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Fresh frozen bone as a viable alternative to autologous bone in large cystic defects: A histopathological, scanning electron microscope and energy dispersive X-ray (EDX) analysis.

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Abstract

Aim: To assess the quality of regenerated bone using fresh frozen bone graft in cystic cavity with the adjacent normal native bone via histological, ultra-structural and energy dispersive X-ray (EDX) analysis.

Materials and methods: Five patients were included in the study. FFB grafting was done following cyst enucleation and re-entry procedure was done after six months of grafting. Bone samples were collected from the site of grafting as well as from the adjacent native normal

bone with the help of 3.0 trephine bur. Bone samples were subjected to ultra-structural and chemical analysis with the help of Scanning Electron Microscope (SEM) / Energy Dispersive X-ray (EDX) detector machine and routine histopathological analysis.

Result: Histological analysis showed signs of healthy osseous tissues with negligible necrotic zone in both regenerated bone as well as the normal native bone samples. Ultra-structural features showed presence of abundant empty lacunae and lacunae with live osteocytes in both the samples, however chemical analysis showed significant increase in weight and atomic percentage of calcium in regenerated bone as compared to that of adjacent normal native bone.

Conclusion: Observing the results of this multi-model approach viz. ultra-structural, chemical and histological comparison between regenerated bone and adjacent native normal bone, we recommend to consider fresh frozen bone as a viable alternative material to autogenous bone in promoting formation of new bone in the pathological defects thus eliminating the donor site morbidity.

Keywords: Cyst enucleation, Fresh frozen bone, SEM/EDX analysis

Introduction

Enucleation of large cyst can lead to a large bone defect. Although large jaw bone defect has a natural potential to heal and regenerate, the healing process after cyst enucleation without any grafting procedure presents a high rate of secondary complications such as weakening of the surrounding anatomical structure and clinically as well as radiologically evident bone defect in the middle of the lesion due to centripetal pattern of the bone regeneration. Moreover, the healing process of large jaw bone defect often leads to bone loss in vertical height as compared to preoperative condition or the contralateral side of the jaw thus reducing the possibility of rehabilitation using dental implants or fixed prostheses in future [1].

Bone availability is the key for successful prosthetic rehabilitation. Thus, techniques of bone reconstruction using bone grafts is being used widely. Different graft materials with different biologic and mechanical properties can be safely and predictably used either alone or combined such as autografts, allografts, xenografts, and with alloplastic materials. Among them, autologous bone has long been considered as the ideal grafting material in bone reconstructive surgery due to its intrinsic properties of osteogenesis, osteoinduction and osteoconduction with lack of host tissue rejection or disease transmission; however, it presents some disadvantages, including donor site morbidity, availability, and unpredictable graft resorption [4].

An alternative for the use of autologous bone is the homologous bone allograft, which are available as freeze-dried bone allograft (FDBA), decalcified FDBA and fresh frozen bone (FFB). The homologous bone acts as a scaffold for deposition of new bone by the host bed. This leads to a process in which the grafted bone undergoes progressive resorption and substitution by new vital bone also known as **Creeping Substitution**. In the past few years, the use of FFB has significantly increased. FFB is harvested aseptically from different anatomical areas of live or cadaveric donors and then immediately frozen and stored at -80°C; where osteoinductive proteins are preserved and in the absence of contraindications emerging from the results of the screening procedures, it can be used for implantation with no additional preparation [11].

The only concern with bone allograft is its antigenicity. The absence of negative reports concerning its antigenicity and the demonstration of reduced immunological reaction in experimental model suggest

that FFB could represent an adequate alternative to autografts. Recently many authors are preferring to use fresh-frozen human bone grafts in oral and maxillofacial surgery for filling bone defects as a consequence of cysts removal and for reconstructive procedures [1].

Aim of the study: The present study aims to assess the quality of bone regenerate in the cystic cavity grafted with FFB.

Materials and methods

This clinical study was conducted in the Dept. of Oral and Maxillofacial surgery, Faculty of Dental Sciences, Ramaiah University of Applied Sciences, Bangalore after being approved by the Institutional Ethics Committee. Informed consent was obtained in accordance with the 1975 Declaration of Helsinki as revised in 2002. Possible risks and benefits of procedure were explained to all the patients in their understandable language.

A total number of five patients were included, among them two were females and three were males with age ranging between 22 to 48 years. In all the patient's mandible was involved with histopathological diagnosis of Odontogenic Keratocyst in two patients and Unicystic Ameloblastoma in three patients. Femoral head fresh frozen bone graft in particulate form was used in all the patients after cyst enucleation.

Following enucleation of the cyst, fresh frozen bone grafting was done (Fig 1 and 2). Re-entry procedure was performed to harvest the bone sample from the regenerated bone and from adjacent normal native bone site under local anaesthesia.

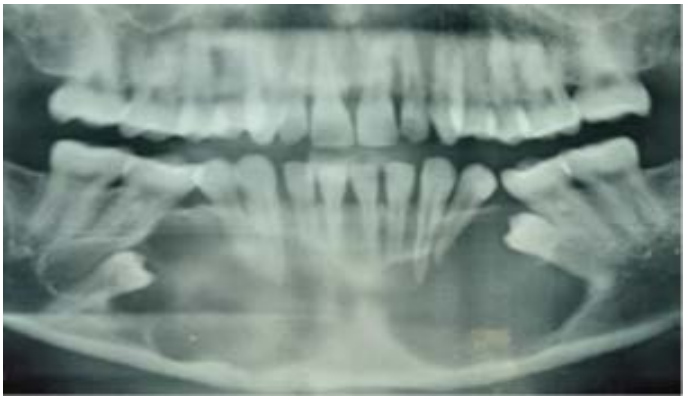

Fig 1: Pre-operative radiograph showing large radiolucent lesions in the anterior mandible bilaterally.

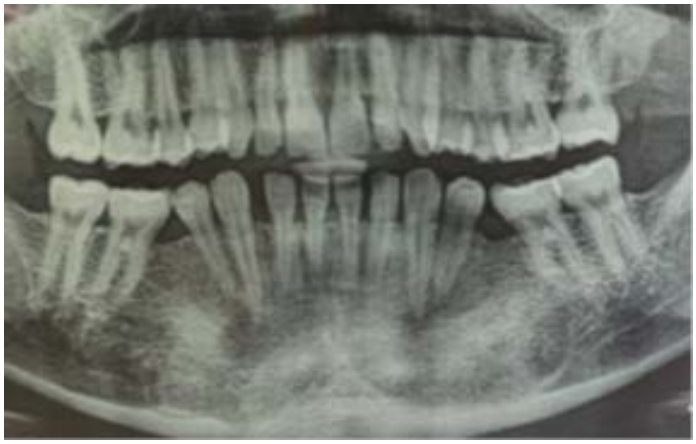

Fig 2: 6 months post FFB grafting demonstrating adequate bone regeneration.

Surgical steps

The re-entry procedure was performed under local anesthesia (2% lignocaine with 1:2,00,000 adrenaline). About 1cm incision was placed on the regenerated bone as well as at adjacent native normal bone and bone samples were harvested from both the sites with 3.0 mm diameter trephine bur and physio dispenser from each patient (Fig 3).

Harvested bone samples were then divided into two segments where one sample from each site was subjected to histopathological assessment in formalin containers labelled separately and other half were subjecting to drying in order to eliminate water content and then submitted for ultrastructural and EDX assessment in a separate sterile container.

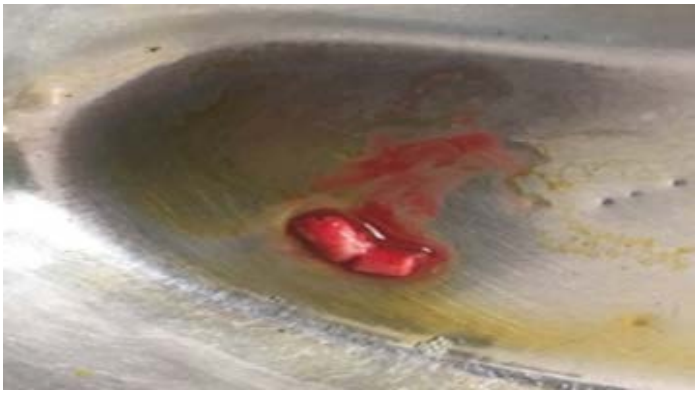

Fig 3: Bone sample harvested with trephine for SCM, EDX and Histopathological analysis.

Processing the bone sample for histopathological analysis

After obtaining a set of bone samples from regenerated site as well from the adjacent normal native bone, fixation was carried out in 10% neutral buffered formalin for 24 hours followed by decalcification with 10% nitric acid for 2 days. After overnight water wash and dehydration with different grades of alcohol, clearing was done with xylene for 45 minutes and paraffin wax blocks sized at 4-5mm were made using hard tissue microtome and 4-5-micrometer sectioning with the help of microtome. Later deparaffinization and rehydration of the slides were carried out and the specimen were stained with haematoxylin and eosin and viewed under the research microscope.

Processing the bone sample for SEM & EDX analysis

Another set of bone sample were fixed in 10% neutral buffered formalin solution and dried under the sunlight. The samples were mounted on the stub and gold coating was done with a sputtering system with thickness of 10nm for 38 seconds. Further the gold coated sample was placed onto the SEM machine [ESCM quanta 2000 by FEI] for viewing at different magnifications. Later the same sample was put through EDX detector in SEM for chemical characterization where calcium and phosphorous contents were recorded.

Statistical analysis

Data was analysed using SPSS software version 18. Median with Interquartile range was used to describe all the variables as the data was skewed. Wilcoxon Signed Rank Test was used to compare medium of Calcium Weight (Ca Wt%), Phosphorus Weight (P Wt%), Calcium Atomic % (Ca At%), and Phosphorus % (P At%) between regenerated bone and adjacent native normal bone where $P< 0.05$ was considered as statistically significant.

Results

Regenerated bone with FFB grafts demonstrated optimal adherence, with no signs of infection, pain and oedema or graft mobility with excellent vascularization during the re-entry procedure. In fact, we felt more resistance and hardness while bone harvesting in the regenerated bone as compared to the adjacent normal native bone with the trephine.

Observations of histopathological characterization

The histologic analysis in the present study demonstrates well: Oriented and uniform bone deposition with viable osteocytes and osteoblasts, highlighting no evidence of necrotic bone in both regenerated bone and normal bone samples. Histological images also showed areas containing lacunae with live osteocytes and also areas containing empty osteocyte lacunae in both regenerated as well as in the normal bone along with evidence of neo-vascularization in all the samples (Fig 4 & 5).

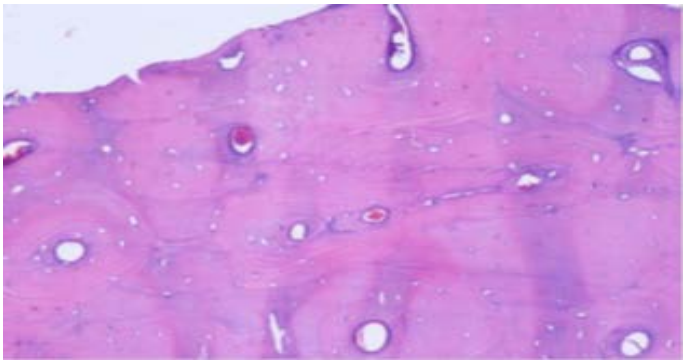

Fig 4: Histopathology of regenerated bone sample

showing bone matrix with filled and empty osteocytic lacunaes.

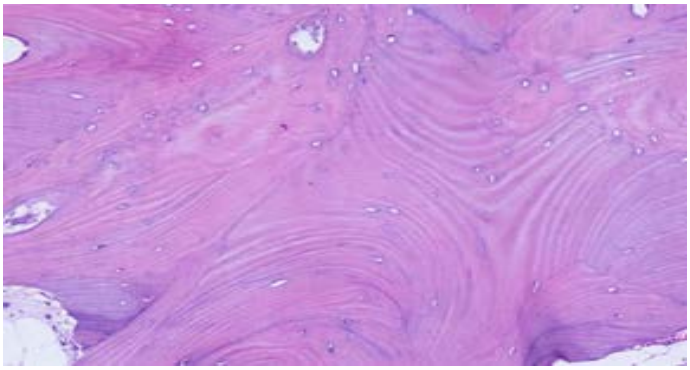

Fig 5: Histopathology of normal native bone sample showing bone matrix with filled and empty osteocytic lacunae.

Observations of Scanning Electron Microscope characterization

Scanning electron microscopy analysis confirmed the results obtained by histopathology, in with particular the presence of filled osteocyte lacunae's, empty osteocyte lacunae and uniform bone deposition (matrix) (Fig 6 & 7).

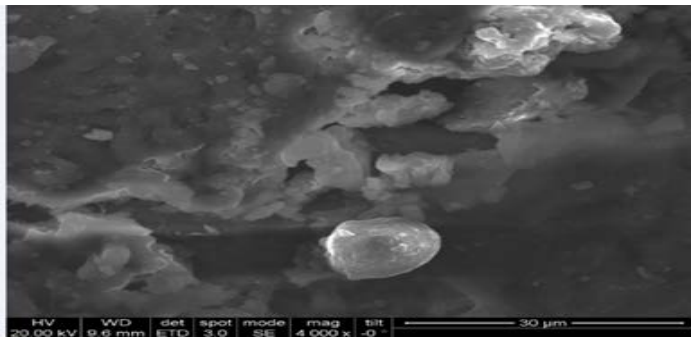

Fig 6: SCM image of regenerated bone demonstrating bone matrix with filled and empty osteocytic lacunaes.

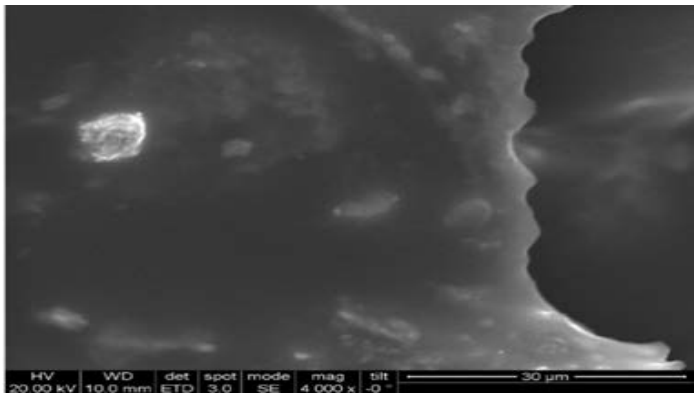

Fig 7: SCM image of normal native bone sample showing bone matrix with filled and empty osteocytic lacunae.

TEST Statistics[a]

Parameters / Test	Total Wt % of Ca in normal bone Total Wt % of Ca in regenerated bone	Total Wt % of P in normal bone Total Wt % of P in regenerated bone	Total At % of Ca in normal bone & Total At % of Ca in regenerated bone	Total At % of P in normal bone Total At% of P in regenerated bone
Z	-2.023[b]	-1.483[b]	-2.032[b]	-1.483[b]
Asymp. Sig. (2- tailed)	0.043	0.138	0.042	0.138

Table 1: Test Statistics where 'a' representing Wilcoxon Signed Ranks Test'b' is based on positive ranks.

Discussion

Integration of bone graft occur by interaction and synergy between bone cells and this is called creeping substitution. Three main process which are involved in these cellular events are osteogenesis (cellular event that promotes the synthesis of bone matrix by osteoblasts), osteoinduction (the capacity to induce the migration of mesenchymal cells and their differentiation into osteoblasts), and osteoconduction (ability of the tissue to serve as a scaffold for cellular processes involved in the repair of bone tissue) [1].

The ideal graft material must satisfy the above-mentioned requirement of cellular events. In addition to these other requirements include biocompatibility, anatomical properties, easy availability, replacement with mature

bone, adequate volume and a satisfactory benefit/cost ratio [5].

Although autologous bone remains the best grafting material owing to its property of osteogenesis and high degree of biocompatibility, it has its own drawbacks such as donor site morbidity, limited availability, operation under general anaesthesia, long term hospital course, possibility of hospital acquired infection and cost [4].

Allograft are derived from humans. The difference is that allograft is harvested from an individual other than the one receiving the graft and it is typically sourced from a bone bank [13]. Bone samples are harvested aseptically from different anatomical areas of live or deceased donors in the absence of contraindications emerging from the results of the screening procedures but more often extracted from the femoral epiphysis [11]. Overlying residual fibrous tissues are removed and the bone samples are immersed in antibiotic solution of vancomycin, polymyxin, ceftazidime, and lincomycin for at least 72 hours at 4°C, followed by freezing at – 80°C, using the cryoprotective DMSO, which allows storage for up to 5 years [9-10].

Use of FFB in dental surgery have become more popular and reliable in recent years for alveolar/jaw bone restoration with better predictable result. FFB is a mineralized and non-irradiated homologous bone [14-15] and its limited processing (i.e., disinfection and deep freezing) guarantees in preserving osteoconductive and potential osteoinductive qualities.

Presence of osteoinductive property in fresh frozen bone is an ongoing debate in literature because previous studies have shown that processing and storage of Fresh frozen bone kills all the viable cells through disruption of the cell membranes as a result of ice crystal formation, which is an important step for preventing any kind of antigenicity. However, Fast freezing using the Dimethyl Sulfoxide (DMSO) has been proven to be a promising cryoprotective substance to improve immune tolerance of allograft bone and to enhance the biologic function by maintaining viable cells capable of giving rise to cell growth [17]. Multiple studies are available on application of FFB but more on implant survival after ridge augmentation. Several authors have reported that none or very few implants were lost in the grafted ridge using FFB [1] [18-19].

Tatiana regina et al revealed no statistically significant difference in the degree of graft volume loss. [5] and supported by others with good percentage of new bone formation on histomorphic evaluation with no statistical significance in volumetric evaluation assessed radiographically between fresh frozen bone and autogenous bone groups in sinus lift procedures [18].

It is suggested that only dense fresh-frozen bone graft with a density > 800 HU are clinically preferable due to their lower degree of resorption as an alternative to autologous bone for horizontal ridge augmentation [14].

Literature search revealed a greater number of studies on histomorphometric assessment with positive correlations among fresh frozen bone graft and autogenous bone graft samples obtained during the re-entry. Some reported active sign of remodelling and confirmed similar collagen pattern with fresh frozen bone to that of autogenous bone and living bone, exhibiting

features of mature and compact osseous tissue surrounded by marrow spaces [1]. Other studies observed histologically similar bone formation pattern among both groups with no significant difference in percentage of density of bone [20]. Cellular response and extracellular matrix mineralization in fresh frozen grafted site were higher in the cultures compared to autogenous grafted site but found no significant difference in osteoblast differentiation [12]. In context to above we the operating surgeons felt more resistance and hardness while harvesting the bone samples during re-entry procedure at

fresh frozen bone grafted site compared to normal adjacent native bone site suggestive of more densified bone with adequate minimal deposition.

The present study demonstrated similar clinical and histopathological features with optimal graft adherence and excellent vascularization during the re-entry procedure. Histologic evidence of uniform bone deposition with no evidence of necrotic bone in any of the bone samples were observed between the allografts and normal native bone. The histological images of both regenerated and normal bone showed similar features of areas containing both filled and empty osteocyte lacunae. Connective tissue cells and neo-vascularisation were evident at the apical end of the biopsy in all the samples.

An invitro study evaluated samples of fresh frozen bone grafts from three tissue banks using optical microscope and scanning electron microscopy that demonstrated organic matter represented by medullary tissue, cellular debris, red blood cells and DNA within the bone tissue. Also highlights the osteocytes occupying individually within their respective lacunae in the bone matrix [7]. Our SCM analysis findings were similar to above mentioned study characters and also confirmed the results obtained by histological observation of our study with respect to presence of filled osteocyte lacunae and empty osteocyte lacunae. As per our knowledge this is the first study comparing the amount of calcium and phosphorous in terms of Wt. % and At % in regenerated and normal bone site, where we observed statistically significant increase in calcium Wt. % and At. % in FFB regenerate as compared to that of adjacent native normal bone with a P-value of 0.04 and 0.138 respectively.

Conclusion

Multi-model assessment such as histological, ultrastructural and chemical characterization between FFB bone regenerate and adjacent native normal bone provided significant evidence of homogeneity in bone architecture with commendable bone matrix mineralization in FFB regenerate. Hence, considering the evidence, we recommend fresh frozen bone graft as a viable alternative to autogenous bone in reconstruction of large cystic defects posing nil donor site morbidity and scarcity of graft material as we encounter in autogenous bone grafts.

However, more studies are needed to explain the excess amount of calcium found in regenerated bone and its clinical significance.

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