Evaluation of the cell viability and *in vivo* biocompatibility of three potential regenerative biomaterials derived from eggshells in Wistar rats

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Abstract. The development of novel bone substitutes for the management of periodontal osseous defects requires assured biological function upon implantation. Chicken egg shells have been introduced for some time in reconstructive surgeries due to their mineral composition. Egg shells are among the most abundant byproducts of food processing waste and appear to have unique biological and chemical properties that support cell differentiation. Additives such as fish collagen and glycerine may enhance their efficacy by improving their regenerative properties, which mimic those of human bone, and increasing their handling characteristics. Biocompatibility and cell viability are two critical factors affecting the ability of any regenerative material to integrate into the body. The aim of the present study was to determine the cell viability and biocompatibility of three egg shell-derived biomaterials, namely, egg shell-derived hydroxyapatite (EHPA), EHPA modified with fish collagen EHPA/Coll, and EHPA/Coll with glycerine. Chicken egg shells were used to synthesise hydroxyapatite via chemical precipitation. The synthesised hydroxyapatite was modified with fish collagen and then with glycerol. All three materials were tested for their viability in mouse fibroblasts and for their *in vivo* biocompatibility in Wistar rats. The results revealed that eggshell-derived hydroxyapatite was highly biocompatible and did not exert any cytotoxic effects. Its modification using fish collagen and glycerine also resulted in derivatives that were biocompatible and noncytotoxic. On the whole, the present study demonstrates that all three materials tested, which were eggshell derivatives, were highly biocompatible and promoted cell growth.

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Introduction

A wide variety of replacement grafts have been attempted in the pursuit of achieving the elusive goal of bone regeneration in osseous defects. These materials are indicated for osseous regeneration in the management of periodontal and peri-implant defects, as are procedures such as socket preservation, ridge augmentation and guided bone regeneration. Biocompatibility and cell viability are critical properties for biomaterials that come in proximity to biological tissues, and represent the capability of a substance to associate with cells in a living system without leading to destruction by unfavourable reactions (1). It is directly related to cytotoxic effects, or the capacity of the material to induce damage to a living system (2).

There are two types of materials that induce bone formation: Grafts and bone substitutes. Both can promote bone regeneration (3). On the basis of their source, these materials with applications in regenerative procedures are autogenous when procured from the same individual, homogenous or allogenic when they are acquired from individuals of the same species, and xenogenic when they are obtained from different species. Alloplastic materials are synthetically manufactured (4).

Alloplasts are prospective bone substitutes, and their outstanding versatility allows them to be manufactured via novel techniques to obtain porous structures that mimic cancellous bone or resemble the fibrillar portion of the extracellular matrix (5,6).

Synthetic materials also provide the advantage of incorporating components with anti-inflammatory and antibiotic effects, thereby reducing the possibility of complications or biological reactions. For example, bone morphogenic protein-9 is used to achieve an osteoinductive effect.

Numerous commercially available osseous grafts currently utilise synthetic materials combined with natural products processed in the laboratory to create biomimetic scaffolds that simulate the extracellular bone matrix (7). Animal bones, fish bones and scales, avian eggshells and the exoskeletons of marine organisms have been used to develop xenogenic materials (8).

Eggshells have been used for various reconstructive surgeries. The mineral composition and high biological activity of these materials render them potentially functional for biological and pharmaceutical applications (9). Surface modifications of eggshell grafts have been attempted to enhance their properties (10). The use of a material derived from eggshells may also have several other benefits due to its obtainability and biodegradability (11,12).

The incorporation of collagen into these scaffolds could improve the efficacy of the biomaterial by contributing to an osteopromoting quality (13). Moreover, it has been reported that hydroxyapatite-collagen scaffolds can have a profound effect on mechanical properties, such as strength, stiffness and pore size (14). Nevertheless, further investigations are required to substantiate the demand for collagen to increase the biocompatibility and stability of the biomaterial without influencing creeping substitution.

Owing to their extensive array of applications, several collagen sources have been investigated. However, the use of collagen, which is mammal-based, has been limited by diseases, such as bovine spongiform encephalopathy and other spiritual constraints (15).

Marine-based collagen is a potential replacement for mammal-based collagen due to its amenable chemical properties, ease of extraction, biocompatibility, low risk of disease transmission and environmental contamination, and few religious and ethical concerns. It has a high collagen content and excellent absorption properties (15).

Glycerol is a non-toxic, biodegradable liquid that the FDA considers 'Generally Recognised as Safe' (GRAS) (16). Hence, it is referred to as an inert carrier and provides improved handling characteristics. Glycerol also provides the advantages of excellent graft containment and flexibility.

Although, there is limited research on HPA-Coll composites (14), the literature on composites derived from hydroxyapatite of egg shell origin and fish collagen is very limited. All the more glycerine has been used as a binder here.

The immune response induced by a biomaterial can influence the healing of biological tissues. Hence, it is a critical property to consider when selecting a regenerative material as a primary anti-inflammatory approach can enhance tissue healing (17).

Before the regenerative capacity of a material can be assessed, its biocompatibility needs to be established to ensure that it does not induce adverse tissue reactions. The present study aimed to examine the biological behaviour of egg shell-derived hydroxyapatite (EHPA), a composite graft prepared using EHPA and fish collagen (EHPA/Coll), and a composite material with glycerol (EHPA/Coll/ Gly) *in vitro* and *in vivo* to demonstrate the cell viability, biocompatibility and inflammatory tissue reactions of these materials in Wistar rats.

Materials and methods

Eggshells and fish collagen. Domestic chicken eggshells of an indigenous breed of hen (Aseel) were obtained from a local poultry farm (Kurupseval farm, Kerala, India). Hydroxyapatite was synthesised from the egg shells via the chemical precipitation method (18). The steps involved cleaning eggshells after removing their membranes, followed by drying, grinding and sintering at high temperature (900˚C in a muffle furnace), resulting in formation of calcium oxide and further calcium hydroxide (following a reaction with atmospheric moisture). Furthermore, the product was reacted with a phosphate source (ammonium phosphate) to give rise to hydroxyapatite.

Fish collagen was extracted at the Central Institute of Fisheries Technology, Kochi, India, via a laboratory-standardised protocol from Rohu fish *(Labeo rohita)* and supplied. The scales were subjected to cleaning and pre-treatment to remove debris, followed by demineralisation (using 0.4M HCl) and further treatment with 0.4 M acetic acid. This was followed by the removal of insoluble portions by filtration and the collection of soluble collagen by salting out the pooled filtrate with NaCl. Centrifugation was performed for 1 h at 8,000 x g and 4˚C, and the collagen was collected as a pellet. Furthermore, the collagen was re-dissolved in 0.5 M acetic acid, after which the salting-in and salting-out processes were repeated twice. The salt was removed from the final suspension via dialysis. The purified collagen was collected by centrifugation forl h at 8,000 x g and 4˚C. and freeze-dried. A temperature of 4˚C was maintained throughout the entire extraction process.

Hydroxyapatite was modified with fish collagen at a ratio of 60:40 at 80˚C, a vacuum level of 0.02 mbar, and a bench-top lyophiliser (Labconco) was used to obtain a sample volume of 5 ml and a flask size of 15 ml. The ratio of 60:40 was selected based on the preliminary optimisation trials where different ratios of hydroxyapatite to collagen 70:30,50:50 and 60:40 were tested. The viability of cells cell exposed to the materials (50 μg/ml) was found to be 121.199±3.336, 128.31±4.090 and 128.58±3.926. All the three ratios were found to be non-cytotoxic. Hence the ratio which mimics the composition of human bone was selected. The procedure was carried out for 24 h.

The modified material, which was in powder form, was converted to an injectable consistency by incorporating glycerol via a mortar and pestle (10 mg of the composite material with 6 ml glycerol).

Cytotoxicity analysis

Cell line and culture conditions. L929 murine fibroblasts (NCCS, Pune, India) were used for the study. They were cultured in Dulbecco's Modified Eagle's medium (DMEM Hi-media) supplemented with 10% FBS, 1% glutamine and 1% antibiotic-antimycotic solution (HiMedia Laboratories, LLC). A humidified atmosphere was maintained throughout the experiment, and the cells were maintained at 37˚C and 5% CO_2.

Assessment of cell viability by MTT assay. The effects of the test compounds (EHPA, EHPA/Coll and EHPA/Coll/Gly) on the cells was assessed using methyl

thiazolyl tetrazolium (MTT) assay, as previously described by Mosmann (19). The seeding of the cells (L929 cell line) was performed in a a 96-well microtiter plate at a density of 5,000 cells/well. The cells were allowed to attach overnight at 37˚C and 5% CO_2. Following adherence, various concentrations (0, 12.5, 25, 50 and 100 μg/ml) of the samples were added to the wells followed by incubation for 24 h at 37˚C and 5% CO_2. The medium was decanted after 24 h of incubation. MTT reagent (HiMedia Laboratories, LLC) 1 mg/ml was added to the wells which were subsequently incubated at 37˚C for 4 h. The MTT solution was removed from the wells, and the formazan crystals formed were solubilised, and the absorbance was recorded at 570 nm using a multimode microplate reader (FluoSTAR Omega, BMG Labtech). The percentage of viable cells in the sample was calculated with respect to that of the untreated cell control cells, as previously described (19).

In vivo biocompatibility. The experiment was conducted on 6 Wistar male albino rats. The animals were 12-15 weeks old and weighed ~250 g. Ethical clearance was obtained from the Institutional Animal Ethics Committee (YU/IAEC/25/2022). Animals were supplied by the Animal House at Yenepoya Research Centre. Pre-operatively, a 10-day preparation time was used for standard rat health tests. All animal welfare considerations were taken care. Animals were provided with special housing conditions such as adequate ventilation, humidity 60-65%, temperature of 22˚C, appropriate cage size, *ad libitum* access to food and water. 6 animals were anaesthetised via an intramuscular injection (80 ml/kg) of a combination of ketamine hydrochloride and xylazine (ketamine at 80 mg/kg body weight and xylazine at 10 mg/kg) for 20-30 min. Once the appropriate level of sedation was obtained, through a 1-cm incision, three subcutaneous pockets were created in the paravertebral region using a BP blade (no. 15). Subdermal implantations were performed using the three materials, namely, EHPA, composite graft (EHPA/Collagen), composite graft with glycerine (EHPA/Coll/Gly), in the left, middle and right subcutaneous pouches, respectively in all 6 animals. Following implantation, the wounds were sutured using four-zero silk sutures. Additionally, 0.1% gentamicin was applied to the wound, and diclofenac 75 mg was injected intramuscularly. Following anaesthesia using an intramuscular injection (80 ml/kg) of combination of ketamine hydrochloride and xylazine (ketamine at 80 mg/kg body weight and xylazine at 10 mg/kg), The wound was sutured with 4-0 silk sutures followed by the application of 0.1% gentamycin and injection of 75 mg diclofenac. The animals were observed twice daily for a period of 3 months. All animal welfare considerations were used to minimize suffering and distress. No death of any animal was reported during this period. Tissue processing procedures were carried out using a tissue processor (Leica Microsystems). The recovered tissue samples (after 14 days) around the implanted sites were procured and stored in buffered formalin for 48 h at room temperature. The tissues were then embedded in paraffin blocks. The samples were cut to a thickness of 6 microns using a microtome (Leica RM 2245).

For histological analyses, the samples were stained using haematoxylin and eosin (Nice Chemicals Private Limited) following deparaffinisation using Xylene for 5 min followed by hydration through graded alcohols. Nuclear staining was performed using Meyer's haematoxylin (2 min) followed by rinsing with water. Cytoplasmic staining was performed with eosin (2 min) at room temperature followed by dehydration, clearing and mounting. The slides were examined under microscope (Olympus CX41 Research Microscope) and microphotographs were obtained. The slides were evaluated by two pathologists who were unaware of the materials used in the experiment. Representative areas with the highest density of inflammatory cells were selected. The inflammatory responses were graded by counting the number of inflammatory cells at high power in the field showing maximum inflammatory cell infiltrate as previously described (20) (Table I).

Table I. Grading of the inflammatory response.

Grade of inflammation	Inflammatory response
Grade 0	Absence of inflammatory cells
Grade 1	Mild inflammation, an average of <25 inflammatory cells per high power field
Grade 2	Moderate inflammation, an average of 25-124 inflammatory cells per high power field
Grade 3	Severe inflammation, an average of ≥125 inflammatory cells per high power field

The information presented in this table has been obtained from a previous study (20).

Statistical analysis. The cell viability assays were performed in triplicate and the data are presented as the mean and standard deviation. The statistical analysis was performed using Statistical Package for the Social Sciences 18.0 (SPSS, Inc.) The Kruskal-Wallis test was used to compare the mean inflammatory scores between the groups followed by Dunn's multiple comparison test. A P-value <0.05 was considered to indicate a statistically significant difference. Spearman's rank correlation analysis was used to assess difference between the inflammatory scores within the same animal.

Results

Cytotoxicity of the materials. All three materials, EHPA, EHPA/Coll and EHPA/Coll/Gly, were found to be non-cytotoxic after 14 days. When the cells were examined under a phase contrast microscope, no morphological alterations were detected (Fig. 1).

The effects of the materials at various concentrations on cell viability are demonstrated in Table II. Although all the materials promoted cell growth, the EHPA/Coll material resulted in greater cell viability than did pure EHPA or EHPA/Coll/Gly at concentrations of 12.5, 25 and 50 μg/ml. However, at a concentration of 100 μg/ml, EHPA promoted the increased growth of murine fibroblasts.

Skin reactions following the placement of the materials. There were no adverse skin reactions noted throughout a period

Table II. Effect of the materials at various concentrations on the viability of mouse fibroblasts.

	Cell viability, %		
Concentration, μg/ml	EHPA	EHPA/Coll	EHPA/Coll/Glyc
12.5	91.01±3.904	116.33±3.202	79.76±1.769
25	96.03±2.766	123.15±3.741	113.49±3.487
50	116.38±3.093	128.58±3.926	121.01±2.998
100	122.82±2.868	117.59±2.648	106.4±2.597

EHPA, egg shell-derived hydroxyapatite; Coll, collagen; Gly, glycerine.

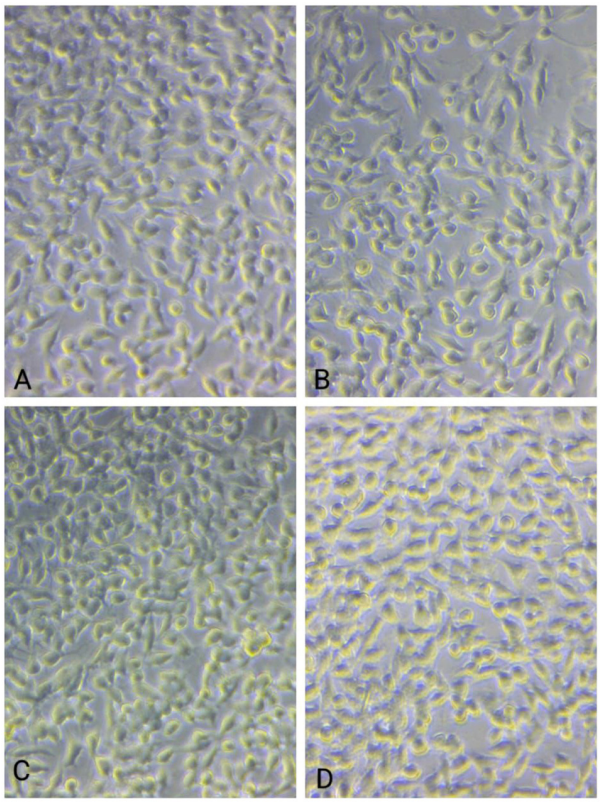

Figure 1. Phase contrast images of the cells treated with the indicated samples at x100 magnification. (A) Control, (B) eggshell-derived hydroxyapatite, (C) composite graft (EHPA/Coll), (D) composite graft with glycerine (EHPA/Coll/Gly). EHPA, egg shell-derived hydroxyapatite; Coll, collagen; Gly, glycerine.

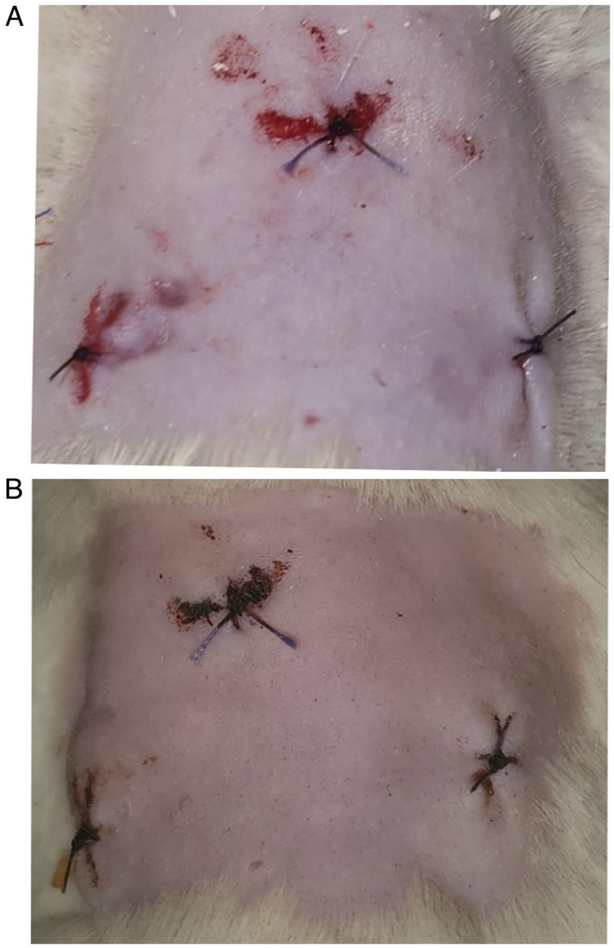

Figure 2. Skin reactions following the placement of materials. (A) Material placed in subcutaneous pouches; (B) 7th day post-operative image (no notable skin reactions such as colour changes, enema, rashes, or pus discharge were observed).

of 14 days after the materials were placed in the dorsal soft tissue pouches of the animals. Given that the initial inflammation caused by surgical trauma is a phase of healing, all sites exhibited a minimal inflammatory response. There were no notable signs of hypersensitivity reactions (Fig. 2). The number of inflammatory cells at a high power field exhibiting the maximum inflammatory cell infiltrate was calculated (Table III). At 14 days post-surgery, the initial phagocytic activities of the cells were noted, followed by the degradation process and the subsequent reabsorption of the material. In the implantation area, the particles were surrounded by collagen fibres, accompanied by a mild inflammatory infiltrate. A sparse number of multinucleated cells and large blood vessels were also observed. Minimum numbers of inflammatory cells were noted at all three sites (Fig. 3).

The Kruskal-Wallis was used to compare the mean inflammatory scores, which revealed that there was no statistically significant difference in the grade of inflammation (number of inflammatory cells per unit area) between the groups at the 2nd week (Fig. 4). Within the same animals, no correlation was found between the inflammatory scores produced by the different materials (Fig. 5). The animals tolerated the surgical procedures well. No adverse reactions occurred during the

Table III. Scoring based on the number of inflammatory cells per unit area recorded.

Sample	Empty control (group A)	EHPA (group B)	Composite graft (EHPA + fish collagen, group C)	Composite graft (EHPA + collagen + glycerine)
Sample 1	1	2	2	2
Sample 2	1	3	2	1
Sample 3	1	2	2	2
Sample 4	1	2	1	1
Sample 5	0	2	2	1
Sample 6	2	2	1	1

EHPA, egg shell-derived hydroxyapatite; Coll, collagen; Gly, glycerine.

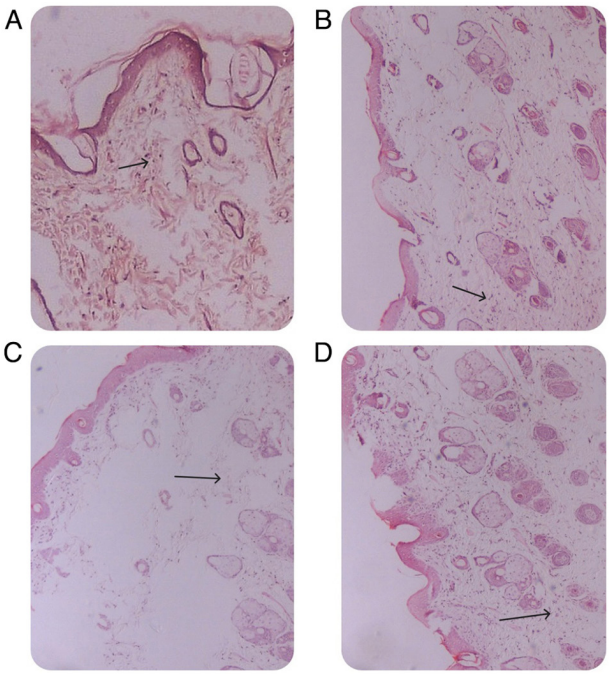

Figure 3. Histological representation of the tissues from the implantation sites after 14 days. (A) Tissue from untreated control sites, (B) tissue from sites treated with the EHPA graft, (C) tissue from sites treated with the composite graft (EHPA/Coll), (D) tissue from sites treated with the composite graft with solvent (EHPA/Coll/Gly). Stereomicroscopy images (at x10 magnification) of the tissues collected from the animals, which included the rat epithelium and connective tissue. The arrows depict the inflammatory cells. EHPA, egg shell-derived hydroxyapatite; Coll, collagen; Gly, glycerine.

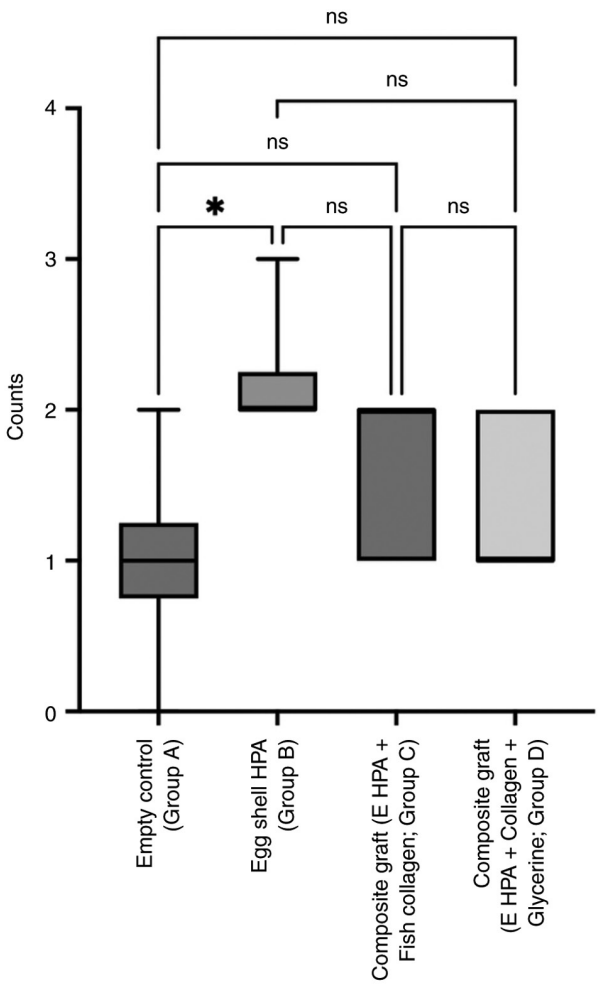

Figure 4. Comparison of the mean inflammatory scores between the groups. The Kruskall-Wallis test revealed no significant difference between the groups (P>0.9999), although slightly higher inflammatory scores were noted in the EHPA group compared to the empty control (P=0.0137). *P<0.05 denotes a significant difference.

14-day study period. There was no difference in the mean inflammatory score between the groups on the 14th day. Further observations for 3 months period also did not reveal any adverse reactions.

Discussion

The evaluation of the cytotoxicity and biocompatibility of regenerative biomaterials is a preliminary and crucial step before their effectiveness in regenerative medicine can be assessed. Cytotoxicity refers to the ability of a material to destroy cells, whereas biocompatibility is the capacity of the material to perform its function without causing detrimental effects in the body.

The three bone substitutes derived from eggshells, eggshell-derived hydroxyapatite, EHPA modified with fish collagen, and EHPA/collagen/Gly were found to be non-cytotoxic and their use led to good cell viability, promoting the growth of L929 fibroblasts. No morphological changes were detected in the cells, and minimal inflammatory infiltration

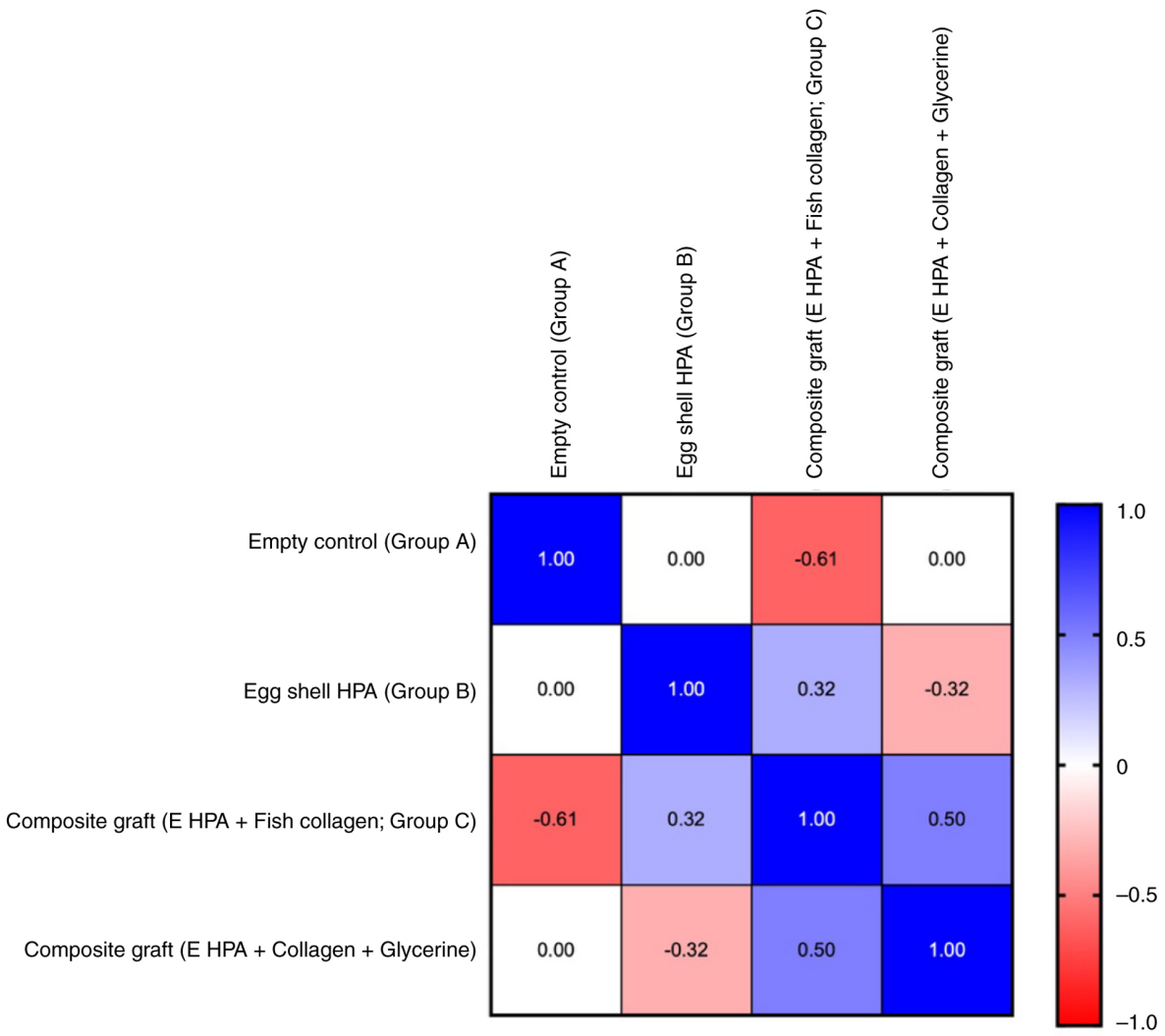

Figure 5. Correlation of the inflammatory scores associated with different materials within the same animal. Spearman's correlation revealed no correlation between the mean inflammatory scores produced by different materials within the same animal.

was noted. Similar observations were noted in other cell lines in other studies, as follows:

In their study, Gutiérrez-Prieto *et al* (21) reported a cell viability of 96% for experimental eggshell-derived hydroxyapatite modified with silicon and poly lactic-co-glycolic (PLGA); 90% for bovine bone-PLGA, and 86% for EHPA/PLGA in osteoblast cell lines. Oladipupo *et al* (22) performed an MTT assay on MG63 cells (osteosarcoma cell lines) and ascertained that, by comparing a sample of EHPA with various concentrations of ammonium bicarbonate, a sample that contained 0% ammonium bicarbonate led to a viability of >85%. By contrast, in EHPA with 40% ammonium bicarbonate, toxicity was detected (22).

In another study, direct-contact assays on hydroxyapatite-β TCP/agarose disks revealed no cytotoxic effects on L929 fibroblasts or human osteosarcoma cells (23). The adherence and proliferation of both cell types on the biomaterial surface, maintaining their characteristic morphology, were observed. Few transient changes in several properties, such as cell cycle, size and complexity were observed. Mild apoptotic changes were induced in Saos-2 osteoblasts but not in fibroblasts (23). These findings support the findings of the present study.

Ma *et al* (24) reported that eggshell-derived amorphous calcium phosphate (ACP) particles displayed better biocompatibility than ACP synthesised from synthetic hydroxyapatite in three-dimensional osteoblastic spheroids. Another study on the effects of eggshell granulate and calcium carbonate on bovine osteoblasts revealed that additives (eggshell-derived calcium carbonate) increased osteoblast activity. The cell cultures treated with eggshells displayed the most potent effects, whereas for hyaluronan, a weaker cell activity was detected (25).

Chuysinuan *et al* (26) conducted a primary cytotoxicity test on MC3T3 cells and reported that the newly fabricated hydrogel, egg shell hydroxyapatite incorporated fibroin alginate hydrogel, was non-cytotoxic. He *et al* (27) also reported that the biomimetic collagen composite matrix-hydroxyapatite scaffolds exhibited high biocompatibility in critical-sized cranial defects in a rat model.

Biocompatibility tests have also revealed that human mesenchymal cells can infiltrate and remain viable after culture on collagen-hydroxyapatite scaffolds and antibiotic-doped substrates (ciprofloxacin and gentamycin) (28). Wang *et al* (29) established that the collagen/glycerol/pullulan gel exhibited

maximum cell attachment and uniform cell distribution, with an improved morphology displaying better extension and three-dimensional characteristics, with sufficiently extended filamentous pseudopods. It was hypothesized that glycerine and pullulan enhanced the physical properties of the gel and also were conducive to cell attachment and proliferation (29). The present study also revealed that the incorporation of glycerine does not negatively impact the cell viability of the material.

In the present study, at 1 week post-implantation, no adverse skin reactions (Fig. 1B) were noted at all three sites. At 2 weeks, the initial phagocytic activities of the cell were noted, followed by the degradation process and subsequent reabsorption of the material. In the implantation area, the particles were surrounded by collagen fibres, with mild inflammatory infiltrate. A sparse number of multinucleated cells and large blood vessels were also observed. The minimum number of inflammatory cells was noted at all three sites.

In the study by Prohl *et al* (30), the histological analysis of inflammatory tissue reactions was compared between a novel material based on a xenogenic bone substitute combined with hyaluronic acid and another xenogenic material of similar composition and a sham operation group. A 2 weeks post-implantation, moderate inflammatory reactions were observed in all three study groups. No differences were found between the groups with respect to pro-inflammatory and anti-inflammatory cells (30).

The study by Markel *et al* (31) revealed that the HPA product generated a low inflammatory reaction compared to demineralised bone matrix. L929 cells, which are mouse fibroblast cell lines, due to their pro-healing properties, are advantageous in regenerative medicine. Hence, their use is highly recommended in studies involving biomaterials, drug delivery and tissue regeneration.

The present study demonstrated that the incorporation of additives, such as fish collagen and glycerol into eggshell-derived hydroxyapatite did not have any negative impact on the cell viability or biocompatibility of the materials. The materials did not lead to any adverse skin reactions in the Wistar rats; hence, their potential as bone regenerative materials should be considered. Further *in vivo* studies on animal models and humans are warranted to establish the regenerative potential of the material.

The present study has certain limitations which should be mentioned. The present study only evaluated the *in vitro* cell viability of the materials and the tissue reactions following their placement in the subcutaneous pouches of Wistar rats. Other mechanical properties, such as compressive strength and injectability, need to be assessed. The regenerative capacity of the materials needs to be confirmed *in vitro* and in animal models before performing any human studies.

In conclusion, in the present study, hydroxyapatite derived from egg shells was used and then modified with fish collagen and glycerine. The samples were evaluated for their cell viability in mouse fibroblasts. All three materials were found to be non-cytotoxic. The biocompatibility was tested in an animal model, which established that the materials were biocompatible. Further studies are required however, to prove their regenerative properties and their application as bone substitutes.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SP was involved in the conception of the study, in acquisition and interpretation of data, and in the drafting of the manuscript. RKS was involved in the conception of the study, in the interpretation of data, in manuscript editing, and in the critical evaluation of the study content. NGT was involved in the conception of the study, in the interpretation of data, and in the evaluation of the study content. SS conducted the experiments and was involved in the acquisition of data. RA was involved in the acquisition of data, in data interpretation, and in manuscript preparation. BPK conducted the experiments and was involved in the conception of the study. PMS was involved in data interpretation and manuscript revision. SS and RA confirm the authenticity of all the raw data. The manuscript has been read and approved by all the authors.

Ethics approval and consent to participate

Ethical approval was obtained from the Institutional Animal Ethics Committee (YU/IAEC/25/2022).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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