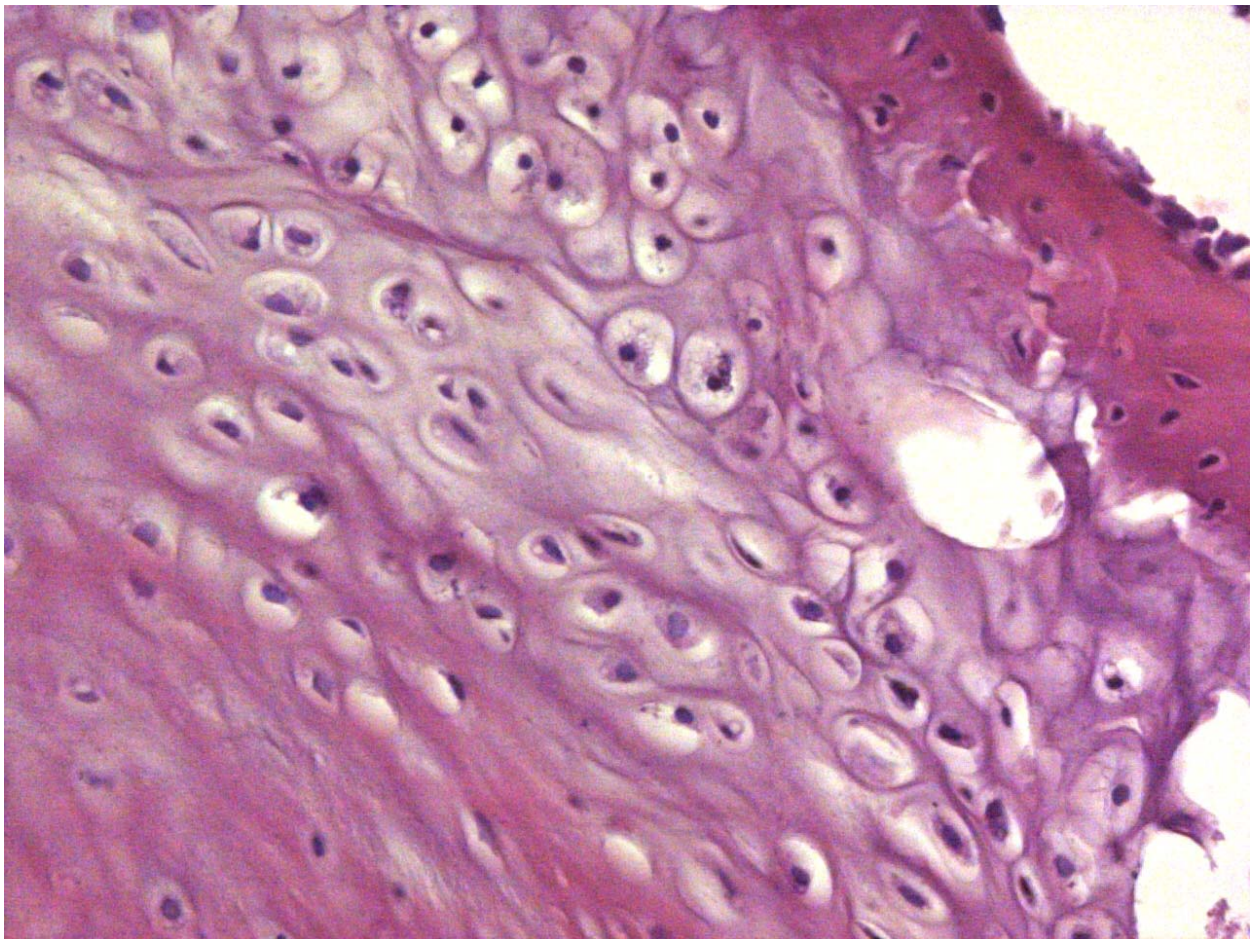

Figure 4.5.1.5: Magnified image of regenerated growth plate with chondrocytes distributed in the extracellular matrix.

4.5.2 Images from safranin O staining

Safranin O staining was also done on the sections obtained at the end of the study. In the case of the safranin O staining the counter staining using fast green did not stain prominently in some sections. This resulted in the presence of only the stained cartilage seen in the images.

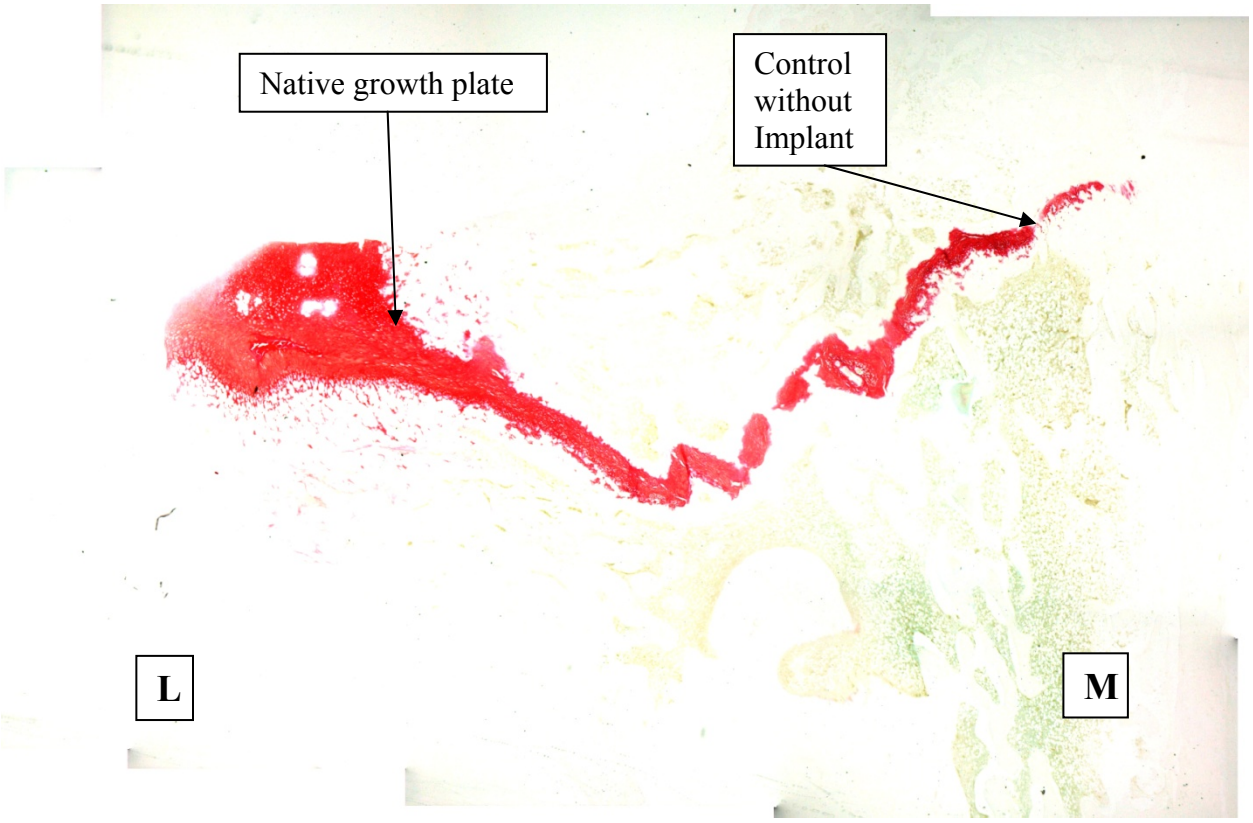

Figure 4.5.2.1: Safranin O stained coronal section of rabbit proximal tibia (control - without scaffold implantation) at the end of the study.

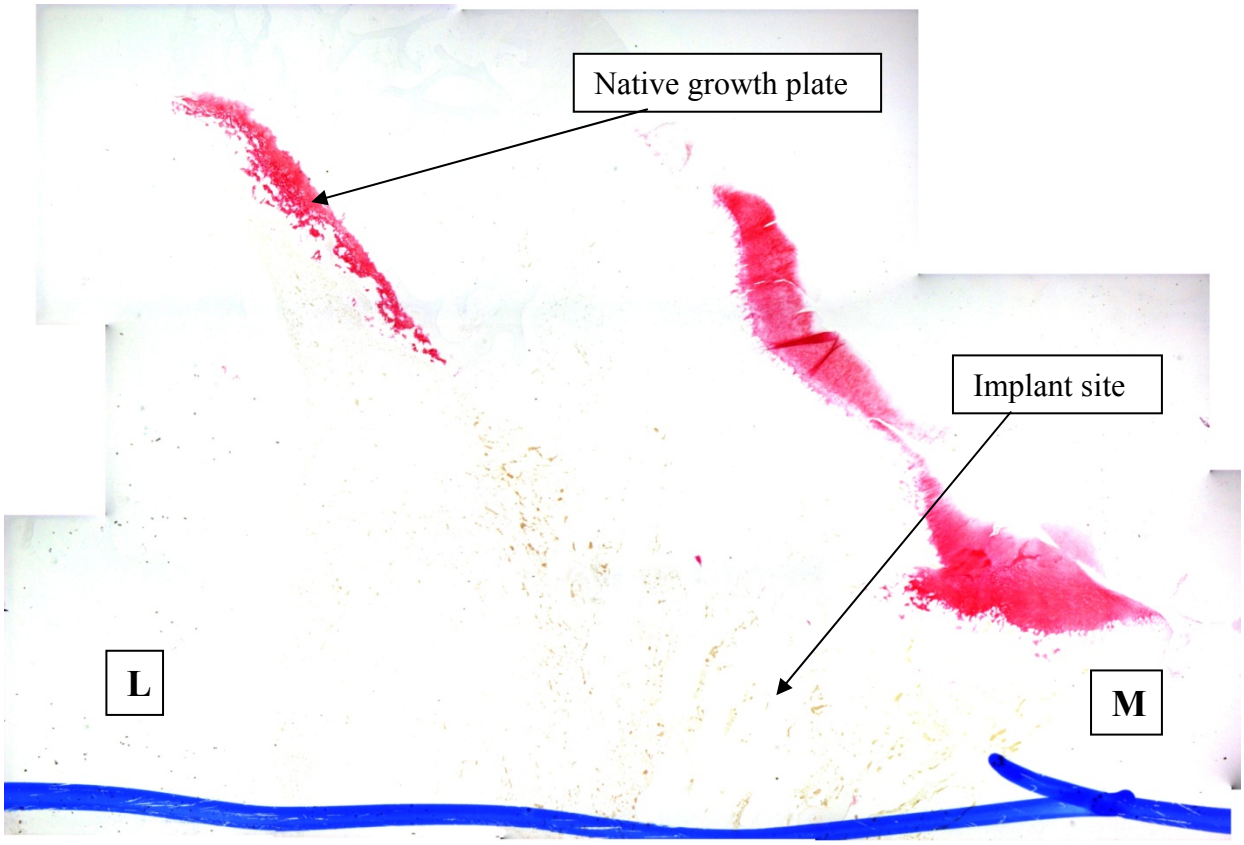

Figure 4.5.2.2: safranin O stained coronal section of rabbit proximal tibia (with implantation of blank scaffold) at the end of the study.

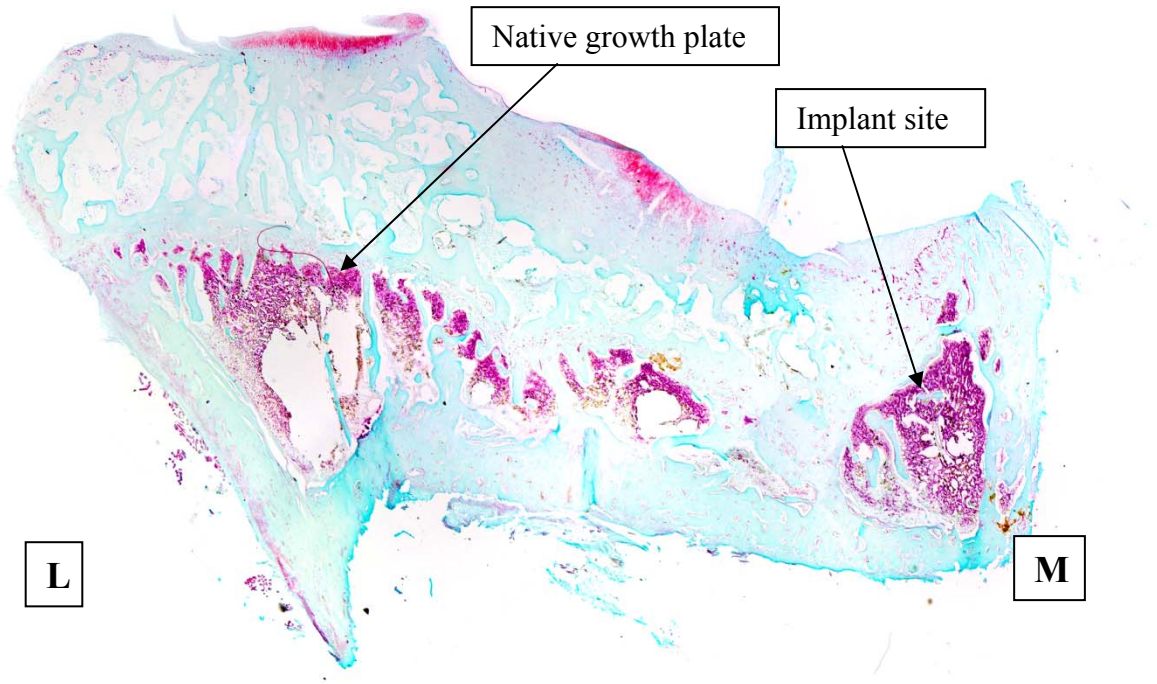

Figure 4.5.2.3: Saffronin O stained coronal section of rabbit proximal tibia (with implantation of IGF-I scaffold) at the end of the study.

5. DISCUSSION

5.1 Release of protein from PLGA scaffold

5.1.1. Release profile of protein encapsulated scaffolds degraded in PBS

Two proteins were used to study the release profile of proteins encapsulated in the PLGA scaffolds. Lysozyme was used as a pilot protein during the initial stages of the release profile study. Release profile of IGF-I (protein of interest) encapsulated in PLGA scaffold was also obtained. Lysozyme is relatively inexpensive when compared to the IGF-I, so it was used to conduct initial pilot studies. The release profiles obtained showed that the release of lysozyme and IGF-I followed a similar biphasic pattern. There was an initial burst (high amount of protein being released in the first 24 – 48 hours) representing the initial diffusion of the proteins from the PLGA scaffold through the pores and the release of proteins from the microspheres present on the surface of the scaffold [17]. Following the initial burst, the amount of protein being released gradually decreased over the next two week time period and slowly stopped after one month. This gradual release of the protein from the scaffold was due to the degradation of PLGA present in the scaffold. The presence of surface pores in the microspheres is the reason the biphasic release that is occurring. The initial phase of release was due to the diffusion of protein through the aqueous pores. The next phase of release was due to the erosion of the PLGA matrix and the release of the proteins encapsulated within the internal pores of the microspheres.

The only difference that was observed when the comparing the release profile and cumulative release of lysozyme and IGF-I was the total amount of protein encapsulated and released. The results show that a greater amount of lysozyme (28 μg) was encapsulated and released when compared to the amount of IGF-I (2.8 μg), even when same amount of IGF-I and lysozyme was used during the encapsulation process. The reason behind this difference in the amount of protein encapsulated and released was mainly due to the size of the protein molecule. The molecular weight of lysozyme (14,700 Daltons) is twice that of IGF-I (7,600 Daltons) [9]. The size of the protein molecule has a major effect on the microencapsulation and its release. Santander-Ortega et al. showed the effect of molecular weight on the protein encapsulation. Being a smaller molecule IGF-I can easily diffuse trough the polymer when compared to a relatively bigger molecule (lysozyme). There are even chances of losing IGF-I during the process of leaching the

IGF-I encapsulated scaffolds in deionised water for creating pores. There are factors like proteins affinity towards the PLGA that might be responsible for reduced amount of IGF-I being encapsulated and released when compared to lysozyme [27]. Despite the loss of IGF-I during the microencapsulation process, the amount that has been encapsulated and released (2.7μg) is physiologically significant. Trippel et al. showed that nanomolar concentrations of IGF-I is sufficient for mitosis and differentiation of growth plate chondrocytes [15].

5.1.2. Release profile of protein adsorbed and protein encapsulated scaffolds

To check the effect of microsphere encapsulation on protein release, protein-encapsulated scaffolds were compared with protein-adsorbed PLGA scaffolds. Since both lysozyme and IGF-I followed a similar release pattern, lysozyme was adsorbed on the surface of the scaffold. The blank scaffolds were immersed in protein solution of desired concentration for different time intervals (one and 24 hours) to adsorb protein on the surface of the PLGA scaffold. The blank scaffolds immersed in lysozyme solution for 24 hours adsorbed more lysozyme when compared to blank scaffolds immersed in lysozyme solution for one hour. Based on this, blank PLGA scaffolds immersed in lysozyme solution for 24 hours were compared with lysozyme-encapsulated PLGA scaffolds. The release profile obtained showed that, unlike the protein encapsulated scaffolds, protein adsorbed scaffolds did not follow a biphasic release pattern. There was an initial burst in the release in the lysozyme adsorbed scaffold, but second phase of release was not observed as in the case of protein-encapsulated scaffold. This is because in the adsorbed scaffolds, the protein was present only on the surface, which was released within the first week. These studies showed that encapsulation of protein within the scaffold increased the amount of drug that can be delivered at the site of interest.

5.1.3. Release profile of protein-encapsulated scaffolds degraded in cell culture medium

IGF-I-encapsulated scaffolds degraded in cell culture medium showed a different kind of release profile. IGF-I was released in a relatively slower manner when the scaffolds were degraded in cell culture medium, compared to the release from the scaffolds degraded in PBS. The statistical analysis also proved that the slopes of the release curves were different between scaffolds degraded in cell culture medium and PBS. The area under the release curve was also measured and it was found that the difference between the area under the release curve obtained

from the scaffolds degraded in PBS and cell culture medium was not statistically significant. This indicates that even though there was a different between the release profiles of IGF-I encapsulated scaffold degraded in PBS and cell culture medium, the amount of protein that has been released at the end of the study has been the same. Faisant et al. showed that the increase in the osmolarity of the release medium had a significant effect on the release profile of drug from PLGA-based microparticles. According to their study an increase in the concentration of the release medium led to the decrease in the drug release rate [19]. The relatively sustained release of IGF-I from the PLGA scaffolds degraded in cell culture medium might be because of a similar effect. In this case the increased presence of various proteins due to the presence of fetal bovine serum (FBS) in the cell culture medium might have decreased the movement of water in and out of the scaffold. This reduces the mobility of the drug (IGF-I) which ultimately affected the release rate. Based on this effect, we can expect a more sustained release of IGF-I from the scaffold in vivo, where the scaffold is placed in a more complex and dynamic environment with enzymes and cells.

5.1.4. Release profile of protein-encapsulated scaffolds degraded in cell culture medium along with macrophages

Scaffolds degraded in cell culture medium along with macrophages seeded on top of them also showed a different kind of release profile. The macrophages were added to further mimic the physiological conditions. Macrophages are one of the dominant types of cells present near the injury site, where their main function is to engulf and digest the foreign bodies that enter the body. Macrophages were seeded on top of the scaffolds to observe their effect on scaffold degradation and protein release. The release profile was significantly different from the release profile of scaffolds degraded in PBS without macrophages. The effects of macrophages on the scaffolds are further discussed in the section 5.2.1 of the discussion.

5.2 Degradation of PLGA scaffolds

5.2.1 Mass loss of scaffolds degraded in PBS

Mass loss studies conducted by degrading blank scaffolds in PBS showed that degradation occurred in a linear fashion. The pores present in the scaffolds were filled with water at first, and the scaffolds started to swell up by absorption of water followed by slow erosion of the polymer. The degradation of the polymer is initiated by the hydrolysis of the ester bonds by the water molecules [18]. Due to the presence of the pores, the scaffold structure is in the form of an interconnected network. Due to this, even as the polymer bonds break, the scaffolds maintain their structure for a period of two to three weeks and then slowly break down. The release profile showed that significant amount of the IGF-I was released within the first three week time period. This shows that before the complete collapse of the scaffold occurred most of the protein encapsulated in the scaffold was released.

5.2.2 Mass loss of scaffolds degraded in cell culture medium

The mass loss profile of the blank scaffolds degraded in cell culture medium showed a similar type of mass loss profile compared to the blank scaffolds degraded in PBS. The mass loss occurred in a linear pattern with respect to time. When the morphology of the scaffolds was studied during various stages of the degradation, the scaffolds increased in size as the degradation proceeded, and then after about three weeks they started to collapse and shrink. The scaffolds swelled up mainly because of the absorption of the water through the pores present in the scaffold. The PBS or the cell culture medium penetrated the pores and started degrading the scaffold's internal structure along with the external surface. The shrinking of the scaffold that was observed after three week time period was mainly due to physical disintegration or fragmentation of the internal structure of the scaffold [28].

This type of change in morphology of the scaffolds due to swelling was observed in both the scaffolds degraded in PBS and cell culture medium, but these morphological changes were more significant in the scaffolds degraded in cell culture medium. The mass loss profile of both the blank scaffolds degraded in PBS and cell culture medium were the same, even though there was difference observed in the morphology during the scaffold degradation in cell culture medium when compared to the scaffolds degraded in PBS.

Mass loss studies were not conducted with macrophages seeded on top of the blank scaffolds degraded in cell culture medium, but the scaffolds seeded with macrophages were observed during the release studies. It was clear that the morphology of the scaffolds was different during the course of degradation with macrophages when compared to the morphology of scaffolds degraded in PBS and cell culture medium. This clearly shows that macrophages have an effect on the scaffold degradation, the scaffolds started shrinking and started to collapse. This may be because of the phagocytic activity of the macrophages that have infiltrated the scaffold, leading to degradation of the polymer scaffold [29]. Pan et al. also showed that the degradation of the PLGA scaffold occurred faster in the presence of macrophages when compared to the PLGA scaffolds degraded in PBS or cell culture medium [30]. This study with macrophages was only a small part of this research. More studies are needed to further explain the effect of macrophages on scaffold degradation and protein release.

5.3 In vitro cell studies

5.3.1 Seeding the cells with fibrin glue

In physiological conditions the cells present near the site of implantation are expected to come in contact with the fibrin. Fibrin is produced as a result of the blood coagulation cascade and is expected to be found at the site of implantation. To mimic this condition in vitro and to keep the BMCs from flowing out of the scaffolds and in to the cell culture medium, fibrin glue was used to clot the cells within the scaffolds. Fibrin glue has been successfully used in several tissue engineering studies for sealing of tissues and cell adhesion [31, 32]. In this research the BMCs were mixed with fibrinogen and seeded on the scaffold followed by addition of thrombin to form a thrombin-fibrinogen clot along with the cells within the scaffold. Yi-xiong et al. showed that fibrin gel used for immobilization of progenitor cells in 3-dimensional PLGA scaffolds resulted in homogeneous distribution of cells [33]. Hybrid scaffolds based on fibrin and PLGA have also been used by Munirah et al. for cartilage tissue engineering [34].

5.3.2 Confocal imaging of BMC-seeded scaffolds

Thevenot et al. showed the ability of cells to infiltrate the 3-dimensional PLGA scaffolds [35]. Studies done by Wang et al. involved the infiltration of BMCs in the pores of PLGA scaffolds. Their studies also showed the ability of BMCs to differentiate within the porous PLGA

scaffold [36]. Confocal images of the scaffolds seeded with BMCs using fibrin glue showed that the cells infiltrated the pores present in the scaffolds. In all the cases the BMCs labeled with fluorescent dye was seen present at the edges of the pores of the scaffold. These images were obtained three weeks after seeding the bone marrow cells on the scaffold which confirms that the cells which infiltrated the pores were held inside them for the required period of time. This also shows that the experimental set up used for the in vitro studies is appropriate.

5.3.3 Analysis of GAG content from the BMCs

GAG assay was performed on the scaffolds seeded with BMCs after three and four weeks of cell culture to estimate the amount of GAG content present. The results showed that the BMCs present on the IGF-I encapsulated scaffolds produced more GAG when compared to the BMCs present on blank scaffolds with IGF-I added externally and the blank control scaffold. The data obtained by GAG assay were normalized to determine the effect of cell proliferation on the amount of GAG produced. The normalization was done by finding the amount of DNA present in each scaffold and dividing the amount of GAG produced by the amount of DNA for the respective scaffolds. The DNA assay showed that the amount of DNA present in the IGF-I-encapsulated scaffolds was higher than that of the control blank scaffold and the blank scaffold with IGF-I added externally. This effect is mainly due to the mitogenic effect of IGF-I on BMCs [8, 12, 37]. The increased number of BMCs in the IGF-I encapsulated scaffold (more DNA) showed the effect of the released IGF-I. The comparison of the normalized data between the control blank, IGF-I externally added and IGF-I encapsulated scaffold at three week and four week time points did not show statistical significance. This was because of increased cell proliferation in the IGF-I encapsulated scaffold and the scaffolds with IGF-I added externally neutralized the amount of GAG produced when normalized. On the whole, the in vitro studies show that the IGF-I released from the encapsulated scaffold led to cell proliferation and production of GAG. With these release profile data, mass loss studies and in vitro cell studies as background, in vivo studies were done by implanting the scaffolds in New Zealand white rabbits.

5.4 In vivo studies

5.4.1 Scaffold used in the animal surgery

The surgery was performed on the medial half of the tibial growth plate of the New Zealand white rabbit. The scaffolds used in the surgery was different in dimensions when compared to the one used in the release studies. Klose et al. showed that the PLGA device geometry did not have any significant effect on the relative release rate of the drug [22]. The drug release from the implanted scaffold must have been similar to that of the scaffold used in the release studies. There might be different release pattern of the drug from the scaffold in vivo, as shown previously in results the release pattern was significantly different based on the medium (PBS or cell culture medium) in which the scaffold is degraded. The rate of scaffold degradation also occurs faster in vivo conditions [18].

5.4.2 Radiographic images

The LDFA and MPTA were measured before creating the growth plate injury, before implantation of the scaffold (after formation of bone bar) and at the end of the treatment period. The idea was to study the bone angles, which serve as the primary means for determining the effect of the implant through the course of treatment. The bone angles measured from the radiographic images showed that there was not significant change in both the LDFA and MPTA during the course of the treatment. There was no change expected in the LDFA as there was no injury created in the femoral part of the leg. As expected LDFA was around 90 degrees at the beginning of the study and continued to be around 90 degrees throughout the course of the treatment without any significant change. Since the injury was induced in the medial tibial region, change in the MPTA was expected during the course of the treatment. The MPTA was around 90 degrees at the beginning of the study and it was expected to go down from 90 degrees after the induction of the growth plate injury. The MPTA was reduced to below 80 degrees in all three cases of control (no scaffold), blank scaffold implanted and IGF-I scaffold implanted cases. This reduction in MPTA was due collapsing of the bone due to the removal of the growth plate. The implantation of the scaffold after removing the bone was expected to stabilize the injury site and deliver the IGF-I encapsulated in the scaffold for cartilage regeneration. The measurement of the MPTA at the end of the treatment showed that there was no significant correction of the

angle observed in the site of scaffold implantation. Future studies must involve a larger sample size and possible improvements in the animal model.

5.4.3 Histology

The images obtained from the tissue sections showed that presence of regenerated cartilage in the cases of the limbs which were implanted with IGF-I-encapsulated scaffolds. This regenerated cartilage was not observed in the case of the controls (animals without implant after induction of growth plate injury) and animals implanted with blank scaffolds in the site of growth plate injury. The regenerated cartilage was observed to be present in the medial half of the tibial growth plate region where the growth plate injury was induced and IGF-I scaffold was placed. The presence of cartilage at this region serves as the evidence for the effect of IGF-I encapsulated scaffold on the regeneration of cartilage at the site of injury. When compared with the native growth plate, however, it was observed that the regenerated growth plate was not identical in its morphological structure with the native growth plate. The chondrocytes in the native growth plate were stacked whereas the chondrocytes present in the regenerated cartilage were not place in a stacked columnar fashion. The chondrocytes were separate from each other and were distributes individually in the extracellular matrix. The IGF-I encapsulated scaffold had random pores distributed homogenously throughout the scaffold. The cells might have entered these pores and started to multiply and differentiate with the aid of the IGF-I that is being released from the scaffold. The random distribution of the cells within the pores of the scaffold might have been the reason for the random organization of the chondrocytes in the regenerated growth plate.

Both the tibias in a rabbit were used for creating injury and used as either control or implanted with blank or IGF-I encapsulated scaffold. This model used for the surgery might have affected the recovery of rabbits as continuous load was applied on both the injury site. This might have also been the reason for the lack of improvement seen in the defective bone angle in spite of the regeneration of the cartilage at the injured site. Previous work done with IGF-I plasmid releasing PLGA scaffolds showed similar type of results with regeneration of chondrocytes at the site of injury without complete correction of the defect in the bone angle. A higher molecular weight

PLGA can be used in future studies to provide prolonged support to the damaged bone and obtain even better tissue regeneration.

6. CONCLUSION

The main aim of this research was to develop a biodegradable drug delivery device for the treatment of the growth plate injury. The regeneration of the growth plate cartilage in the site of injury has confirmed that the IGF-I was successfully released from the porous PLGA scaffold and induced the formation of chondrocytes and extracellular matrix. The release studies showed that microencapsulation of IGF-I was a suitable method for storage of the drug within the scaffold that is going to be released. The release studies and the in vitro cell studies confirmed that the device was capable of releasing the drug in a required pattern and bioactive state. The IGF-I encapsulated porous PLGA scaffold implanted in the rabbit model for treatment of growth plate injury has successfully regenerated cartilage at the site of injury, even though it was not effective in correcting the defect in the bone angle that was caused due to the injury. The results of this research has shown that using porous PLGA scaffolds with IGF-I encapsulated in the form of microspheres will serve as an appropriate drug delivery system for the cartilage regeneration. This work will be a significant step forward in the tissue engineering of growth plate cartilage.

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