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ORIGINAL ARTICLE

How is Biodegradable Scaffold Effective in Gap Non-union? Insights from an Experiment

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Abstract

Objective To evaluate the role of composite (Chitosan/Chondroitin sulphate/gelatin/nano-bioglass) scaffold in the union of critical size bone defect created in the rabbit's ulna.

Methods The composite (Chitosan/Chondroitin sulphate/gelatin/nano-bioglass) scaffold was fabricated using the freeze-drying technique under standard laboratory conditions. The scaffold was cut into the appropriate size and transferred into the defect created (critical bone size defect 1 cm) over the right ulna in the rabbit. The scaffold was not implanted on the left side thus the left side ulna served as control. Results were assessed on serial radiological examination. Rabbits were sacrificed at 20 weeks for histopathological examination (Haematoxylin–Eosin staining and Mason's trichrome staining) and scanning electron microscope observation. Radiological scoring was done by Lane and Sandhu's scoring.

Results Among 12 rabbits, 10 could complete the follow-up. Among those 10 rabbits, 8 among the test group showed good evidence of bone formation at the gap non-union scaffold implanted site. Histological evidence of new bone formation, collagen synthesis, scaffold resorption, minimal chondrogenesis was evident by 20 weeks in the test group. Two rabbits had poor bone formation.

Conclusion The chitosan-chondroitin sulphate-gelatin-nano-bioglass composite scaffold is efficient in osteoconduction and osteoinduction in the gap non-union model as it is biocompatible, bioactive, and non-immunogenic as well.

Keywords Tissue engineering · Nano-bioglass scaffold · Gap non-union model · Osteogenesis · Composite substitute · Biodegradable

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Introduction

Since the time of Hippocrates and Galen bone has been studied for its ability to self –regenerate [1]. Large bone defects as a result of trauma, infection, tumour excision, congenital malformation, stress shielding in prosthesis often require bone transplantation. Bone grafting is one of the most commonly performed surgeries accounting for half a million procedures per year within the United States and more than 2 million in the world. Although autograft is associated with donor site morbidity and limited availability, it is most often treated as gold-standard in these procedures because of its ideal bone graft characteristics [2–4].

Rarely, large bone defect heal by itself, moreover, critical size defect is defined as, the minimal length /segment which fails to unite by itself in a lifetime [5]. These bone defects can be filled by many different materials such as autograft, allograft, xenograft, or bone substitutes.

Ideal bone graft material to fill these defects should be osteoconductive, osteoinductive, biocompatible, bioresorbable, mechanically resistant, and easily available to use. It should be cost-effective to make it widely available. The sole substitute to fulfill all specifications is autologous bone but, it has its own share of disadvantages of donor site morbidity and limited availability [6, 7]. Although allograft had emerged as a suitable alternative to autograft, its use got limited due to potential risk of infection, limited osteoconduction, laborious harvesting & preserving procedures and necessity of a bone bank [2].

Bone substitutes which are defined as "synthetic, inorganic or biologically organic combinations can be implanted for the treatment of a bone defect instead of autogenous or allogenous bone." [8–10]. However, the majority of the available materials are osteoconductive but very few are osteoinductive. Few Bone substitutes in current use are demineralized bone matrix, Hydroxyapatite (HA), corals, β-tri-calcium phosphate (β-TCP) (Ca3 (PO4)2), Biphasic calcium phosphates (HA and β-TCP ceramics), calcium sulphate (CaSO4), Calcium phosphate cements (CPCs), bioactive glasses, Polymer-based bone substitutes [8].

Very recently composite substitutes have given promising results in animal trials as an emerging field of regenerative medicine and tissue engineering. Composite substitutes are the one with a combination of more than one biomaterial which add their advantages synergistically [2].

In this study, we are presenting our results of a composite (CH/CS/G/nBG) material as a scaffold to fill an artificially created bone gap (more than critical size defect) in an experiment on the rabbit and decipher its efficacy in being osteoconductive, osteoinductive and bio-compatible.

Materials and Methods

The present study was conducted in the Department of Orthopaedics, School of Biochemical Engineering, Centre of Experimental Medicine and Surgery from November 2016 to July 2018. Approval of CPCSEA was obtained before the start of the experiment.

4 months old healthy white 12 male adult rabbits of average 2–3 kg weight were chosen and left for 1 week before starting the experiment. The principle of laboratory care, feeding, and sacrifice was followed as per ICMR guidelines on the care of experimental animals.

Characteristics and Preparation of Scaffold

To prepare a scaffold, the biomaterial used should be non-toxic, easily available, biodegradable, and non-immunogenic. Chitosan, chondroitin sulphate, gelatin, nano-bioglass (CH/CS/G/nBG) scaffold was chosen and fabricated using Freeze-drying technique. Briefly, the nBG nanocrystal powder was dispersed in a CH/G/CS solution by sonication. The produced composite mixture was put in a Teflon vial to be sonicated again and then transferred into a freezer maintained at – 20 ℃, which induced solid–liquid phase separation. Separated water was replaced with the gelatin to enhance the mechanical strength of the scaffold. The solidified mixture was kept at that temperature for 12 h and then transferred into a freeze-drying container maintained at – 40 ℃. The samples were then freeze-dried for 2–3 days under vacuum (0.5 mmHg). Finally, the obtained scaffolds were cross-linked and used for in-vitro and in-vivo study for new bone tissue regeneration [11, 12].

Transplantation

The definitive procedure for implantation of the composite scaffold (Fig. 1) in rabbits was performed under anaesthesia using an I/M injection of titrated doses of ketamine and midazolam. In each rabbit, both forearms were shaved and disinfected with spirit and betadine. Defect (critical bone size defect 1 cm) was artificially created in the midshaft of the bilateral ulna. The periosteum at the defect site was removed to mimic clinical non-union and to avoid the formation of synostosis (Figs. 2 and 3). The 3-dimensional composite scaffold was cut into the appropriate size and transferred into the defect (critical bone size defect 1 cm) over the right ulna, namely the test side (Fig. 4). The scaffold was not implanted on the left side thus the left side ulna served as control. Intact radius served as an internal splint for the press-fit scaffold on the test side, while in controls it helped in bearing weight partially. Both the forearm wounds were closed in layers.

Follow-up of rabbits was done after a period of 4, 8, 12, and 20 weeks by radiographic examination (Table 1). Histopathological examination was done after sacrifice at 20 weeks by H&E and Mason's trichrome staining (to assess collagen content in the matrix formed). Scanning Electron

Fig. 1 Composite scaffold

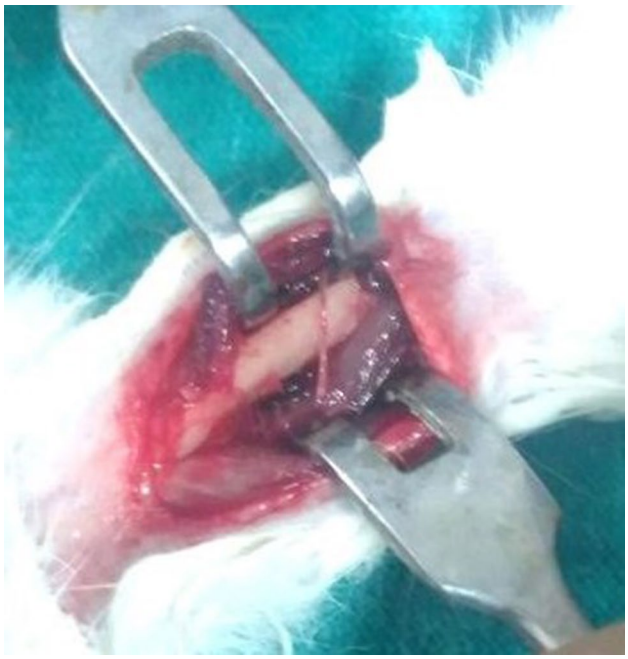

Fig. 2 Exposure of the ulna

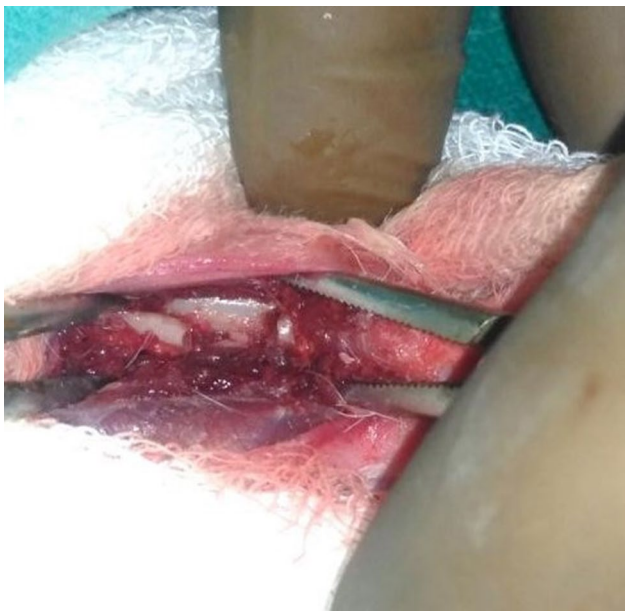

Fig. 3 Creation of critical bone defect

Microscope examination was done to look for scaffold integration, scaffold porosity, colonization, and growth of osteoblast by the end of 20 weeks.

Statistical Analysis

Descriptive statistics was applied for quantitative data, to test for normality of data, a Shapiro–Wilk test was applied.

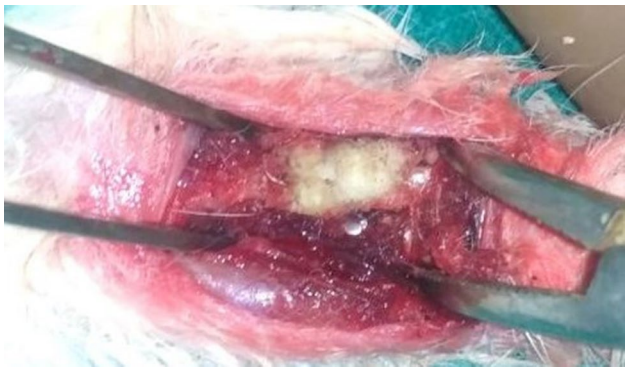

Fig. 4 Scaffolds implanted at defect site

Table 1 Number of rabbits which underwent radiological follow-up

Radiological follow up rabbits	Number of test limb (Right -implanted)	Number of control limb (Left)
4 weeks	10	10
8 weeks	10	10
12 weeks	10	10
20 weeks	10	10

Quantitative variables were evaluated by the student's two-tailed *t*-test amongst various subgroups (follow up period). p-value < 0.05 was taken as statistically significant.

Results

Among the 12 rabbits studied, 2 rabbits died in the immediate postoperative period. Clinical and histopathological examination of the fracture site did not show any signs of inflammation. The cause of death was concluded due to anaesthetic overdose or adverse drug reaction. 10 rabbits were subjected to radiological assessment as per Table 1 every 4, 8, 12, and 20 weeks by Lane and Sandhu's scoring (Table 2) [13].

At the end of 20 weeks, rabbits were sacrificed by giving lethal doses of intramuscular midazolam and subjected to gross and histopathological examination.

For histopathological examination, en-bloc resection of ulna containing bone defect site with or without scaffold was done (Figs. 5 and 6). Each specimen was decalcified and embedded in paraffin. Sections 4 µm thick were prepared and stained with haematoxylin and eosin stain and Mason's trichrome staining.

Among 10 rabbits, 8 rabbits showed good evidence of bone formation at the gap non-union site (Figs. 7 and 8). Table 3 showing union as per Lane and Sandhu scoring in test limb with a significant p-value. Histological

Table 2 Lane and Sandhu scoring for radiological assessment of bone union

Score	Interpretation
0	No evidence of new bone formation
1	Little amount of callus formation
2	All around the margin of the scaffold there is increase in the radio opacity: calcification
3	Formation of bridging mass in the created defect: increased radio density
4	Increase in the girth / density reaching up to the periphery of the defect

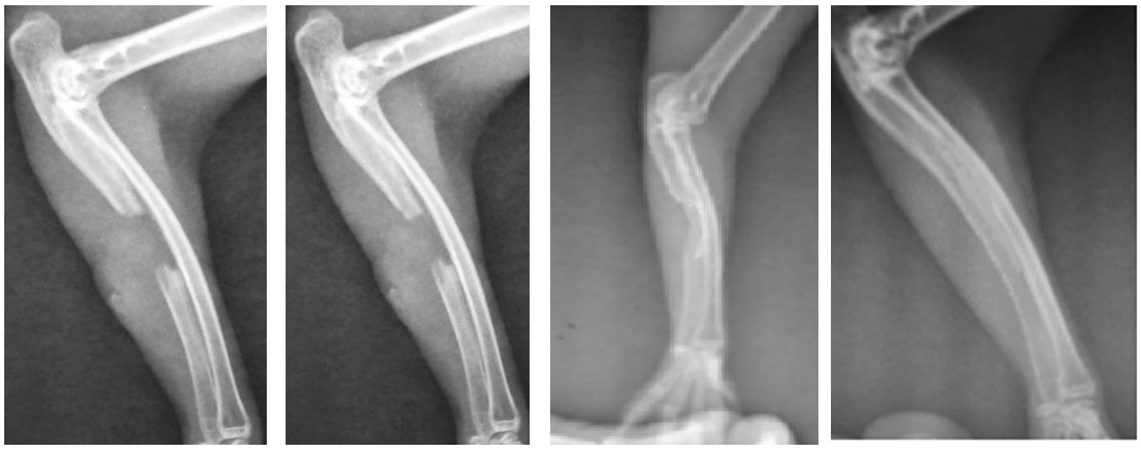

Fig. 5 Serial x rays at 0, 4, 8 and 12 weeks showing new bone formation at defect site in right ulna

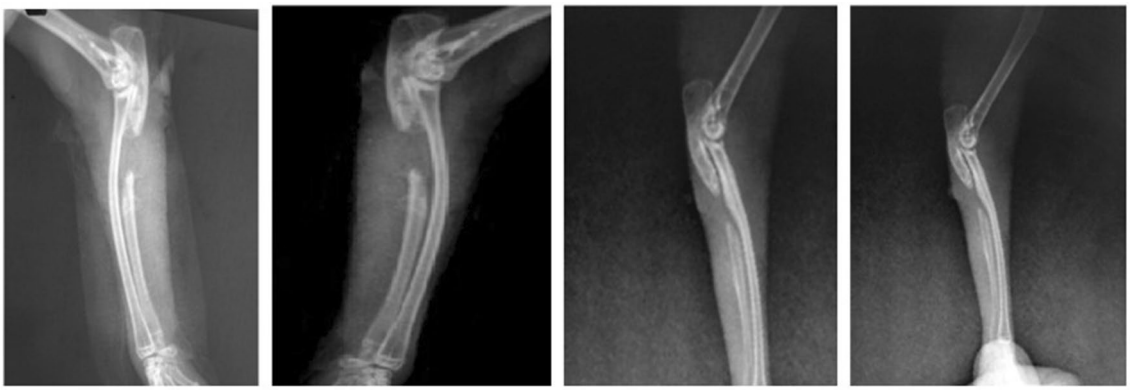

Fig. 6 Serial x rays at 0, 4, 8 and 12 weeks showing no bone formation at defect site in left ulna

evidence of new bone formation, collagen synthesis, scaffold resorption, minimal chondrogenesis, was evident by 12 weeks in the test group (Figs. 9a, b, 10a, b). Two rabbits showed poor bone formation.

Scanning Electron Microscope examination of the retained scaffold showed good integration between the scaffold and host bone and porosity of implant, cell adherence (Fig. 11).

Discussion

Even though autograft has been the gold standard for bone grafting procedures, it is an additional procedure for the patients. There are studies reporting donor site complications in up to 20.6% cases [6, 7]. Hence it has always been

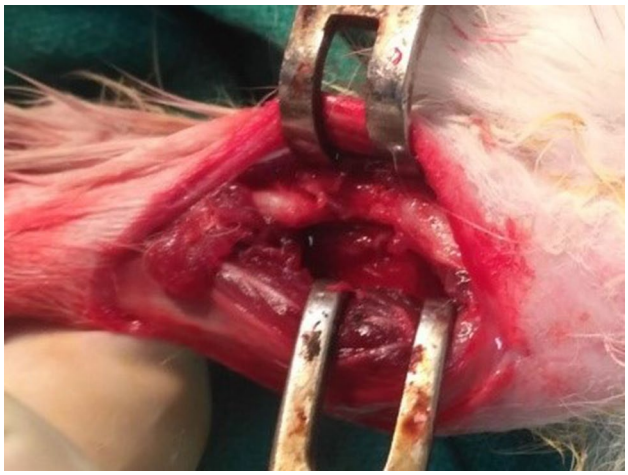

Fig. 7 Gross examination of bone defect of right side at 20 weeks

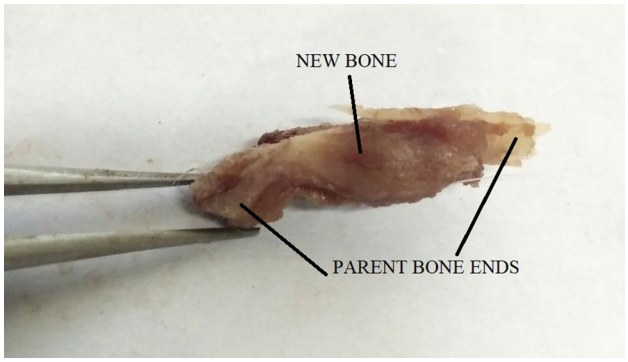

Fig. 8 En-block resection of right ulna containing bone defect site and adjacent bone

the source of inquisitiveness for the researchers to find a better alternative for it.

Ideal material for the scaffold to promote new bone growth and integration with host tissue should have the following properties. First, it should be conducive for rapid vascular growth. Second, since the radiological identification of newly formed bone is easier, scaffold material should be radiolucent. Third, the scaffold material should be self-resorptive to create space for new bone. Fourth, a composite scaffold combination should be pliable for handling in a clinical setting. Finally, the material should promote osteoconductive bridging between the host bone and the new bone.

The results of this experiment demonstrate that new-bone formation is elicited in critical-sized defects in the ulna of a rabbit by the implantation of a novel 3D biodegradable scaffold. Under the conditions utilized in this study, the implantation of the composite scaffold led to the formation of new bone. The new bone wasn't uniformly distributed throughout the cell–matrix implant but integrated comprehensively with the host bone. Gross examination of the specimen revealed that there was progressive resorption of the implant by 16 weeks. Previously, similar studies done using low or poor biodegradable implants did not show any change in the consistency of the implant even after 20 weeks of follow up, since none of the implants were biodegradable [14–16]. Hence the biodegradability of the implant was desired and advantageous which was observed in our study and the sequential resorption of the implant associated with a corresponding new bone formation appreciated radiologically (Fig. 5).

Moreover, during postoperative follow-up, there was no sign of any immunological reaction either during clinical or histological examination heralding the biocompatibility of the scaffold.

The observation in this study suggests that the presence of new bone formation at the defect site indicates the osteoinductive, osteoconductive, and osteogenic properties of the composite scaffold (CH/CS/G/nBG). Also, avoidance of secondary fixation of the scaffold to bone provides indirect evidence that the composite achieved stability by rapidly adhering to the non-uniting ends.

Among various biopolymers, chitosan (CH) has shown promising results in osteoconductive properties [17]. It is positively charged at physiological pH and requires anionic polymer to form a stable complex. Chondroitin sulphate (CS) is an anionic polysaccharide that can stabilize chitosan. It has been proved to possess anti-inflammatory and tissue regenerating properties [18, 19]. CS, also facilitates mineralization of the newly forming bone by being an anionic polymer in the composite attracting cationic calcium ions [20]. The gelatin in this study was obtained by partial hydrolysis of the collagen. It showed significantly decreased immunogenicity as compared to

Table 3 Average of Lane and Sandhu scoring in test and control limbs of rabbits

Mean Follow up	Test (Right ulna with scaffold) X ray score	Control (left ulna with bone gap) X ray score	*P*-value(student's 2-tailed *t*-test
4 weeks	1	0	> 0.05
8 weeks	2	0	> 0.05
12 weeks	2.5	1	0.012
20 weeks	3.5	1	0.002

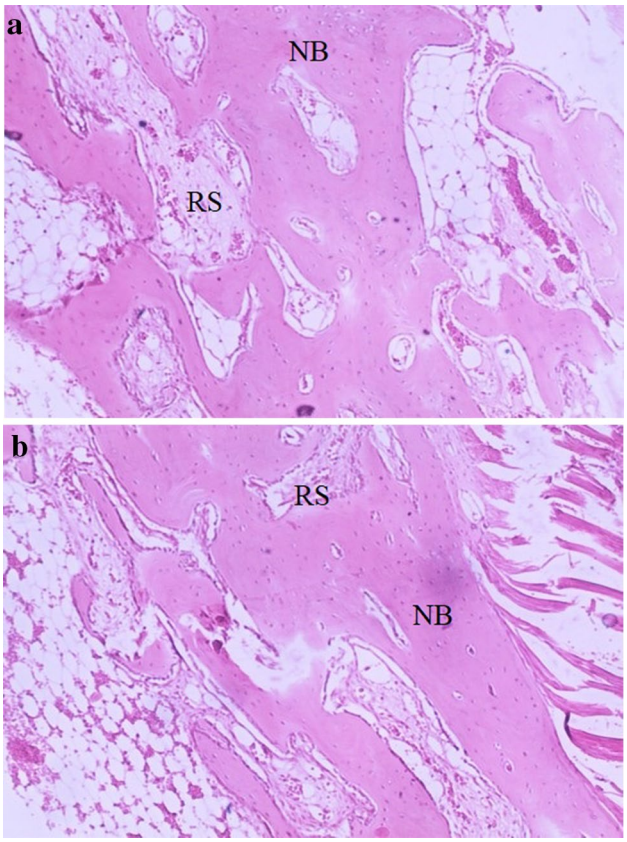

Fig. 9 **a**, **b** Bony trabeculae lined by osteoblasts along with presence of fatty marrow elements (NB- new bone, RS- resorbing scaffold) (Haematoxylin and eosin staining, 100 X)

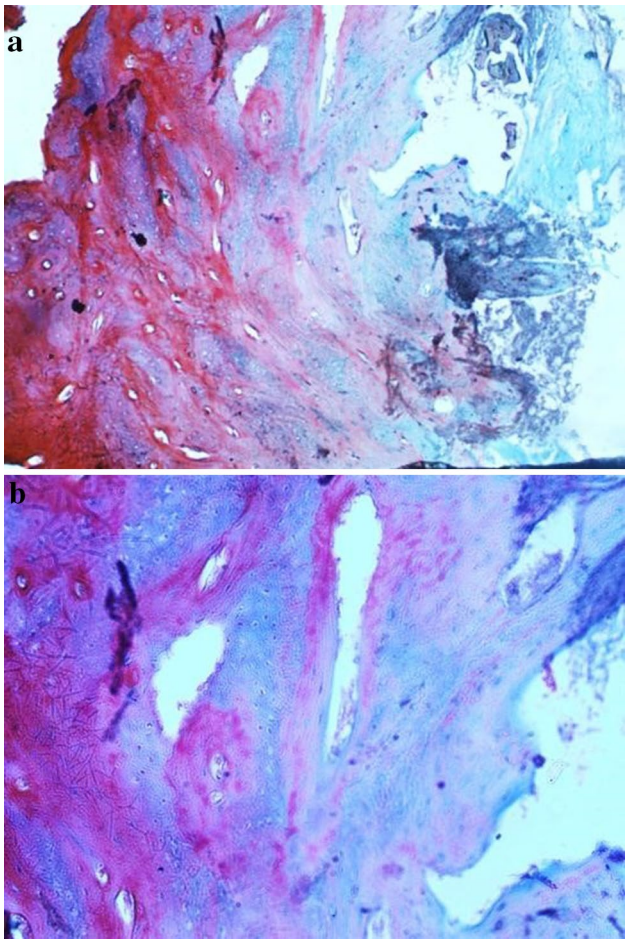

Fig. 10 **a**, **b** Showing newly formed bone cells and connective tissue (blue colour)—Masson's trichrome staining

collagen. Moreover, gelatin has been shown to provide skeletal support for cellular adhesion, migration, and proliferation [21].

Studies have demonstrated that nano-bioglass has more osteogenic potential [22, 23]. As nBG helps in attaining appropriate pore size, both microporous and macroporous by liquid–liquid phase separation, the present study describes a freeze-drying technique to fabricate polymer/nBG-composite scaffolds with high porosity and controlled pore architecture [12].

The chitosan-based scaffold is known to have decreased mechanical strength and structural stability. To overcome this limitation, referring to the study by Singh et al. the nBG incorporated into the composite scaffold through polyelectrolyte complexation (PEC) was subjected to phase separation. Separated water was replaced with gelatin enhancing the mechanical strength of the scaffold. This sequential processing, use of PEC during scaffold preparation, incorporation of nBG increased the compressive strength of the scaffold [11, 12, 24].

In one study, bioactive glass (BG) was mixed with a collagen solution to create a composite scaffold with or without phosphatidylserine (PS). Rat MSCs were used in this study. Results indicated PS promoted attachment and proliferation of MSCs in the scaffold. Alkaline phosphatase, osteocalcin, and osteopontin were secreted more by MSCs in the COL-BG-PS composite. Similar results were observed in the rat femur defect model showing better bone formation in rats with COL-BG-PS/MSC composite scaffold as compared to COL-BG/MSC, and cell-free COL-BG-PS scaffolds at the defect [25]. The result of this study suggested that the addition of PS to scaffold has a synergistic effect on bone formation in scaffolds containing stem cells with its own set of limitations in certain mechanical properties such as low strength, toughness, and reliability [26, 27].

The gelatin, bioglass, chitosan, chondroitin sulphate scaffold used in this study degrades slowly in the initial hours, and the rate of degradation increases with time [28, 29]. The composite scaffold has a three-dimensional architectural similarity to that of natural bone, thus the scaffold that we provide as our implant should help in the new bone formation in a better way. The aligned structure should help the

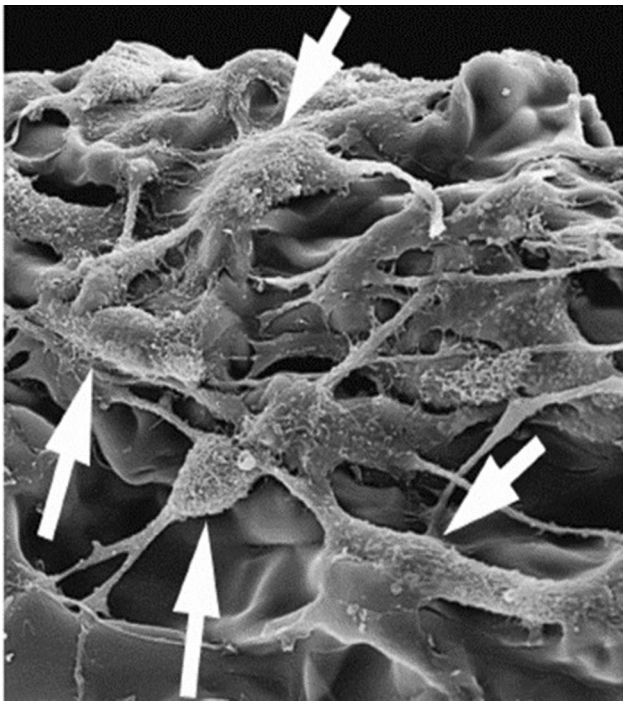

Fig. 11 Scanning electron microscope showing scaffold porosity, cell adherence and osteogenesis

mineralization of the newly formed bone in an oriented fashion thus hastening the process of bone remodelling at a faster rate. This is due to two broad reasons, first, the time taken for the reorientation of the newly formed bone to that of the natural bone texture will almost vanish and the presence of Hydroxyapatite at the site of new bone formation will help the healing process of bone regeneration.

Easy availability, large size, easy handling due to their docile nature, and suitable anatomy for the present study made rabbits as the most suitable candidates for this study. The ulna bone in rabbits is easily palpable, hence easy for surgical creation of bone defect and the anatomy of the intraosseous membrane in between the radius and ulna provides appreciable support for the implanted scaffold.

It can be criticized that the implant did provide a suitable scaffold for migration of bone-forming cells; as evidenced by the callus at the implant-bone interface. Absence of any frank signs of infection at the implanted site during the gross examination, clinical well-being of rabbit during follow up suggesting the good compatibility of the scaffold with the experimental subject. Intradermal immunological sensitivity testing revealed no signs of sensitivity on clinical grounds both at the implanted and skin tests sites.

In summary, the (CH/CS/G/nBG) scaffold is easy to handle, nearly radiolucent, biodegradable, and non-immunogenic. It is important to note that there were both macroscopic and microscopic evidence of new bone formation as per radiological and histological examination. Even though our study showed promising results to promote composite scaffold to fill defects, it has its share of limitations. Require a large multicentric study with an increased sample size for further confirmation of our results and the need for accessory fixation for larger tubular defects and load-bearing capabilities of the scaffold. Also, successful implantation of viable or lyophilized osteoblasts might be an avenue to work up.

Conclusion

We can conclude that the chitosan-chondroitin sulphate-gelatin-nano-bioglass scaffold is efficient in osteoconduction and osteoinduction in the gap non-union model and it is biocompatible, bioactive, and non-immunogenic as well.

Compliance with Ethical Standards

Conflict of interest We hereby declare that we do not have any sort of conflict of interest with any person or authority.

Ethical standard statement Approval of CPCSEA was obtained for animal experiment.

Informed consent None.

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