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Original Research

Eggshell Derived Hydroxyapatite as Bone Graft Substitute in the Healing of Maxillary Cystic Bone Defects: A Preliminary Report

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Abstract:

Background: Since ancient times, use of graft materials to promote healing of defects of bone is well-known. Traditionally, missing bone is replaced with material from either patient or donor. Multiple sources of bone grafts have been used to graft bone defects to stimulate bone healing. Hydroxyapatite is naturally occurring mineral component of bone, which is osteoconductive. This versatile biomaterial is derived from many sources. The aim of this study is to evaluate the efficacy of eggshell derived hydroxyapatite (EHA) in the bone regeneration of human maxillary cystic bone defects secondary to cystic removal/apicoectomy and compare the material properties of EHA *in vitro*.

Materials and Methods: A total of eight maxillary bone defects were grafted after cystic enucleation and/or apicoectomy in the year 2008 and completed the study at 1 year. The patients were followed-up 2 weeks after surgery for signs and symptoms of infection or any other complications that may have been related to surgical procedure. Follow-up radiographs were obtained immediately after surgery followed by 1, 2, and 3 months to assess the efficacy of EHA in bone healing. Physicochemical characterization of the EHA was carried out in comparison with synthetic hydroxyapatite (SHA), also compared the biocompatibility of EHA using in vitro cytotoxicity test.

Results: By the end of the 8th week, the defects grafted with EHA showed complete bone formation. However, bone formation in non-grafted sites was insignificant. The values of density measurements were equal or more than that of surrounding normal bone. These results indicate that the osseous regeneration of the bone defect filled with EHA is significant. EHA showed the superior material properties in comparison with SHA.

Conclusion: EHA is a versatile novel bone graft substitute that yielded promising results. Because of its biocompatibility, lack of disease transfer risks, ease of use and unlimited availability, EHA remains a viable choice as regenerative material. EHA is very cost-effective, efficient bone graft substitute, which can be prepared in a very economical way. It is a worthwhile bone substitute because it is safe and easily available material.

Key Words: Bone regeneration, bone substitute, eggshell derived hydroxyapatite, hen's eggshell, hydroxyapatite, osteoconductivity, synthetic hydroxyapatite

Introduction

Since ancient times use of graft material to promote healing of large defects of bone is well-known.[1-3] Traditionally, missing bone is replaced with allograft.[1-3] Because of morbidity of donor site, second surgery to harvest graft, quantity required, and so many other factors made the clinicians and scientists to search for alternative bone graft substitutes.[4-9] Recently, use of processed or synthetic bone graft substitute has gained popularity over traditional methods.[3,7-9]

Hydroxyapatite (HA) is a naturally occurring mineral component of bone. HA is osteoconductive.[3,7,8] Few studies shown that nanocrystalline HA is osteoinductive in nature[10,11] and stimulates cells for periodontal tissue regeneration.[12] This versatile biomaterial derived from many sources, e.g., bone, corals, synthetic, etc. HA is used to graft bone defects to stimulate bone healing.[2,3,7-9] The aim of our study is to evaluate the efficacy of indigenously prepared eggshell derived hydroxyapatite (EHA) in bone healing and compare the material properties with synthetic hydroxyapatite (SHA).

Materials and Methods

Sample preparation and characterization

Hen's eggshells washed thoroughly and heated in box furnace at 900°C for 2 hrs to decompose organic matter and convert it to calcium hydroxide after exposure to the atmosphere. The product was finely ground in an agate pestle and

mortar. Calcium hydroxide weighed and mixed with distilled water to form 0.3 M suspension and reacted with 0.5 M di ammonium hydrogen phosphate solution corresponding to the stoichiometric ratio of Ca/p = 1.67. The mixed reactants were irradiated in a domestic microwave oven (BPL India, 245 GHz, 800 W). The product was then washed repeatedly with distilled water to remove unwanted ions and dried overnight in an oven at 100°C to produce EHA.[13] The procedure was repeated to check the reproducibility. The SHA was prepared in an identical manner by heat processing using synthetic calcium hydroxide (analytical grade, Merck, Germany). A small amount of both the samples were heated at 900°C for 2 hrs. Followed by cooling to improve the crystallinity and to check the purity.[13]

Cell culture and cell viability assay

The *in vitro* cytotoxicity test was performed as per direct contact method[13] (CISO 10993-5, 1999) using osteoblast cells maintained in minimum essential media supplemented with fetal bovine serum. These cells were seeded onto sintered HA pallets of 4 mm diameter and 2 mm thickness for 24 hr, which were viewed under an optical microscope. High density polyethylene and copper were used as negative and positive control samples, respectively.[13]

Clinical and radiological evaluation

The total of eight patients treated in the year 2008 for periapical lesions such as residual and radicular cysts were included in this study. All the patients were grafted with EHA after cystic enucleation and/or apicoectomy. Clinically, the wound healed uneventfully in all cases. Suture removal was done on the 7th day after surgery. Patients were excluded if the graft was lost or infected or were lost during the follow-up examination. The patients were followed-up at 1st and 2nd week after surgery for signs and symptoms of infection or any other complications that may have been related to the surgical procedures. At 1st, 2nd, and 3rd month and later 6th month radiographs were obtained to assess the amount of osseous fill. Mucosal color, post-operative pain and swelling was noted during clinical evaluation. Visual analog scale was used for clinical pain measurements.

Observer strategy was modified (Figures 1 and 2) to assess the efficacy of EHA in the healing of bone after grafting of cystic defects.[14-18] The radiographic measures were collected at the following times: (1) Presurgically, (2) immediately after surgery, (3) 1st month after surgery, (4) 2nd month after surgery, (5) 3rd month after surgery, and (6) 6th month after surgery. Radiographs were evaluated and documented for (1) change in the surgical site outline, (2) change in the internal portion of surgical site, and (3) the density of bone formation. All the radiographs were examined blindly by two examiners. In case of any gross inconsistency with observations, the third examiner observed the radiographs to prevent bias and the results were tabulated. Density was noted in comparison with surrounding normal bone, as the surrounding bone density is considered as reference since beginning of the study and correlated until last follow-up. The comparison of the images were performed with variable intensity light. The radiographic changes in surgical site outline, internal portion of surgical site after surgery correlated with density using Mann–Whitney U-test and Wilcoxon matched paired test.

Results

All the cases healed well with no significant adverse clinical observations. The bone density had reached that of surrounding normal bone or more in all cases by the end of 8 weeks indicating the bone regeneration. Significant density changes were observed between 1st, 2nd, and 3rd month as summarized in Table 1 and Figure 3. Density remained steady afterwards indicating complete bone healing. Control group showed very less bone regeneration and density measurements were insignificant

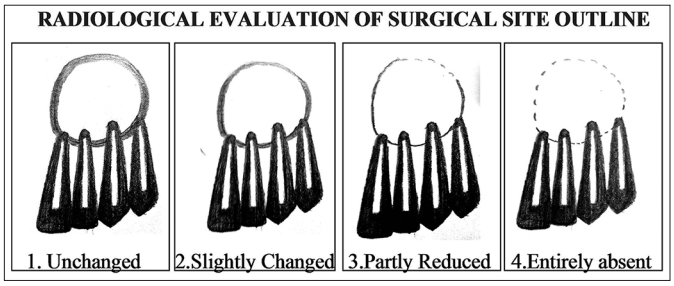

Figure 1: Schematic diagram showing radiological evaluation of surgical site outline.

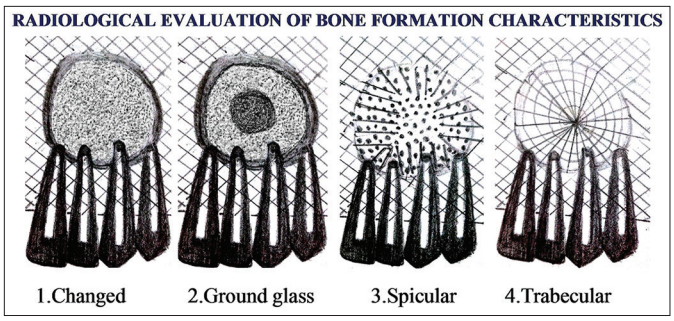

Figure 2: Schematic diagram showing radiological evaluation of bone formation characteristics.

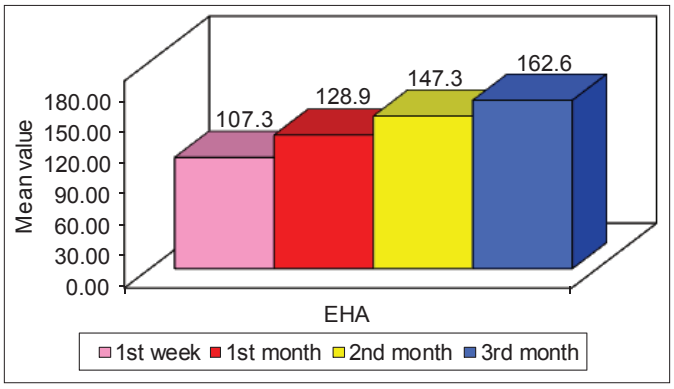

Figure 3: Graph showing measurements of mean bone density at 1st week, 1st month, 2nd month, and 3rd month.

for correlation. The margin blending with material margin was progressive indicating the bone regeneration from the periphery to the center. These changes observed at 1st, 2nd, and 3rd month were significant as summarized in Table 2 and were well-correlated with density changes (Figure 4). The internal portion of surgical site was trabecular or specular in all the cases at the end of 2nd month after surgery showing successful healing and osteoid regeneration as summarized in Table 3. The *in vitro* cell viability test showed the material is biocompatible. The material characterization revealed the EHA is superior compared to SHA as summarized in Table 4.

Discussion

Currently in the United States alone, number of bone graft procedures done per year exceeds 500,000 and approximately 2.2 million worldwide.[19] The estimated cost of these procedures approaches $2.5 billion per year. Harvesting the autograft requires an additional surgery that can result in its own complications such as inflammation, infection and chronic pain that occasionally outlasts the pain of the original surgical procedure.[4-6] Quantities of bone tissue that can be harvested is also limited, thus creating a supply problem.[2-6,19,20] Risk of human immunodeficiency virus transmission with allograft was reported to be one case in 1.6 million population.[20,21] Cases of hepatitis transmission and development of septic arthritis from the donor tissue have been reported with allograft.[22-24] The complement-dependent cytotoxicity reported[25] other cases of allograft-related infection or illness and death of patient due to *Clostridium sordellii*.[25]

The limitations[4-6] of autografts, and allografts have necessitated the pursuit of alternatives.[2,3,7-9] Two basic criteria for successful grafting (i.e., osteoconduction and osteoinduction) were used by investigators and several alternatives were also developed; some of which are available for clinical use and others are still in the developmental stage.[2,3,7-9] Many of these alternatives use a variety of materials, including natural and synthetic polymers, ceramics and composites, whereas others have incorporated factor and cell-based strategies that are used either alone or in combination.[2-3,7-9,26-29] This article introduces the EHA as a novel bone graft material.

The formulations of eggshell are being used as mineral and trace element supplying agent.[29,30] The various formulations comprising eggshell powder have been examined in rats.[29-32] In recent times, this eggshell derived material has been introduced as bone graft substitute.[29] There are few studies with surface modified eggshell as osteoconductive bone filling material for bone regeneration with variable benefit.[31,32] After histomorphometrical evaluation at 4 and 8 weeks interval, it was confirmed that the eggshell-derived powders have excellent new bone formation ability.[31-33] This has led to the curiosity to prepare the EHA from eggshell waste in a very economical way.[34] Even the material properties are superior to the commercially available graft materials.[13] The material is chemically pure form of nanocrystalline HA with eggshell origin alike any other SHA.[13,34] The different forms of HA and origin are in use as bone graft substitute since long time.[7-10]

Table 1: Comparison at 1st week, 1st month, 2rd month, and 3rd month with mean bone density by paired *t*-test in EHA.

Treatment durations	Mean	Standard deviation	Mean difference	SD difference	Paired *t* value	*P* value
1st week	107.3333	5.6782	−21.5833	7.4524	−10.0326	0.0000*
1st month	128.9167	5.9001				
1st week	107.3333	5.6782	−39.9167	13.3652	−10.3459	0.0000*
2nd month	147.2500	10.6269				
1st week	107.3333	5.6782	−55.2500	22.3653	−8.5575	0.0000*
3rd month	162.5833	20.2460				
1st month	128.9167	5.9001	−18.3333	11.8807	−5.3455	0.0002*
2nd month	147.2500	10.6269				
1st month	128.9167	5.9001	−33.6667	20.7247	−5.6273	0.0002*
3rd month	162.5833	20.2460				
2nd month	147.2500	10.6269	−15.3333	11.2439	−4.7240	0.0006*
3rd month	162.5833	20.2460				

*Significant at 5% level ($P<0.05$). EHA: Eggshell derived hydroxyapatite

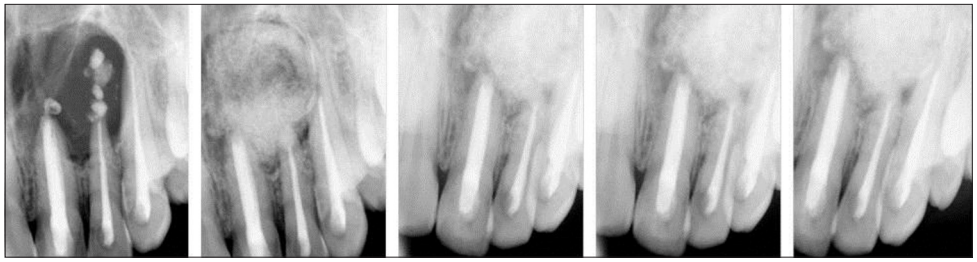

Figure 4: Intraoral periapiacal radiographs showing radiological evaluation of surgical site outline and bone formation characteristics.

Table 2: Comparison at 1st week, 1st month, 2nd month, and 3rd month with radiological evaluation of surgical site outline by Wilcoxon matched pairs test by ranks in EHA.

Treatment durations	*t* value	*Z* value	*P* level
1st week-1st month	0.0000	3.0594	0.0022*
1st week-2nd month	0.0000	3.0594	0.0022*
1st week-3rd month	0.0000	3.0594	0.0022*
1st month-2nd month	0.0000	2.9341	0.0033*
1st month-3rd month	0.0000	2.9341	0.0033*
2nd month-3rd month	0.0000	1.8257	0.0679

*Significant at 5% level (P<0.05). EHA: Eggshell derived hydroxyapatite

Table 3: Comparison at 1st week, 1st month, 2nd month, and 3rd months with radiological evaluation of bone formation by Wilcoxon matched pairs test by ranks in EHA.

Treatment durations	*t* value	*Z* value	*P* level
1st week-1st month	0.0000	3.0594	0.0022*
1st week-2nd month	0.0000	3.0594	0.0022*
1st week-3rd month	0.0000	3.0594	0.0022*
1st month-2nd month	0.0000	3.0594	0.0022*
1st month-3rd month	0.0000	3.0594	0.0022*
2nd month-3rd month	0.0000	-	-

*Significant at 5% level (P<0.05). EHA: Eggshell derived hydroxyapatite

Table 4: Comparison of physicochemical properties of EHA and SHA.

Sample	Crystalline size	Surface area (m^2/g)	Density (g/cm^2)	Cytotoxicity
EHA	21	106	3.12	Noncytotoxic
SHA	19	104	3.06	Noncytotoxic

EHA: Eggshell derived hydroxyapatite, SHA: Synthetic hydroxyapatite

In our study 10 patients enrolled, over the course of 1 year, two patients were lost follow-up. However, all 10 patients were followed-up through first 2 months. All attempts to contact the two patients failed in locating them. If the patient could not be followed-up for the remaining period, his or her data were not included for the purpose of reporting levels of osseous fill and bone healing. At the initial post-operative visits at 1 and 2 weeks, there are no signs of infection. At the 1-week follow-up visit, all patients reported less pain associated with grafted site than with the non-grafted site.

Radiographic changes in surgical site outline and bone formation characteristics were significant between 1st week and 1st month (P < 0.05) showing specular or ground glass appearance with merging of material and bone margin.[18] The radiographic bone healing observed in all the patients confirms findings reported by other authors [29-32] that EHA is biocompatible and well-tolerated by oral tissues in humans. Baliga *et al.*[33] have shown the biocompatibility of surface modified eggshell material in cystic cavities of jaw bones with centripetal ossification, which occurred within 6 weeks. The enhancement of bone regeneration could be explained by the ability of the HA to facilitate bone adsorption and calcium release, which stimulates osteoblast differentiation and bone formation.[35] Compared to SHA, the EHA seems to have better morphology, stoichiometry, sinterability, stability at high temperatures and an osteoblast adhesion.[13,34]

Eggshell HA seems to be promising graft material with excellent properties for grafting. EHA is hydrophilic, absorbing surrounding fluids and blood, making it easy to handle and place it in the surgical site. This study did not include collection of tissue for histologic examination because of ethical considerations. The histologic examination of EHA has been studied in animal models[32] and surface modified eggshell in humans showing early bone regeneration.[33]

The EHA showed biocompatibility and good *in vitro* material properties. It is available in unlimited quantity. It can be easily sterilized by autoclaving without altering its biological properties.[30] EHA can be used as graft material for grafting of the bone defects secondary to periodontal diseases, trauma, tooth extractions, developmental imperfections, intra bony defects, sinus lift procedures, and so on.

Conclusion

EHA is a versatile novel bone graft substitute that yielded promising results. Because of its biocompatibility, lack of disease transfer risks, ease of use and unlimited availability, EHA remains a viable choice as regenerative material. EHA is very cost effective, efficient bone graft substitute which can be prepared in a very economical way. It is a worthwhile bone substitute because it is safe and easily available material.

Many products are being marketed today as bone grafts. Several of these products capitalize on the necessities of an ideal substitute. As more materials are adapted and discovered, pre-existing products are finding new applications and effectiveness in combination with newly emerging technology. In addition, further research is going on to use it in combination with collagen for bone repair. It would be valuable to study a larger sample size and with variable age group to test hypothesis including hisomorphometry to confirm its nature of bone regeneration. The future of EHA graft material continues to be an expanding topic.

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