ORIGINAL RESEARCH PAPER
VOLUME-6 | ISSUE-5 | MAY - 2017 | ISSN No 2277 - 8179 | IF : 4.176 | IC Value : 78.46
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

Evaluating the osteogenic potential of CHT/HAP/PCL biocomposites in bone tissue engineering: *An in vivo* study

Orthopaedics

Krishan Gopal Jain Stem Cell Facility, All India Institute of Medical Sciences, New Delhi, India
Manisha Singh Stem Cell Facility, All India Institute of Medical Sciences, New Delhi, India
Anupama Kakkar Stem Cell Facility, All India Institute of Medical Sciences, New Delhi, India
Rajesh Malhotra Department of Orthopaedics, All India Institute of Medical Sciences, New Delhi, India
Vineeta Batra Department of Pathology, G. B. Pant Hospital, New Delhi, India
Alok Ray Centre for Biomedical Engineering, Indian Institute of Technology, New Delhi, India
Balram Airan Department of Cardiothoracic Vascular Surgery, All India Institute of Medical Sciences, New Delhi, India
Sujata Mohanty Stem Cell Facility, All India Institute of Medical Sciences, New Delhi, India

ABSTRACT

This study was performed to investigate about the osteo- conductive nature of PCL, HAP and CHT, when transplanted at the ectopic site in Wistar rats. Biocomposite materials were prepared and characterized as per our previously reported study. Three study groups were planned: a) Scaffolds without hBMSC, (b) Scaffolds with undifferentiated hBMSC and (c) Scaffolds with hBMSC primed into bone forming cells (osteocytes). These scaffolds were implanted at ectopic site in Wistar rats and studied for osteogenesis at 2, 4 and 6 weeks of implantation by X-ray imaging and immunohistochemistry analysis. It was observed that maximum mineralization was there in implanted scaffolds with osteoinduced scaffolds, followed by those in first and second group. There was increase in bone spicules formation and decrease in inflammatory cells over a period of time. Implanted scaffolds have osteoinductive properties and may have scope in bone tissue engineering. For this, transplantation studies need to be performed in bone defect animal model.

KEYWORDS:

Bone tissue engineering, biocomposites, osteoinductive scaffolds

Introduction:

With the increasing incidences of diseases related to bone tissue and accidental cases, there is an emerging need of developing bone substitutes as a part of bone tissue engineering to help in bone regeneration. Developing countries like India, where medical facilities are not available readily to everyone, especially in remote areas, bone tissue engineering may prove to be worthwhile. Developing scaffolds using biocomposites for bone regeneration will not only make the treatment available off the shelf, but also help in faster recovery of the patient.

There are various types of osteo-conductive and osteo-inductive materials available which are FDA approved and hence, can be used for medical purpose. Some of the examples are polycaprolactone (PCL), hydroxyapatite (HAP), chitosan (CHT), etc. These substances, if blended in appropriate ratio (called biocomposite), may lead to the concurrence with the properties of the natural bone tissue. Some of the important properties of the biocomposite that should be kept into consideration while preparing any scaffold for bone tissue engineering are pore size, porosity, mechanical properties like compressive strength and compressive modulus, biodegradability, biocompatibility, cell attachment and osteogenic potential (1-4).

There have been lots of compositions proposed by various researchers for treating bone defects. A thorough literature review led us to study a variety of compositions of these biomaterials having maximum similarities with the natural bone.

We have mixed polycaprolactone, hydroxyapatite and chitosan in various ratios and prepared the scaffold using freeze dry method; and studied for all the above mentioned properties and found that 25CHT/HAP/PCL (25 parts per hundred resin filler and HAP AND PCL in the ratio of 1:1) showed better biochemical and osteoinductive properties (4). 25CHT/HAP/PCL scaffold was selected for *in vivo* studies on the basis of the *in vitro* characterization and biocompatibility studies.

To validate our *in vitro* findings further, we performed animal experiments, wherein we transplanted the biocomposite scaffold (25CHT/HAP/PCL) at the ectopic or sub- cutaneous site of Wistar rats. The outcome was analysed on the basis of biocompatibility, biodegradability, bone tissue forming capacity of the implanted scaffold and immune response by the host at the site of implantation.

Materials and Methods:

All the experiments and procedures were performed after obtaining ethical clearance from Institutional Committee for Stem Cell Research and Institutional Animal Ethics Committee, AIIMS, New Delhi.

Scaffolds Preparation for In Vivo Studies

For *in vivo* study we chose 25CHT/HAP/PCL biocomposite scaffold because of its superior mechanical strength, optimum pore size and porosity, required degradability, cell biocompatibility, cell proliferation and osteo-inductivity over other scaffolds compositions. Scaffolds with dimensions of 10 mm in diameter and 2 mm in thickness were used for the implantation. Scaffolds of group 1 were immersed in cell expansion medium (DMEM-LG medium with 10% FBS and Penstrep) for 14 days. Scaffolds of group 2 were seeded with a suspension of hBMSCs (1×10^5/20 µl/disc) and cultured for 14 days. Group 3 was seeded with a suspension of hBMSCs (1×10^5/20 µl/disc) and cultured in osteogenic medium for 14 days (4). After having been cultured, the scaffolds were washed in sterile PBS three times before implantation (Figure 1).

Surgical Procedure and Scaffold Implantation

Surgery was performed three days after the beginning of

immunosuppressive therapy. Rats were anaesthetized with intra-peritoneal injections of ketamine (140mg/Kg) and xylazine (7 mg/Kg). The operational skin area was shaved and disinfected with povidone-iodine solution (10% w/v, Johnson & Johnson, India). One longitudinal incision was made in the midline of the dorsal skin exposing the connective tissue. A pocket was made by blunt dissection in subcutaneous tissue, and scaffolds with or without cells, were immediately implanted into the pockets. Each animal received one implant of a specific group described above. Three samples were implanted for each implantation time and a total of 3 animals for each group were studied. Subsequently, skin wounds were closed with resorbable sutures (Mersutures 4-0, Ethicon, Johnson & Johnson, India) and the surgical area was submitted again to asepsis. The rats were housed single after surgery and received humane care. The rats were euthanized with overdosed pentobarbital (100 mg/kg) to harvest the implanted scaffolds 2, 4 and 6 weeks after surgery. Implants were retrieved with surrounding tissues and used further for histology. All surgical procedures were performed under aseptic conditions using dual access animal handling station (ESCO, Singapore) (Figure 2 & 3).

Histological analysis and mineral detection

Scaffolds removed from the back of the rats 2, 4 and 6 weeks after implantation were immediately photographed and assessed for opacity and formation of any bone like tissues radiographically. Retrieved implants were fixed in 10% formalin, dehydrated in ascending series of alcohol and embedded in paraffin. Tissue blocks were sectioned at 5 μm thickness, positioned on glass slide and stained by hematoxylin and eosin (H&E). The stained sections were observed under optical microscope and analysed to evaluate host tissue response in terms of inflammation, fibrosis, necrosiss, vascularisation, scaffold degradation and tissue ingrowth in scaffolds (5). The specimens were analyzed by a pathologist blinded to the identity of each specimen.

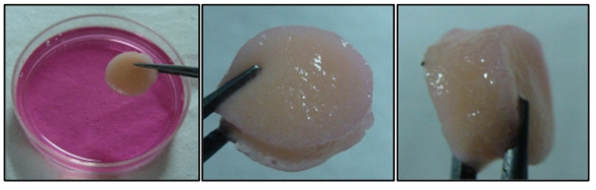

Figure 1: Digital images of the 25CHT/HAP/PCL biocomposite scaffold before implantation.

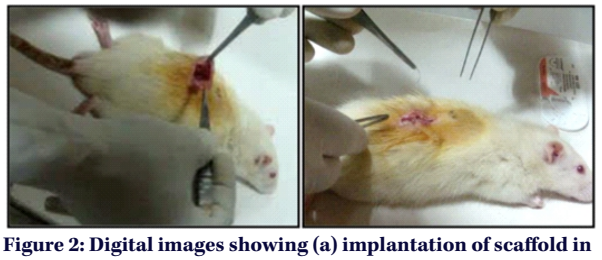

Figure 2: Digital images showing (a) implantation of scaffold in rat model at ectopic site (sub- cutaneously) and (b) closing of the wounded skin by suture after scaffold implantation.

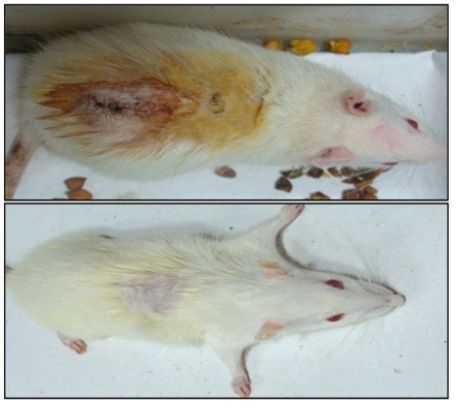

Figure 3: Digital images showing wound healing after (a) 2 hours (b) 2 weeks of scaffold implantation

Radiographic Imaging:

Ossification in the implanted scaffolds was studied by X-ray imaging. Wistar rats were anesthesized using ketamine and xylazine, placed in the dark chamber of IVIS machine (Perkin Elmer, USA) and X- ray imaging was done.

Results:

Animal Sacrificing and extraction of implanted scaffolds:

On termination of various time points, i.e., 2 weeks, 4 weeks and 6 weeks, respectively, the implanted scaffolds were extracted from the ectopic site and processed further for X- ray and histology studies. All the rats were observed under *in vivo* imaging system (IVIS) machine to monitor mineralization in the implanted scaffolds (Figure 4). X-ray studies of the extracted scaffolds show mineralization of the scaffolds (Figure 5 & 6). Both the studies show that mineralization at the scaffold site started at 4 weeks of implantation. Hematoxylin and Eosin staining also show formation of bone spicules in the scaffolds. The control group in which no cells were seeded on the scaffold, also showed basal level of calcification.

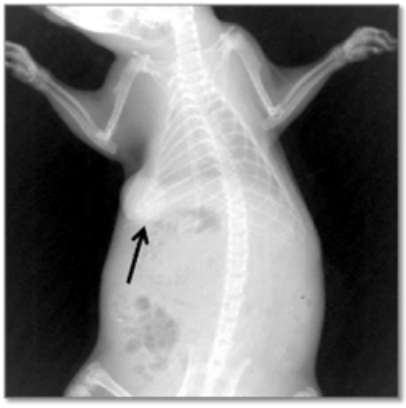

Figure 4: Radiographic image, taken from IVIS machine, showing mineralization of scaffold after implantation before sacrificing the rats for histological examination

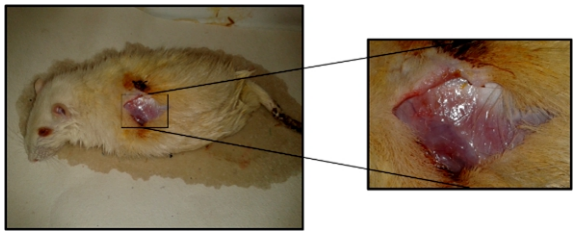

Figure 5: Digital images showing extraction of scaffold from the ectopic site of implantation

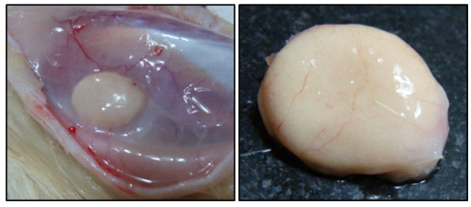

Figure 6: Digital images showing (a) implanted scaffold in the dorsal subcutaneous tissue (b) implant harvested with surrounding tissue

Mineral detection of the implants

Determination of mineral formation and deposition in the implanted scaffolds was done by using radiographs of the retrieved implants. All biocomposite scaffolds showed opacity from week 2 because of its

HAP content. Radiographic surveys of the implants showed that calcification occurred in the scaffolds after 4 weeks and increased with the duration of implantation up to 6 weeks. Control implants (without hBMSCs) also demonstrated increase in mineral density with increase in implant duration. The radiographic appearance of the implant was consistent with the histological findings (Figure 7).

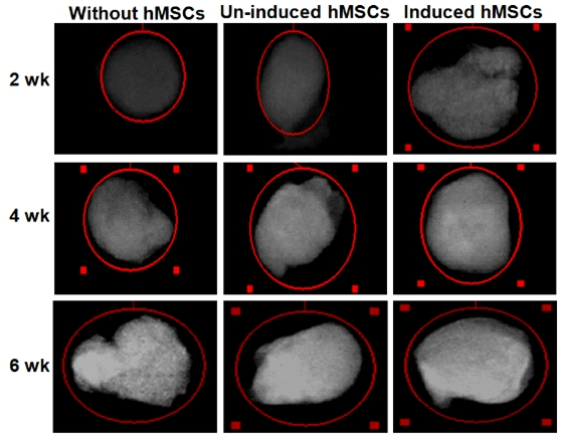

Figure 7: Radiographic images of implants harvested after at 2, 4 and 6 weeks, showing mineralization in the scaffolds. The mineralization started after 4 weeks of implantation and was observed more in case of scaffolds seeded with hBMSCs

Histological evaluation

Figure 8 shows host tissue response to the biocomposite scaffolds with or without cells after 2, 4 and 6, weeks of implantation. It was observed that neutrophils migrated from blood into the scaffolds. The formation of blood vessels was clearly seen at week 2.

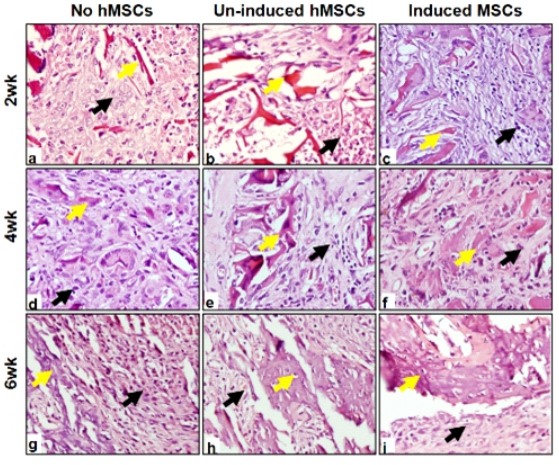

Figure 8. Photomicrographs of biocomposite scaffolds implanted in rat subcutaneous tissue (original magnification ×20). (a, b, c) Two weeks after the implantation; yellow arrow- chitosan, black arrow- inflammatory cells (d, e, f) Four weeks after the implantation; yellow arrow- bone spicules, black arrow- inflammatory cells (g, h, i) Six weeks after the implantation; yellow arrow- bone spicules, black arrow- inflammatory cells (a, d, g) Scaffolds not colonized by cells (control group); (b, e, h) scaffolds colonized by undifferentiated hBMSC; (c, f, i) scaffolds colonized by differentiated hBMSC.

Histological Analysis after two weeks

After 2 weeks of implantation, all the implants (with and without hBMSCs) were surrounded by a thin fibrous capsule. Histological examination of control group (scaffolds without hBMSCs) implants showed poorly vascularised fibrous connective tissue filled with inflammatory cells (macrophages and lymphocytes) and few foreign body giant cells surrounding the implant and empty spaces in the centre of the scaffolds. In the peripheral regions fibrosis was identified and no osteoid like tissue formation was observed. The same occurrence was observed in cell seeded groups (scaffolds Group 2 & Group 3) (Figure 8 a,b,c).

Histological Analysis after four weeks

After 4 weeks of implantation, all the implants were also surrounded by a thin fibrous capsule. Histological examination of control group (scaffolds without hBMSCs) implants showed bony spicules (yellow coloured arrows) interspersed with collagen fibers. The bone spicules showed calcium deposition and closely related osteoclasts and osteoblasts.

In study groups with hBMSCs (osteo- induced and uninduced), bony spicules were seen surrounded by osteoclasts and osteoblasts. Some foreign material was also present surrounded by foreign body giant cells. All groups showed fibrous connective tissue with inflammatory cells (macrophages and lymphocytes) and few foreign body giant cells surrounding the implant and empty spaces in the centre of the scaffolds (Figure 8 d,e,f).

Histological Analysis after six weeks

After 6 weeks of implantation, the appearance of the fibrous capsule around the implants had not changed as compared with the three week implants. Histological examination of control group (scaffolds without hBMSCs) implants showed bony spicules (yellow coloured arrows) interspersed with collagen fibers. The bone spicules showed calcium deposition and closely related osteoclasts and osteoblasts.

In group with uninduced hBMSCs, bony spicules were seen bordered by collagen, fibrosis and dense chronic inflammatory cells infilterate. The amount of bone like tissue slightly increased from 4 to 6 weeks. The same occurrence was observed in group with osteo- induced hBMSCs. Well formed bone spicules were present. The amount of connective tissue increased and filled the pores of scaffolds. Inflammatory cells along with foreign body giant cells were also observed (Figure 8 g,h,i).

Thus, from our present study, we may conclude that after 4 weeks of scaffolds implantation, all study groups showed meagre level of bone formation reaching around 10-15% of the implant area. At 6 weeks, the bone formation increased to 30- 40% in each group. Bone formation or mineralization was least in the group where scaffolds were seeded without hBMSC.

Discussion:

The results of *in vitro* comparative studies of all the scaffolds showed that the novel 25CHT/HAP/PCL biocomposite scaffold had good mechanical strength, optimum pore size and porosity, required degradability, cell biocompatibility, cell proliferation and osteo-inductivity (4). Therefore, we used this biocomposite scaffold for in vivo studies to evaluate the host tissue response, degradation and bone tissue formation. In this study, we standardized the implantation of hBMSCs seeded scaffolds in immunosuppressed Wistar rat model for the study of ectopic bone formation. In previous studies nude mice were used for the xenogenic transplantation (6,7). However, because of limited settings, we could not use nude mice, therefore we have used immunosuppressed Wistar rats in this study.

The results of radiological evaluation were consistent with histological findings. All acellular implants had lower radiological opacity than cellular implants, suggesting that newly formed bone like tissue contributed to the increasing mineral densities. Our results are in line with the findings of previous studies (8,9).

Histopathological results showed an acute inflammatory reaction in all groups studied. The inflammatory reaction may be attributed to the immune response of the rat immune system against xenogenic hMSCs. However, other authors have also reported a chemotactic effect of chitosan on neutrophils (10,11). Pamela et al. reported that, neutrophilic migration to the material was due to the specific

interactions of chitosan or its oligosaccharides with neutrophil receptors such as the selectins (12). A large number of macrophages, neutrophils and few foreign body giant cells infiltrate the implants and were observed throughout the study period. However, it did not prevent the osseous tissue formation.

Regardless of the experimental group, the invasion of tissue starts in the border of the scaffold toward its centre. This tissue was made up of connective fibers, mononucleated cells and multinucleated giant cells. In the implant groups colonized by cells, some cells with aspects of necrosis could be observed in the central area.

In implants, harvested after 2 weeks well-vascularized fibrous connective tissue and inflammatory cells were observed. Few multinucleated giant cells were also present. No bone like tissue formation was identified. After 4 weeks, osteoid formation was observed in all implants seeded with or without hMSCs. The bone spicules were found interspersed with collagen in the central area of the implants and were bordered by osteoclasts and osteoblasts. In group 2 and group 3 implants, some foreign material surrounded by foreign body giant cells was also observed which could not be identified.

In vivo bone formation involves recruitment of osteo-progenitors by chemotaxis and their subsequent proliferation and differentiation into osteoblasts (13). Since, CHT and HAP are osteoinductive and osteocoductive materials, respectively; it may be possible that biocomposite scaffolds had recruited circulatory osteoprogenitor cells from the host. In a previous study it was shown that bioceramics, can induce bone formation without prior cell seeding. It was suggested that this strategy could only be used for small defects, where a small amount of circulating MSCs and osteoprogenitors would be needed (14). This technique would not be successful in the case of large bone defects, due to the high number of those cells that would be needed (15). Our results also showed that the amount of bone like tissue was less in group1 implants as compared to group 2 and group 3 implants. However, these findings are in contrast to the recent study of Gomide et al., where they showed no osteoid tissue formation in scaffold implanted without and with un-induced cells (16). By observing histological sections, we found that most of the scaffolds biodegraded after 4 weeks, which results from the biodegradable component of CHT, and it is useful to blood vessels and new tissues in growth. Moreover, the surrounding host tissue had no inflammation, which shows the scaffold in itself and degradation products are all nontoxic. In addition, most of the scaffolds were covered by collagen, suggesting the scaffolds had osteoinduction to some extent due to the CHT content of composite scaffolds.

After 6 weeks, the implants showed increase in the amount of osseous tissue formed as compared to tissue formed in 4 weeks. The implant group with differentiated MSCs showed broad spicules as compared to other two groups. Group 3 showed 40% of its area filled by bone spicules lined with osteoblasts and osteoclasts. All the bone spicules were surrounded by well vascularised denser connective tissue. In central area more empty spaces were observed which may be produced by the scaffold degradation and were not completely filled by the connective tissue.

This study is a prospective one towards bone tissue engineering. While we have proposed the newer and convenient method of scaffold preparation, i.e., freeze drying method in our previously reported study, we have also compared various compositions of CHT/HAP/PCL to yield best scaffold in terms of maximum similarity with the natural bone properties (4). This study leads to further research prospects in the area of bone tissue engineering. More blends using biocomposites can be devised using the composition reported by us for treating large bone segmental defects in future. Scaffolds with better mechanical strength, biodegradability and biocompatibility can be formed, taken clues from our present study. Also, the compatibility and usage of these scaffolds for large bone segmental defects have to be established by expanding this study further. In developing countries like India, where the cases of accidents and traumatic bone injury are increasing and the treatment is not readily available, our current study will help in developing off- the shelf therapy for such bone diseases, which is easy and economical to prepare.

Acknowledgement:
This study was supported by Indian Council of Medical Research (ICMR) and Department of Biotechnology, Government of India.

References:

1. Anto´nio J. Salgado, Olga P. Coutinho, Reis RL: Bone Tissue Engineering: State of the Art and Future Trends. Macromol Biosci 2004, 4:743-765.
2. Aylin Sendemir-Urkmez, Russell DJ: The addition of biphasic calcium phosphate to porous chitosan scaffolds enhances bone tissue development in vitro. Inc J Biomed Mater Res 2007, 81A:624-633.
3. Aurelio Salerno, Daniela Guarnieri, Maria Iannone, Stefania Zeppetelli, Netti PA: Effect of Micro- and Macroporosity of Bone Tissue Three-Dimensional-Poly(e-Caprolactone) Scaffold on Human Mesenchymal Stem Cells Invasion, Proliferation, and Differentiation In Vitro. Tissue Engineering: Part A 2010, 16(8):22661-22673.
4. Parhi. Purnendu, A. Ramanan, Alok RR: Nanocrystalline Powders of Alkaline-Earth Phosphates as Precursors for Bioceramics. American Journal of Biochemistry and Biotechnology 2006, 2(2):61-65.
5. Krishan G. Jain, Sujata Mohanty, Alok R. Ray, Rajesh Malhotra & Balram Airan. Culture & differentiation of mesenchymal stem cell into osteoblast on degradable biomedical composite scaffold: In vitro study. Indian J Med Res 142, December 2015, pp 747-758.
6. Shweta Sharma, Radhika Tandon, Sujata Mohanty, Seema Kashyap, Murugesan Vanathi. Phenotypic Evaluation of Severely Damaged Ocular Surface after Reconstruction by Cultured Limbal Epithelial Cell Transplantation. Ophthalmic Res. 2013;50(1):59-64. doi: 10.1159/000346868.
7. Sergei A. Kuznetsov, Paul H. Krebsbach, Kazuhito Satomura, Janet Kerr, Mara Riminucci, Dafna Benayahu, Robey PG: Single Colony Derived Strains of Human Marrow Stromal Fibroblasts Form Bone After Transplantation In Vivo. J Bone Miner Res 1997, 12(9):1335-1347.
8. Eun J. Gang, Darko Bosnakovski, Camila A. Figueiredo, Jan W. Visser, Perlingeiro RCR: SSEA-4 identifies mesenchymal stem cells from bone marrow. Blood 2007, 109:1743-1751.
9. Weinand C, Gupta R, Huang AY, Weinberg E, Madisch I, Qudsi RA, Neville CM, Pomerantseva I, Vacanti JP: Comparison of hydrogels in the in vivo formation of tissue-engineered bone using mesenchymal stem cells and beta-tricalcium phosphate. Tissue Eng 2007, 13(4):757-765.
10. Hattori H, Sato M, Masuoka K, Ishihara M, Kikuchi T, Matsui T, Takase B, Ishizuka T, Kikuchi M, Fujikawa K et al: Osteogenic potential of human adipose tissue-derived stromal cells as an alternative stem cell source. Cells Tissues Organs 2004, 178(1):2-12.
11. Usami Y, Okamoto Y, Minami S, Matsuhashi A, Kumazawa NH, Tanioka S, Shigemasa Y: Migration of canine neutrophils to chitin and chitosan. J Vet Med Sci 1994, 56:1215-1216.
12. Kosaka T, Kaneko Y, Nakada Y, Matsuura M, Tanaka S: Effect of chitosan implantation on activation of canine macrophages and polymorphonuclear cells after surgical stress. J Vet Med Sci 1996, 58:963-967.
13. Pamela J. VandeVord, Howard W. T. Matthew, Stephen P. DeSilva, Lois Mayton, Bin Wu, Paul HW: Evaluation of the biocompatibility of a chitosan scaffold in mice. Inc J Biomed Mater Res 2002, 59:585-590.
14. Alonso M, Claros S, Becerra J, Andrades J: The effect of type I collagen on osteochondrogenic differentiation in adipose-derived stromal cells in vivo. Cytotherapy 2008, 10(6):597-610.
15. Anto´nio J. Salgado, Olga P. Coutinho, Reis RL: Bone Tissue Engineering: State of the Art and Future Trends. Macromol Biosci 2004, 4:743-765.
16. Perka C, Schultz O, Spitzer RS, Lindenhayn K, Burmester GR, M S: Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. Biomaterials 2000, 21(11):1145-1153.
17. Gomide VS, Zonari A, Ocarino NM, Goes AM, Serakides R, Pereira MM: In vitro and in vivo osteogenic potential of bioactive glass-PVA hybrid scaffolds colonized by mesenchymal stem cells. Biomed Mater 2012, 7(1):1-10.