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ORIGINAL ARTICLE

Zinc nanoparticles induced eggshell collagen membrane used for guided bone regeneration: A novel approach in rabbit models

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Abstract

Conventional methods of guided bone regeneration (GBR) in bone tissue engineering frequently encounter challenges in attaining adequate antibacterial and osteogenic qualities owing to intrinsic limits. The purpose of this preclinical study was to assess the effectiveness of two distinct membranes, Healiguide® and a unique GBR membrane produced from eggshell membranes (ESM), in combination with bone graft replacement in animal models. The objective of this study was to evaluate the efficacy of a new guided bone regeneration membrane developed from eggshell membrane that incorporates zinc nanoparticles for the regeneration of bone tissue in rabbit models. Groups A (no membrane), B (new ESM GBR membrane), and C (Healiguide® membrane) were assigned to three groups, with eight male New Zealand rabbits weighing two–three kilograms each. Standardized surgical procedures were implemented, and histological analysis along with radiographic examination was used for follow-up assessments six and 12 weeks after surgery. Radiographic examination and histological sectioning revealed differences in bone density and quality between the groups. Group B showed the highest level of bone regeneration, followed by Groups C and A. Statistical analysis confirmed a significant difference between the groups ($p < 0.05$). The eggshell membrane showed encouraging results in the enhancement of bone regeneration and integration in rabbit models. These results suggest its potential as a viable alternative to GBR in bone tissue engineering, with promising prospects for improving clinical outcomes. However, additional studies and clinical trials are required to confirm its safety and effectiveness for medical use.

Keywords GBR · Eggshell membrane · Antibacterial GBR · Zinc nanoparticle

Abbreviations

GBR	Guided bone regeneration
ESM	Eggshell membrane
CBCT	Cone beam computed tomography
ZnNPs	Zinc nanoparticles

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Introduction

Regenerative techniques such as sinus augmentation, ridge preservation, periodontal regeneration, and guided bone regeneration (GBR) have revolutionized dentistry and orthopedic biomedical applications [1, 2]. These procedures use biomaterials as scaffolds to support bone repair, offering a physical structure for bone regeneration and facilitating cellular adhesion, protein synthesis, and tissue growth [3, 4]. GBR has successively emerged as an important technique for resolution and bone tissue repair or reintroduction. The solution to many challenges in clinical practice should be widely applied in advanced reconstructive surgeries. The barrier membrane is a key component of GBR; it prevents soft tissue infiltration into bone defects while allowing bone substitutes to integrate into the bone. This creates a favorable environment for osseous growth and remodelling [5, 6]. Although non-resorbable membranes, such as dense polytetrafluoroethylene (d-PTFE), expanded polytetrafluoroethylene (e-PTFE), and titanium-reinforced membranes,

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have structural stability and wound stabilization, there can be drawbacks such as infection risk, handling difficulty, and patient discomfort [7–12]. Non-resorbable or bioabsorbable membranes are crucial for effectively directing regeneration. Bioresorbable membranes composed of collagen are utilized in clinical practice because of their considerable functional and morphological capabilities, allowing the investigation of cellular behavior across various domains. In contrast, bioresorbable collagen membranes derived from bovine or porcine pericardium or tendons are of great importance because of their biocompatibility, natural degradation by host matrix metalloproteinases (MMP), and their capacity to promote connective tissue regeneration without encouraging epithelial migration [2, 13]. These highly biocompatible membranes are further enhanced by decellularization processes that remove antigenic epitopes, retain the collagen structure, and allow cellular interactions [4]. These membranes have been shown to promote osteogenesis and angiogenesis through interactions with periodontal ligament stem cells [14]. However, collagen-based membranes suffer from disadvantages including rapid degradation, unevenness, disintegration, and insufficient spatial stability [2, 15–18]. Bacterial infections on membrane surfaces may compromise GBR outcomes, resulting in GBR material degradation and clinical failure. Kim et al. (2020) noted an around 77.8% implant success rate for GBR therapy over an 8-year follow-up and emphasized the need for improved materials [19]. Advances in biomaterials have allowed the integration and enhancement of growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor β1 (TGF—β1), which stimulate cellular migration, adhesion, and differentiation [3, 4]. Platelet concentrates, such as platelet-rich plasma (PRP) and platelet-rich fibrin (PRF), have now become an essential part of enhancing the efficacy of GBRs. GTR membranes are commercially viable and predominantly collagen-derived from tissue sources. Although the GTR technique produces good clinical outcomes for the treatment of periodontitis, its wide clinical use is limited by its high cost, non-antibacterial nature, scarce supply in large amounts, and compromised degradation kinetics of collagen membranes, especially in light of extreme periodontitis cases involving multiple teeth where larger membranes are required. Eggshell membrane (ESM) has recently garnered attention as a potential alternative because of its high protein content, resemblance to the extracellular matrix, and facilitation of cellular adhesion and proliferation. ESM is composed of interwoven fibers with 80–85% proteins, 10% collagen (types I, V, and X), and glycoproteins [20–22] and exhibits adaptability, high porosity, and perfect water-retaining capacity, making it suitable for regenerative tissue applications. Further property enhancement is obtained by incorporating biologically significant metal nanoparticles (e.g., zinc) into the collagen frameworks. Synthetic zinc has been well documented as having antimicrobial properties and as a pro-osteogenesis/pro-angiogenesis molecule, making it a valuable entity to include in GBR therapies [23–25]. In this study, ESM was doped with Zn to create a novel GBR membrane. Preliminary in vitro characterization showed promising antibacterial, osteogenic, and angiogenic properties of zinc-doped ESM membranes. We propose that this barrier can inhibit soft tissue invasion for beyond 12 weeks. Furthermore, employing a tibial defect model in rabbits, we expect that the breakdown of the innovative GBR membrane would promote smooth integration without eliciting an immune response in the tissue This novel approach to GBR membrane development aims to overcome the limitations of existing materials, paving the way for enhanced clinical outcomes in regenerative dentistry and orthopaedics.

Methodology

The fabrication of gelatin-coated eggshell membranes (ESM-G) involved immersing eggshell membranes (ESMs) in a gelatin solution at room temperature while subjecting them to continuous stirring. This technique evenly coated ESMs with gelatin, creating composite gelatin-coated membranes (ESM-G strips).

A 5 mM zinc oxide (ZnO) solution was prepared using highly purified (18.2 MΩ) deionised water to incorporate zinc nanoparticles into ESM-G for four hours, the solution was maintained at a temperature of 4 degrees Celsius. Following that, the ESM-G strips were submerged in this ZnO nanoparticle solution, which enabled the nanoparticles to become embedded inside the membrane structure. Following the combination of gelatin with zinc nanoparticles, the result was an ESM-G composite that was enriched with zinc. This composite exhibits potential as a guided bone regeneration (GBR) membrane for bone tissue engineering applications.

Characterisation and in vitro evaluation: The novel ESM GBR underwent comprehensive characterization through SEM to meticulously examine the surface morphology. Following this, it underwent detailed EDX analysis to precisely evaluate the presence of Zn nanoparticles on the GBR membrane. (Fig. 1,2). To evaluate the antimicrobial activity of the GBR membrane, the Kirby-Bauer disc diffusion technique was utilised to assess the antibacterial characteristics of the membrane against Porphyromonas gingivalis. Following the incubation period, the inhibition zones that surrounded the membrane samples were assessed to determine the extent of the antibacterial action represented in Fig. 3. To determine whether or not the MG-63 cells that were cultured on the GBR membrane were biocompatible, the MTT test was utilised to examine the proliferation of these cells. Using absorbance at 570 nm, we were able to determine the

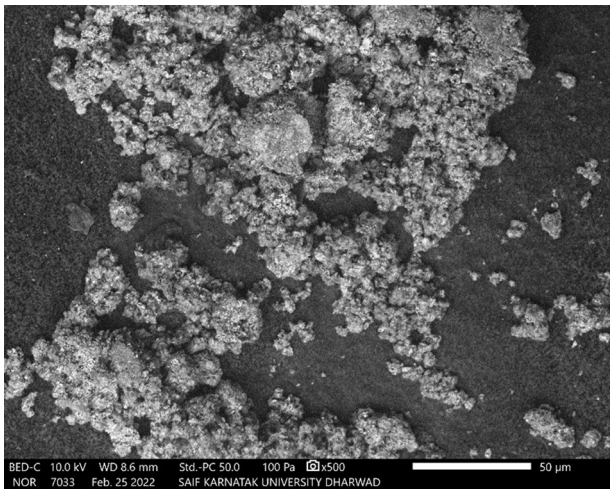

Fig. 1 SEM image at the magnification of 500X of ESM doped with zinc nanoparticles and gelatin

growth and survival of the cells by analysing the metabolic activity of the cells (Fig. 4).

The Von Kossa staining technique was utilised to evaluate the calcium deposition that occurred in MG-63 cells that were grown on GBR sheets. The osteogenic potential of the membrane was demonstrated by the observation of mineralised regions that indicated calcium buildup under optical microscopy after fourteen days (Fig. 5).

Chorioallantoic Membrane (CAM) test: The CAM test was utilised to evaluate the angiogenic potential of the GBR membranes. Over seventy-two hours, the membranes were positioned on the chorioallantoic membrane of fertilised chicken eggs, and the creation of blood vessels in the direction of the sample was seen (Figs. 6 and 7).

This methodological approach provides critical insights into the possible application of the GBR membrane in bone regeneration by conducting a complete evaluation of the surface characteristics, degradation profile, antibacterial activity, cellular response, and capacity to induce angiogenesis of the GBR membrane.

In vivo *study: experimental subjects*

Eight male New Zealand rabbits, each ranging approximately two and three kilogrammes, were employed for preclinical testing. Animals were obtained and maintained at the veterinary centre of Rajrajeshwari Medical College and Hospital. In line with standard procedures, the animals were housed with a day-night cycle and unrestricted access to food and water. The BOCI ethical committee approved the study before its initiation, as shown by decision number BOCI/EC22/2. All experimental methods adhered to the 1960 Prevention of Cruelty to Animals Act. Standard preoperative and postoperative therapies were delivered. The study encompassed eight rabbits, each possessing three osteotomy sites.

Three cohorts of rabbits were established: Group A (lacking membrane), Group B (new ESM GBR membrane), and Group C (commercially available type I collagen membrane Healiguide®).

Preoperative animal preparation

The hair on the selected leg was trimmed one day before to the procedure. Sterile gauze saturated with 2% chlorhexidine solution was employed to cleanse the surgical site and eliminate any debris.

Surgical Procedure: The rabbits were positioned in the surgical room subsequent to subcutaneous sedation with 0.1 mg/kg medetomidine while in their cages. Ketamine and xylazine were administered intramuscularly at doses of 35 mg/kg and 50 mg/kg, respectively, to induce anaesthesia.

The medial part of the limb, over both the femoral and tibial heads, was cut 3 cm to reveal the skeleton. Thereafter,

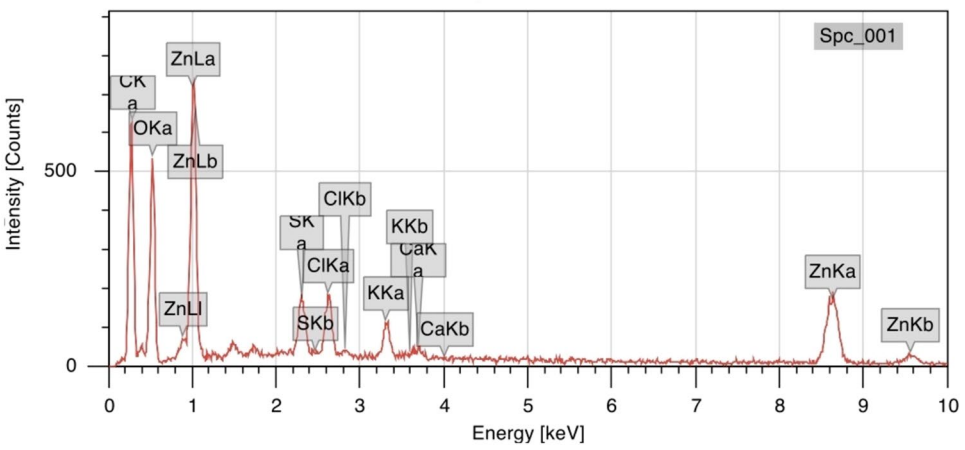

Fig. 2 Energy-dispersive X-ray spectroscopy (EDX) analysis confirming the presence of zinc

Fig. 3 GBR film exhibiting a broader inhibitory zone in the Kirby-Bauer test

a full-thickness flap was used to reveal the tibial and femoral heads.

Three osteotomies, each measuring 4 mm × 4 mm, were created under continuous saline irrigation (Fig. 8). The surgical technique was same across all rabbits. Osseograft replacements were applied at each spot as designated. Group A Group A was administered no membrane, Group B was administered the NOVEL ESM GBR membrane, and Group C was administered the Healiguide® membrane.

Subsequent to the implantation of the allograft and membranes, the surrounding soft tissues were meticulously sutured in layers. Closure of the subcutaneous fascia was accomplished using 4–0 absorbable sutures, whereas skin closure utilised 3–0 silk sutures. The rabbits received post-operative treatment and were monitored at designated intervals for evaluation.

Meloxicam (0.05 mg/kg) was given subcutaneously at regular intervals for 5 days to manage postoperative pain. Taximia (125 mg) was injected subcutaneously every 24 h for five days post-surgery to avert infection. Rabbits were observed daily during the 12 week trial duration.

Euthanasia: The rabbits were euthanised at six and twelve weeks of age. Pentobarbital (100 mg/ml) was administered via the ear vein following the application of a local anaesthetic cream.

Analysis: Histological sections and follow-up (CBCTs) were acquired six and twelve weeks after surgery. Bone density and quality were evaluated in greyscale. The survey data were imported into a Microsoft Excel spreadsheet, and statistical analysis was conducted using SPSS version 20 software.

Results

In terms of characterisation, scanning electron microscopy showed a porous structure with three dimensions and rough surfaces resembling craters. Figure 1 also shows that the Zn nanoparticles clumped together on the surface. Figure 2 shows that the EDX analysis verified the presence of zinc with a mass percentage of 22.14 ± 0.46 percent.

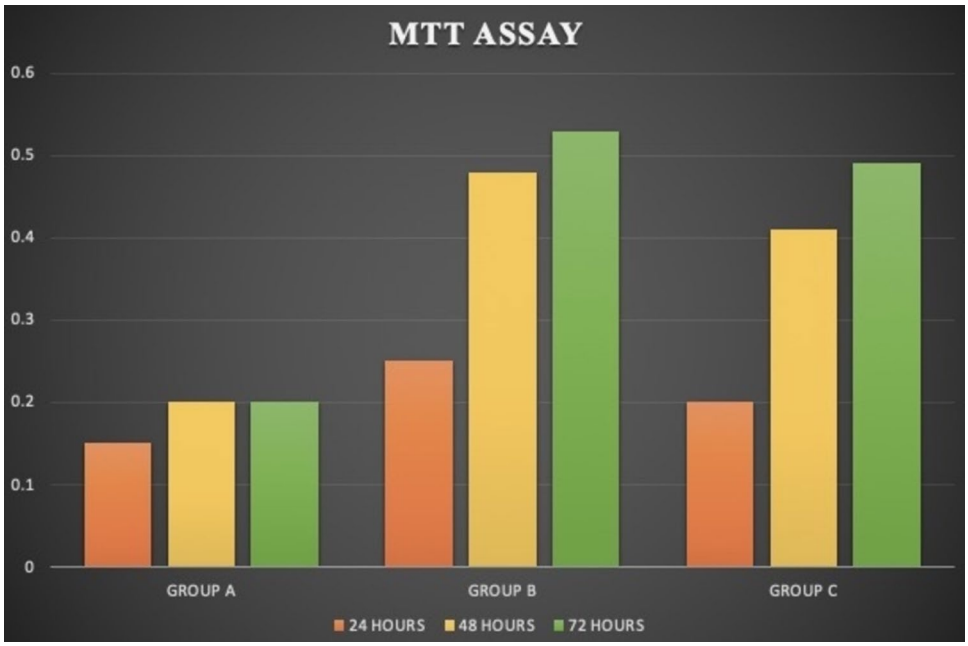

Fig. 4 Cell Proliferation Assay. The statistical test used was Repeated Measures ANOVA, where $*P \leq 0.05$ is considered statistically significant

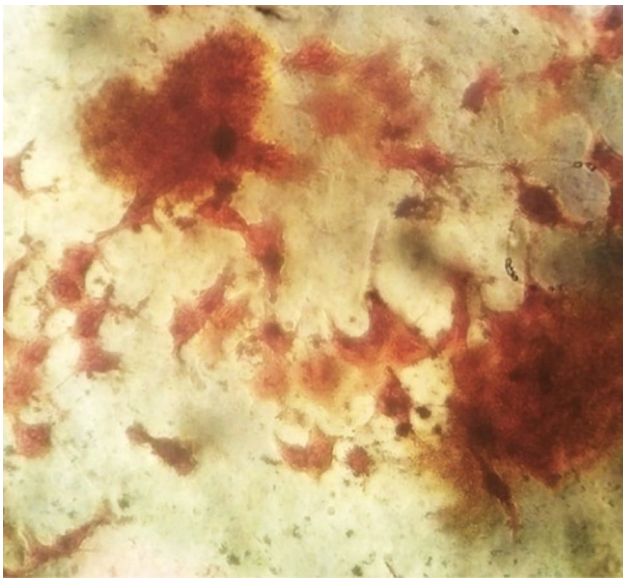

Fig. 5 Von Kossa staining at the end of the 14 days revealed increased calcium accumulation in the cell's cytoplasm(40X magnification)

Fig. 6 Chorioallantoic membrane (CAM) analysis

Hydrolytic degradation: The unique GBR membrane made from type X collagen maintained its resorbability for 45 days.

Tensile strength: Compared to the standard membrane's 13.3 Mpa tensile strength, the GBR membrane made from type X collagen demonstrated a sufficient 14.3 MPa, yielding a statistically significant result $*P < 0.05$.

Kirby-Bauer test: The test group exhibited a zone of inhibition of 24 mm, whereas the control group showed a zone of inhibition measuring 5 mm against P. gingivalis. Standard deviation (SD) and mean were used to represent the values of the inhibitory zone. Using the Mann–Whitney U Test, we obtained a statistically significant result ($*P < 0.05$) (Fig. 3).

MTT assay: A new GBR membrane made of type X collagen was visible (2.57 OD), and there were notable changes in the proliferation of MG 63 cells, according to the MTT assay. Statistical significance was defined as $P < 0.05$ (Fig. 4). Moreover, von Kossa staining of MG63 cells revealed the most prominent osteogenic nodules (Fig. 5). This application facilitates the formation of an extracellular mineralized matrix.

CAM Assay: The bioengineered GBR membrane was evaluated macroscopically on a chick CAM sample and found to be encased in CAM vessels with allantoic vessels radiating outward in a "spoke-wheel" configuration. (Figs. 6,7). Statistical significance was determined using the Kruskal–Wallis test, with a significance level of $*P < 0.05$.

In vivo *assessment*

Histopathological examination

Group A (Control).

Six Weeks Post-Implantation, mature connective tissue was observed on one side, and mineralized bone tissue was characterized by thin trabeculae, large osteocyte lacunae, and basic fuchsin staining on the other side (Fig. 9A).

Twelve weeks post-implantation: As shown in Fig. 9B, there was mature cortical bone and little or no marrow spaces, revealing that the graft was well incorporated as the underlying bone had formed well.

Group B (test: Novel GBR Membrane).

Six Weeks Post-Implantation: Endothelial cells and residual membranes showed mild fibrosis and a mild inflammatory reaction with lymphocyte, granulocyte, and macrophage infiltration. Masson's trichrome staining indicated newly formed bone, and there was no feature of necrosis or reactive infiltration in the later follow-up.

Twelve Weeks Post-Implantation Remodeling was remarkable with mature woven bone, labelled osteoblastic interface, presence of osteocyte lacunae, and collagen fibers connecting the membrane to the bone. No inflammatory changes were observed (Fig. 10A, B).

Group C (Positive Control, Commercial GBR Membrane).

Six Weeks Post-Implantation: There slight decline in the extent of fibrosis and the proportion was accompanied by an increase in the density of new blood vessels. In the fresh trabecular bone, multiple osteocyte lacunae were observed

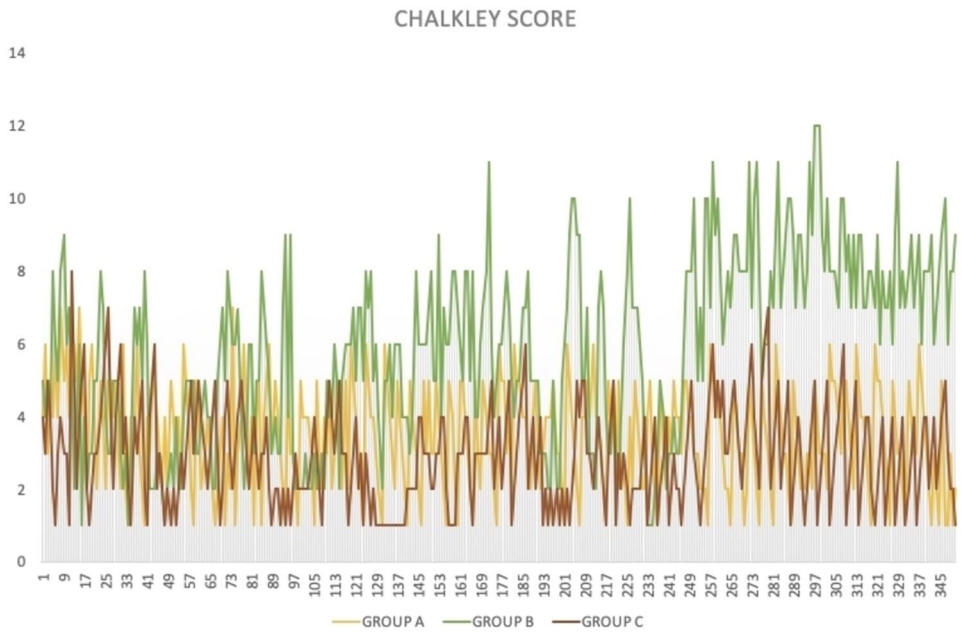

Fig. 7 Graph showing chorioallantoic membrane (CAM) analysis with increased angiogenesis

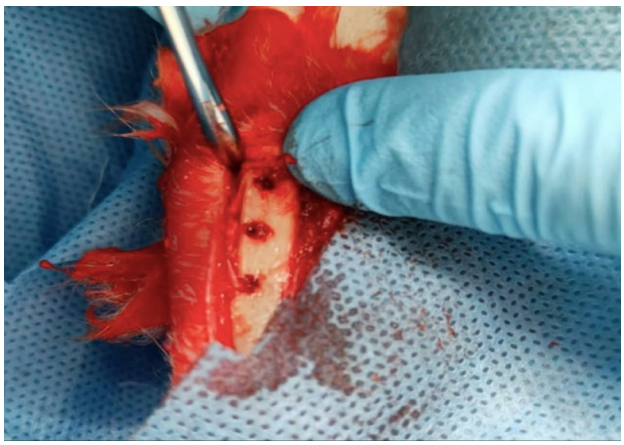

Fig. 8 Surgical representation of the three osteotomy sites divided into Groups A, B, and C

at a given site without any osteogenic, necrotic, or reactive tissue transformation.

Twelve Weeks Post-Implantation: The process of healing at the osteotomy site reached the state of ossification, and the new bone at the fracture line was characterized by dense mature woven bone. Bone-to-membrane bonding was confirmed through collagen fibers linking both structures, although there were no signs of inflammation (Fig. 11A, B).

Radiographic examination

Six weeks post-implantation:

Group A showed cortical plate disruption at the graft level (Fig. 12).

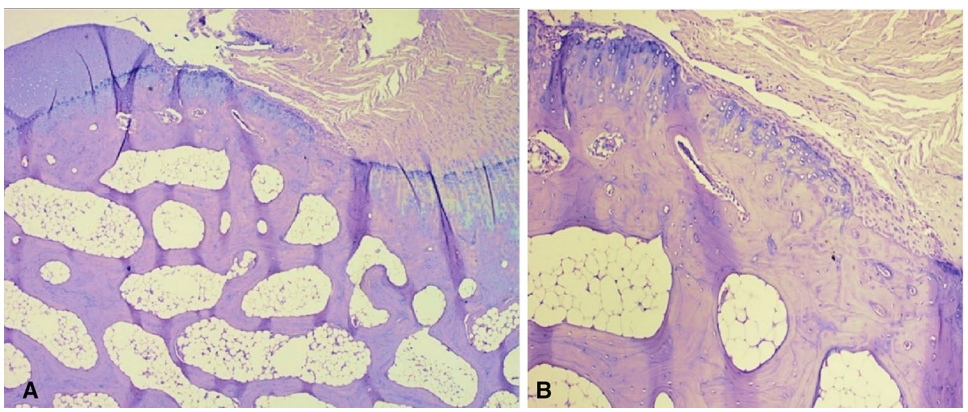

Fig. 9 Group A At six weeks, twelve histological examinations revealed connective tissue on one side of the slides and the formation of new bone characterized by thin trabeculae on the other side(80X magnification)

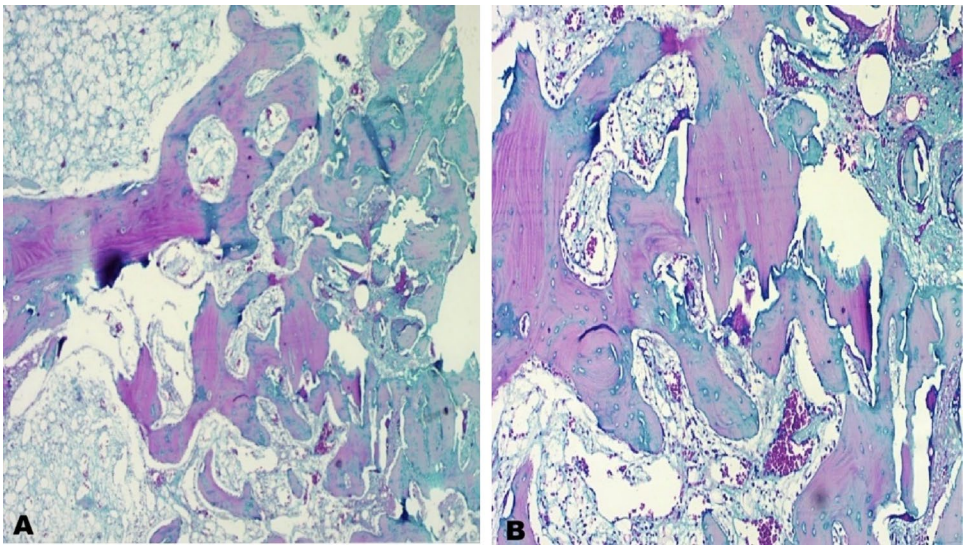

Fig. 10 Group B At six weeks, twelve histological examinations exhibited complete healing, with the presence of thick cortical bone and minimal marrow gaps(80X magnification)

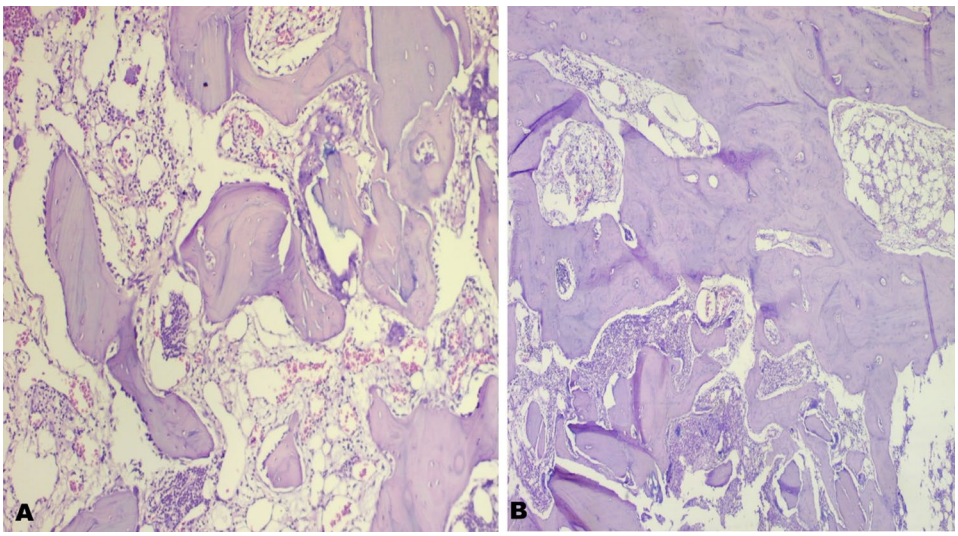

Fig. 11 Group C At six weeks, twelve histological examinations exhibited Collagen fibres between the membrane and bone, with no inflammatory cells detected(80X magnification)

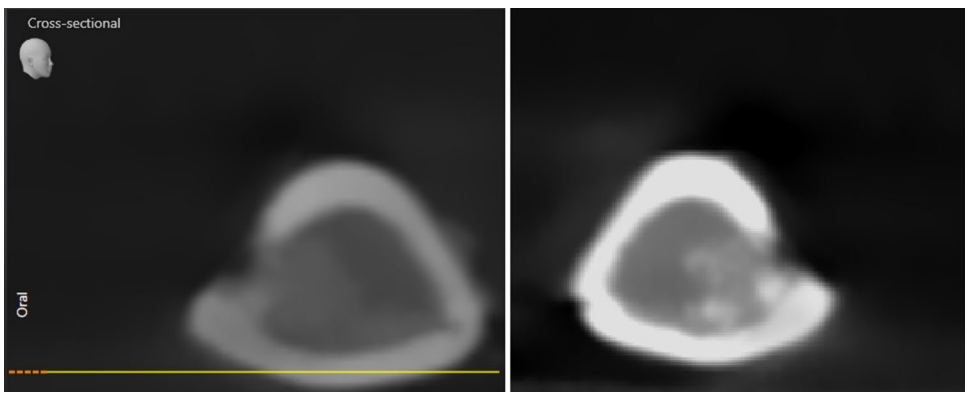

Fig. 12 Group A Six weeks, Twelve radiological examinations

As shown in Fig. 13, Group B confirmed that the cortical plate was continuous, indicating a faster rate of bone

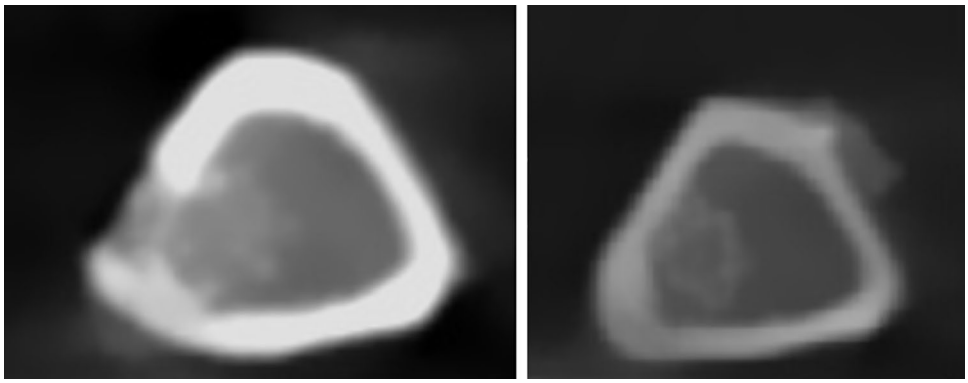

Fig. 13 Group B Six weeks, Twelve radiological examinations

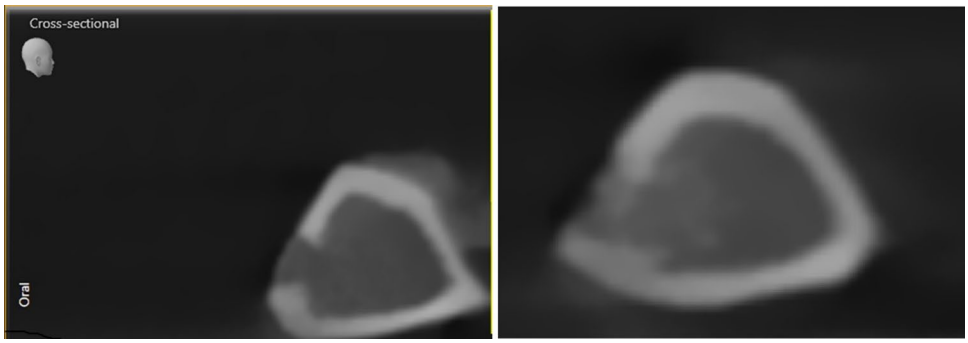

Fig. 14 Group C Six weeks, Twelve radiological examinations

regeneration.

Group C had a defect that resembled a trench at the osteotomy level (Fig. 14).

Twelve weeks post-implantation:

Group B showed fully regenerated cortical bones with better radiopacity than Groups A and C (Fig. 14).

Discussion

The purpose of this study was to determine whether a new GBR membrane that was developed from eggshell membrane (ESM) and contained zinc nanoparticles (ZnNPs) was effective in regenerating bone tissue. These findings provide insight into the advantages and disadvantages of this novel technique, which has several implications for further development of clinical applications and future research. Examination of the morphology and composition of the surface using a scanning electron microscope (SEM) revealed that the surface was both rough and porous, as shown in Fig. 1. The findings of Aprile et al. [26], who demonstrated that porous membranes increased cell adhesion and tissue integration, are consistent with our conclusion. EDX analysis, which revealed the presence of zinc nanoparticles ($22.14 \pm 0.46\%$ by weight), is in agreement with studies that have demonstrated the ability of zinc nanoparticles to positively affect bone regeneration because of their antibacterial and osteogenic capabilities [25]. Zn nanoparticles, on the other hand, have a rather high weight percentage, which may give rise to worries over their possible cytotoxicity. Even though the findings of our MTT experiment suggest increased cellular proliferation, it is essential to keep in mind that an excessive amount of zinc can hurt the health of cells. [27]. To prevent the possibility of harmful consequences, future research should properly calibrate the amount of ZnNP. The degradation characteristics demonstrated a substantial reduction in weight over 45 d ($p < 0.005$), indicating that the degradation rate was suitable for tissue regeneration. This is in agreement with the findings of Wang [28], who pointed out that the regulated breakdown of GBR membranes is necessary for successful bone regeneration. With potent antibacterial activity against Porphyromonas gingivalis, Zn nanoparticles, as reported by Toledano [25], have the potential to aid in the prevention of infections. Based on the results of the MTT assay, which demonstrated a considerable increase in the number of MG-63 cells, it can be inferred that the GBR membrane assisted cells in growing and remaining alive (Fig. 4). Similar studies have demonstrated that collagen-based membranes promote osteoblast development [26, 29]. This discovery lends credence to the findings of these studies. Von Kossa staining provided additional confirmation of the enhanced calcium buildup, which substantiates the osteogenic potential of the membrane (Fig. 5). However, research conducted by Chung et al. [29]

suggests that collagen-based GBR membranes do not always offer the best possible mechanical support for bone regeneration compared to synthetic alternatives. As a result, even though the new GBR membrane has potential, its mechanical qualities require further investigation. The results of the CAM analysis of angiogenesis showed an increase in the number of blood vessels reaching the membrane samples, which is evidence of an angioproliferative impact (Figs. 6 and 7). Hue et al. 2024 [30] observed that GBR membranes containing bioactive compounds improved angiogenesis, which is important for efficient bone repair and is congruent with the data obtained. However, the use of complementary and alternative medicine (CAM) analysis has several drawbacks, as it might not perfectly reproduce the intricate human physiological environment. In vivo, histological examination demonstrated encouraging tissue responses at both six and 12 weeks after implantation. At the six-week mark, there was a limited amount of fibrosis, minor inflammation characterized by immune cell infiltration, and active neovascularization, all of which demonstrated that the membrane was highly biocompatible (Fig. 10A). Previous studies have highlighted the significance of a regulated inflammatory response in ineffective tissue integration (Pereira et al., 2019 [31]). These findings are in line with these conclusions, which are compatible with those of earlier investigations. The fact that there were no instances of necrosis or responses involving foreign bodies provides additional evidence that ESM-derived GBR membranes containing Zn NPs are biocompatible, as reported by Abdelaziz et al. [32]. Masson's trichrome staining, which demonstrates the appearance of nascent bone tissue, is a clear indication of the membrane's propensity to be osteogenic. This is compatible with the idea that a barrier membrane should exhibit both biocompatibility and osteoconductivity, as demonstrated by this observation. [32, 33]3Abdelaziz et al., JinWook et al., and several others. Figure 10B shows that after 12 weeks, there was a large amount of bone growth, with well-defined osteoblastic boundaries and an intimate integration of collagen fibers with the newly produced bone. These findings provide evidence that tissue repair and maturation occur gradually, supporting the function of membranes in directing bone regeneration. Previous studies that highlighted the regeneration ability of collagen-based membranes (Zubery et al., 2007 [34]) revealed that the histological outcomes were consistent with those of the current study. The radiographic examination provided further evidence to support these histological findings. The creation of a consistent cortical plate at the experimental location as early as six weeks revealed that the bone had already begun to stabilize (Fig. 1). The findings of this study are consistent with those of earlier clinical trials that have produced comparable findings. [35] Zubery et al. found in 2008. Furthermore, the existence of mature cortical bone with enhanced radiodensity by the time the animal was 12 weeks old provided further evidence that the membrane was effective in aiding bone regeneration. Despite the widespread view that highly bioactive membranes are necessary for effective bone regeneration, our findings call into question this notion. K. Kang and others [38. Both histologically and radiographically, the observed bone repair and remodelling in the ESM-derived GBR membrane containing Zn NPs demonstrates the therapeutic potential of this membrane. To further confirm the efficiency of the membrane in promoting favorable bone regeneration, Liao et al. [37] found statistically significant changes in bone production when compared to the control group. Although the mechanisms underlying bone regeneration are complicated, it is believed that several elements contribute to the membrane's effectiveness.

The persistent release of zinc ions from the membrane may play a significant role in the stimulation of osteogenic differentiation by activating certain signalling pathways [23–25]. Additionally, the biomimetic nature of the matrix produced from ESM has the potential to facilitate cell adhesion, proliferation, and differentiation by providing a favorable scaffold. To gain a better understanding of the mechanisms that are at play, it is necessary to conduct more research on the exact molecular interactions that occur between the membrane and host tissue.

Implications for therapeutic practice

The zinc-doped eggshell membrane presents a promising advancement in guided bone regeneration (GBR) techniques, offering several clinically significant benefits.

Patient outcomes

Using these technologies can reduce infection risks, speed healing times, and directly benefit patient recovery, as well as the longevity of dental implants or prosthetic rehabilitation. Such improved vascularization at defect sites could provide superior graft integration and fewer complications, and therefore, predictable results in complex cases.

Enhanced Osteogenesis: The osteogenic activity enabled by zinc nanoparticles is due to increased proliferation and differentiation of osteoblasts. This property supports robust bone regeneration, and is thus suitable for ridge preservation, sinus elevation, and peri-implant defect management.

Superior antimicrobial properties

The intrinsic antimicrobial effect of zinc inhibits Porphyromonas gingivalis, a common cause of peri-implantitis and periodontal disease. This reduces the risk of infection, thereby facilitating a favorable healing environment and long-term implant success.

Regulatory challenges and manufacturing considerations

However, the true problem of scaling up the mass production of eggshell-derived membranes with consistent quality and desired performance characteristics has not been resolved. For widespread clinical adoption, it is critical to ensure the bioactivity and structural integrity of the material while being cost-effective and accessible.

Future applications

The zinc-doped eggshell membrane has the potential for widespread application in various dental and maxillofacial procedures, including the treatment of large bone defects. Further research on combining membranes with growth factors or other bioactive chemicals may boost their medicinal potential. Finally, clinical trials are needed to assess membrane effectiveness in humans and their effects on patient outcomes.

Conclusion

This study found that eggshell membrane-infused zinc nanoparticle-infused GBR membranes could promote rabbit bone regeneration and integration. Good histopathological and radiographic results show that it is efficacious and biocompatible, making it a promising clinical option. Its use in bone regeneration treatments and human benefits require additional studies and clinical investigations.

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Declarations

Conflict of interest No conflict of interest.

Ethical approval BRANEMARK OSSEOINTEGRATION CENTRE INDIA: BOCI/EC22/2.

Informed consent "Not Applicable" in this section.

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