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Development of Biomimetic Mouldable Osseous Scaffold Engineered from Bioactive Decellularized Extracellular Matrix-polymer-gel Composite: A potential bone substitute

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Abstract

Critical-sized bone defects remain a major clinical challenge in veterinary orthopaedics due to limited intrinsic regenerative capacity and the drawbacks of conventional grafting options. This study reports the development and systematic evaluation of a novel bioinspired osseous scaffold engineered from decellularized bubaline cancellous bone granules, collagen gel, and polycaprolactone, with optional enrichment using autologous adipose-derived stromal vascular fraction. Scaffold physicochemical characteristics—including porosity, material density, and ultrastructure—were assessed through liquid displacement assays, histomorphometry, and scanning electron microscopy, confirming complete decellularization, interconnected porosity, and favourable matrix architecture. Mechanical testing demonstrated significantly enhanced tensile strength in the composite scaffold compared with plain acellular bone–collagen constructs. *In vitro* biocompatibility was validated by adipose derived mesenchymal stem cell adhesion, proliferation, and migration, with DAPI staining confirming uniform cellular distribution and robust scaffold–cell interactions, along with Alizarin Red S Staining for assessment of osteogenic mineralization.

Keywords: Bioinspired scaffold, Decellularized extracellular matrix (dECM), Adipose-derived stromal volume fraction (ADSCc), critical-sized defect (CSDs), polycaprolactone (PCL), stromal vascular fraction (SVF)

Introduction

Bones are living organs that provide the body's structural framework: they give shape and support, protect vital organs, and enable movement. They also serve as reservoirs of minerals (e.g. calcium) and contribute to blood cell production and metabolic regulation [1]. Although bone has intrinsic healing ability, defects that exceed its self-repair capacity will not heal on their own. Critical-sized bone defects (CSDs) – by definition, the smallest intraosseous injuries that will fail to heal spontaneously within an organism's lifetime – thus require clinical intervention [2]. Such large bone gaps can result from severe trauma (including fractures with non-union or infection), tumor resection, congenital malformations, or extensive osteolysis. In veterinary patients, as in humans, CSDs compromise skeletal integrity and function and pose a major clinical challenge [2,3]. The Current reconstructive approaches for segmental bone loss include: Autologous bone grafts which involves transplanting the patient's own cancellous bone (e.g. from the iliac crest or fibula) remains the clinical gold standard because it provides live osteogenic cells and native matrix cues. However, autografts are limited in supply and cause donor-site morbidity, pain, and prolonged recovery [4]. Secondly, Allografts/Xenografts. Banked bone from donors (allograft) or other species (xenograft) can supply an osteoconductive scaffold. Yet these grafts are only osteoconductive (they lack viable cells), and they carry risks of immune reaction or disease transmission [5]. Even with current sterilization methods, residual antigens in allogeneic or xenogeneic bone can provoke inflammation or rejection. Lastly, synthetic substitutes which includes metallic implants, bone cements (e.g. PMMA), ceramics or polymers provide immediate structural support, but they do not remodel into living bone [6]. In practice, synthetic materials can lead to complications (e.g. stress shielding, inflammatory reactions, thermal necrosis) and are often non-degradable, necessitating eventual removal [5,6]. Because of these limitations, there is intense research into tissue-engineered bone substitutes that combine biocompatibility with regenerative potential. In particular, scaffolds derived from decellularized extracellular matrix (dECM) aim to recapitulate the native bone microenvironment. Such natural scaffolds retain native bone proteins, mineral structures, and growth factors while being non-immunogenic. Recent reviews have shown that decellularized bone matrices are highly osteoconductive and well tolerated by host tissue. In fact, decellularized xenograft cancellous bone scaffolds have been proposed as viable alternatives to autograft bone, with studies reporting “high biocompatibility with little or no host response” and robust new bone formation [7].

In a veterinary context, one attractive ECM source is bubaline (water buffalo) bone. Buffalo-derived ECM scaffolds combine the advantages of preserved bone architecture with cultural and safety benefits. For example, bubaline tissues avoid some zoonotic risks of bovine sources and are acceptable in regions where cattle products are restricted [8]. Recent work has shown that buffalo-derived collagen matrices and bone ECM maintain their native ultrastructure and support tissue regeneration to enhance scaffold mechanics and handling, synthetic polymers such as polycaprolactone (PCL) can be incorporated. PCL is a biocompatible, slowly degradable polyester often used to reinforce bone grafts. For instance, Wang et al. fabricated porous PCL scaffolds infused with decellularized bone matrix and found that the composite closely matched the mechanical properties of native bone [9,10]. Importantly, the presence of bone ECM on the PCL dramatically enhanced osteogenic cell behaviour: stem-cell adhesion, proliferation and differentiation were markedly increased, and mineral deposition was far greater than on PCL alone [10]. In that study, the dECM/PCL

scaffolds significantly boosted bone matrix formation, underscoring the value of combining natural ECM cues with a supportive polymer framework [9].

Another strategy is to augment scaffolds with cells. Autologous stromal vascular fraction (SVF) the heterogeneous cell population obtained from a small sample of the patient's adipose tissue – has shown promise in tissue repair. Adipose-derived SVF contains a rich mixture of progenitor and accessory cells (adipocyte precursors, endothelial and pericyte cells, immune cells, etc.) along with growth factors. This complex cell pool provides a broad regenerative stimulus and can enhance angiogenesis and osteogenesis when delivered with scaffolds [11].

In the present study, we have therefore developed a novel mouldable composite scaffold for treating CSDs in veterinary orthopaedics. Our scaffold is built from pulverized decellularized bubaline cancellous bone granules embedded in a type I collagen gel and reinforced with a porous PCL framework. Type I collagen (the predominant organic matrix in bone) provides a favourable cell substrate, while the bone granules supply native mineral and matrix signals. The PCL structure gives the scaffold mechanical integrity and allows it to be shaped intraoperatively. We hypothesize that this bioinspired composite will mimic the architecture of trabecular bone (complete decellularization, interconnected porosity) and will support host cell invasion and new bone formation. In brief, we designed this scaffold to combine two naturally derived bone components (mineralized ECM chips and collagen) with a synthetic polymer to overcome the weaknesses of each material alone. To our knowledge, no prior report describes a ready-to-use, mouldable bone graft composed of decellularized bone particles plus collagen and PCL. We aim to demonstrate that this scaffold, optionally seeded with autologous adipose SVF, can provide a stable, osteoconductive matrix for repairing critical-sized defects.

Materials and Methods

Ethical approval

All experimental procedures were approved by the Institute Animal Ethics Committee, Indian Council of Agricultural Research (ICAR)-Indian Veterinary Research Institute.

Study period and location

The study was conducted from February 2024 to December 2024 at the Division of Surgery, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.

Experimental animals approval

Ten clinically healthy New Zealand White rabbits (*Oryctolagus cuniculus*), aged 8–12 months and weighing 2.3–2.8 kg (6 males, 4 females), were enrolled after Institute Animal Ethical Committee (IAEC) approval. Animals were housed individually under standard laboratory conditions and fed a balanced diet with ad libitum water. A 2-week acclimatization period was provided prior to experimentation, with a controlled 12-h light/dark cycle maintained throughout the study.

Decellularization of Scaffolds

Decellularization of Bubaline bone and tendon

Cancellous femoral bone blocks of bubaline origin were processed using a previously validated decellularization protocol [12] with minor modifications. Samples were cut into trabecular blocks, subjected to sequential chemical treatments to remove cellular components, and lyophilized. The decellularized extracellular matrix (dECM) was fragmented into chips and homogenized to obtain uniform granules (Fig. 1A–C)

Collagen Extraction from Tendon

Decellularized bubaline Achilles tendon was processed to produce collagen gel, which served as the organic phase for scaffold fabrication (Fig. 1D)

Development and standardisation with optimization of Bioinspired composite osseous scaffolds

A bioinspired scaffold was engineered using pulverized dECM granules, collagen gel, and polycaprolactone (PCL) to mimic the biochemical and biomechanical properties of native bone. Multiple combinations of dECM (6–80 weight %), collagen gel (5–35 weight %), and PCL (0.5–5 weight %) were tested to optimize mouldability, handling characteristics, binding efficiency, and mechanical strength for segmental defect repair (Fig. 1C–F)

To establish the ideal formulation, multiple combinations of dECM granules (6%, 8%, 10%, 14%, 18%, 20%, 50%, 70%, and 80% w/w), collagen gel (5%, 10%, 15%, 20%, 25%, and 35% w/w), and PCL (0.5%, 1%, 1.5%, 2%, 3%, 4%, and 5% w/w) were systematically evaluated. Each formulation was assessed for mouldability, mechanical integrity, handling characteristics, and binding capacity to identify a scaffold suitable for segmental bone defect repair.

On the basis of these optimization trials, three scaffold variants were standardized:

Type-I: Highly mouldable injectable filler:

dECM granules (30 weight%), collagen gel (65 weight%), and PCL (5 weight%).

This formulation yielded a soft, easily mouldable scaffold suitable for filling irregular or small voids.

Type-II: Balanced mouldable scaffold:

dECM granules (50 weight%), collagen gel (45 weight%), and PCL (5 weight%).

This composite provided a balance between pliability and mechanical stability, appropriate for moderate load-bearing sites.

Type-III: High-stiffness scaffold:

dECM granules (70 weight%), collagen gel (25 weight%), and PCL (5 weight%).

This variant demonstrated increased rigidity and was intended for larger defects requiring a firmer, more supportive structure. Hence Type-III was selected for further *in vivo* studies in managing critical sized bone defects.

These optimized compositions formed the basis for the bioinspired osseous scaffolds evaluated in the present study.

Sterilization of bone grafts

Scaffolds were thoroughly rinsed with 1× PBS 10–15 times to remove residual chemicals, followed by a PBS wash containing antibiotics. Constructs were then immersed in DMEM and

incubated overnight for sterilization and equilibration. Prior to the application on patients, grafts were exposed to Ultraviolet (UV) sterilization for 30 minutes.

Experimental design

Ten clinically healthy adult New Zealand White rabbits (*Oryctolagus cuniculus*) were procured from the Laboratory Animal Resources Section, ICAR–Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India. The animals were 9-10 months of age, with an average body weight of 2.17 ± 0.27 kg. All rabbits were acclimatized to the experimental environment for 15 days prior to the commencement of the study. All the rabbits were provided with 12 hours dark/light cycle.

Anaesthesia and restraint

The surgical procedures include adipose tissue collection for extracting stromal volume fraction (SVF), the animals were anesthetized using xylazine hydrochloride (Indian immunological limited, India) at the rate of 6 mg/kg body weight intramuscularly and ketamine hydrochloride (KETAPIL, Pil, India) at the rate of 60 mg/kg body weight intramuscularly as per [13], keeping a gap of 10 minutes between the time of injection of two drugs.

Adipose tissue collection

Rabbits were positioned in sternal recumbency, and the dorso-thoracic region was aseptically prepared. A 2–3 cm longitudinal incision was made over the interscapular fat pad, and approximately 5-6 g of adipose tissue was dissected and collected in sterile PBS with antibiotics (Fig. 2A–C). Wound closure was performed using 3-0 polyglactin 910 (subcutaneous) and 3-0 polyamide (skin) sutures

Isolation, culture and characterisation of adipose derived SVF

Adipose tissue was washed in antibiotic-supplemented PBS and centrifuged at 2000 rpm for 2–3 minutes to eliminate debris. Samples were finely minced and centrifuged again. The tissue was then digested in 0.1% collagenase at 37 °C for 1 hour with gentle agitation (Fig. 3A & B). Digestion was neutralized using complete DMEM (10% FBS, 1% penicillin–streptomycin), filtered through a nylon strainer, and centrifuged at 2000 rpm for 10 minutes. The resulting SVF pellet was resuspended in 0.5 mL complete medium (Fig. 3C).

***In vitro* Mechanical strength testing of scaffold**

The mechanical properties of the acellular bone scaffold and two formulations of the bioinspired composite scaffold—

- (i) dECM granules 50 weight% + collagen gel 45 weight% + PCL 5 weight%, and
- (ii) dECM granules 70 weight% + collagen gel 25 weight% + PCL 5 weight%

were evaluated using a 25 kN universal tensile testing machine (Model AMT-SC; S. No. 01521). Each sample was mounted securely in the machine grips and subjected to uniaxial tensile loading at a constant crosshead speed of 5 mm/min until failure. Tensile strength was assessed by applying force parallel to the longitudinal axis of the graft, and supplementary diagonal axis measurements were also recorded. Load–displacement curves were generated for all specimens, and failure points were identified using a strain threshold mode set at 50% strain.

The mechanical behaviour of the two composite scaffold variants was compared with that of the decellularized bone graft.

Material density test

Material density was measured by ethanol liquid displacement following as per [14]. One-way ANOVA with Tukey's HSD was used to evaluate differences between native bone, dECM, and composite scaffolds

Apparent density

Apparent density was calculated using: **Density (d) = Mass / (length × breadth × thickness)** Dimensions were measured using vernier calipers and a screw gauge. Statistical comparisons used one-way ANOVA with Tukey's HSD

Porosity testing

The percentage of pores (empty space) in a scaffold or solid surface is defined as porosity. Porosity (%) was measured using ethanol displacement as per [15]. Differences among native bone, dECM, and composite scaffolds were analyzed using one-way ANOVA ($p < 0.05$)

SVF Adsorption onto Composite Scaffolds

The composite scaffold (dECM + collagen gel + PCL) was seeded with autologous SVF using a multiple-injection technique to ensure uniform cell distribution across its structure.

Assessment of Cell Seeding Uniformity

Microscopic Evaluation

Scaffold-cell interaction was assessed on the basis of ADSC attachment, proliferation, and viability were evaluated at days 3, 7, and 14 using DAPI nuclear staining (Fig 4 A-C). Scaffolds were cultured in DMEM with 15% FBS and 100 U/mL penicillin–streptomycin under 5% CO₂ and 85% humidity. Early attachment was assessed after 4 hours of incubation.

Histomorphometric analysis of scaffolds

Native bone, acellular bone, composite scaffolds, and ADSC-seeded scaffolds (Fig.5 A-C) were trimmed to 1 × 1 cm, fixed in 10% neutral buffered formalin (48–72 h), and decalcified using Goodling and Stewart's fluid (formic acid–formalin mixture). Decalcification progress was monitored by flexibility, transparency, and pin penetration tests. Tissues were processed routinely, sectioned at 4 μm, and stained with hematoxylin and eosin following the protocol's as per [16]. Statistical comparison was performed with one-way ANOVA and Dunnett's test.

Scanning electron microscopy (SEM)

SEM was performed on dECM scaffolds, composite scaffolds, and ADSC-seeded scaffolds to evaluate surface morphology, pore architecture, and cell distribution. Samples were fixed in 2.5% glutaraldehyde overnight at 4 °C, washed in PBS, dehydrated through graded ethanol (30–100%), and subjected to critical-point drying using CO₂. Specimens were mounted on aluminium stubs, coated with hexamethyldisilazane (HMDS), and sputter-coated with gold/palladium (7–10 mA, 1–2 kV, 15 min) before imaging (Model JFC 1600). (Fig.6 A-C)

Cell Scaffold Interaction

Twelve-well multi-well plates were filled with bioinspired scaffolds. The scaffolds were incubated with a culture medium in a CO₂ incubator for one night prior to cell seeding. The scaffolds were then gradually loaded with cells in the centre at a concentration of 1.0×10^6 cells per scaffold in a total volume of 50–75 μ l after the medium was aspirated out and repeated after 6 hours with same number of cells. The multi-well plates were then gently incubated for the first two to three hours, without being moved, to promote cell adhesion. The cell-seeded scaffolds were then subsequently incubated for two to three days for cell growth after the final culture volume was increased to 1-2 ml. Then, scaffolds were seeded with cells and the scaffolds were positioned on fresh culture wells. Subsequently, the scaffolds underwent three meticulous washings in sterile PBS, after this initial incubation, the scaffolds were transferred to fresh culture wells and underwent three meticulous washes with sterile PBS (Fig.7).

Alizarin Red S Staining for Assessment of Osteogenic Mineralization

Alizarin Red S (ARS) staining was employed to evaluate extracellular matrix mineralization as an indicator of osteogenic differentiation. ARS is a calcium-binding dye that selectively complexes with calcium salts deposited during late-stage osteoblast maturation, thereby serving as a reliable marker for mineralized nodule formation, on day 3, 7 and 21 (Fig.8 A-C).

Briefly, differentiated osteogenic cells cultured on scaffolds were fixed with 4% paraformaldehyde for 15–20 min at room temperature and washed thoroughly with phosphate-buffered saline (PBS). Fixed samples were incubated with 2% (w/v) Alizarin Red S solution (pH adjusted to 4.1–4.3) for 20–30 min under gentle agitation. Excess stain was removed by repeated washing with distilled water until a clear background was obtained as per [17]. The stained samples were examined under an inverted light microscope, and mineralized regions were identified by the presence of intense red–orange deposits corresponding to calcium-rich nodules. This protocol is widely accepted for qualitative and semi-quantitative assessment of osteogenic mineralization *in vitro*.

Results

The present study was conducted to develop and evaluate bioinspired and composite osseous scaffolds. The purpose of this study was to investigate relative application for clinical healing in critical sized bone defect. The technique of seeding ADSC on bioinspired composite scaffold was optimized and *in vitro* evaluation of grafts for clinical application using a set of relevant parameters were carried out at Division of Surgery ICAR-IVRI, Izatnagar.

Stromal Vascular Fraction (SVF) Yield and Cell Morphology

Adipose tissue harvested from the interscapular depot yielded a consistent and viable population of stromal vascular fraction (SVF) cells. Manual counts using a Neubauer haemocytometer demonstrated an average concentration of $2.98 \pm 0.15 \times 10^4$ cells/ μ L, corresponding to a yield of $2.98 \pm 0.15 \times 10^6$ cells/g of adipose tissue (Fig.9). These findings align with previous reports highlighting the interscapular region as a reliable adipose source in rabbits [18]. Microscopic evaluation confirmed the presence of spherical, uniformly dispersed SVF cells with clear nuclear boundaries, indicating minimal shear-induced damage during processing. This high-yield isolation validated the suitability of the adopted enzymatic digestion protocol for generating ADSC-rich SVF for scaffold seeding.

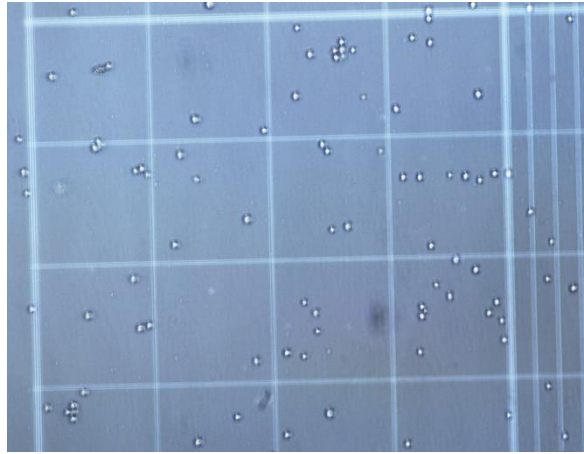


Figure 9 Cells present in the adipose-derived stromal vascular fraction (SVF) observed in the Neubauer's chamber of the hemocytometer (40 $\times$).

Quantification of Total SVF Cell Population

The Neubauer's chamber, was employed to assess the overall count of Adipose-Derived SVF in 5-7 grams of adipose tissue. This method, widely recognized as the gold standard for cell counting, is both straightforward and easily accessible as per [18]. The total cell count in the ADSC was determined by averaging cell counts within specified grid areas and incorporating the dilution factor. In this particular study, the dilution factor was considered as 1, as the ADSC was used without any further dilution. A total of nine rabbit samples were examined to evaluate the autologous adipose derived SVF in this research. As depicted in (Fig.4.)

2. Physicochemical Evaluation of Scaffolds

2.1 Material Density

Material density values varied significantly ($p < 0.0001$) across native bone, acellular scaffolds, and composite scaffolds ($F(2,27) = 74.3$). The acellular scaffold exhibited higher density than the composite scaffold but approached the values of native bone, suggesting effective decellularization without compromising mineral integrity. This corresponded well with SEM observations showing preserved trabecular microarchitecture.

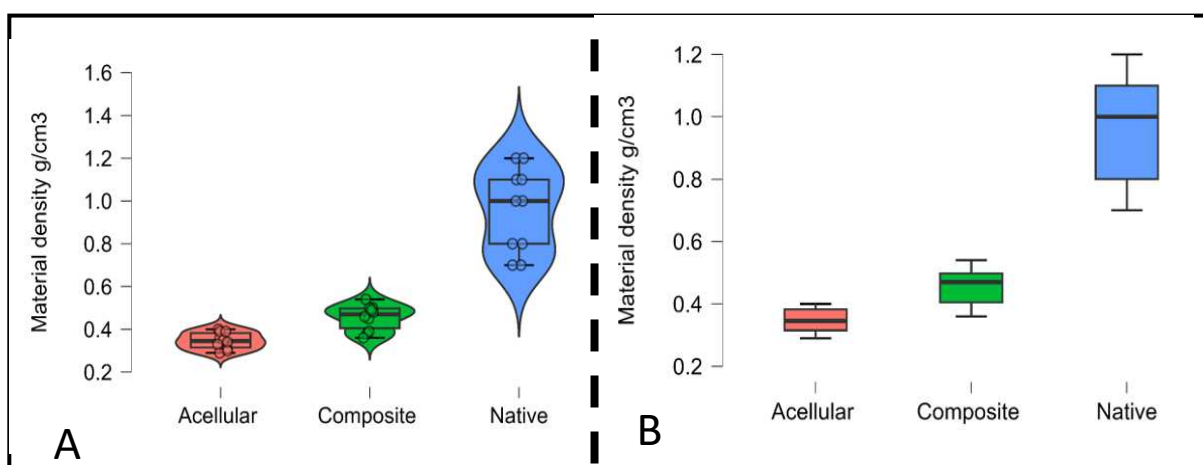


Figure 10 Comparison of **material density (g/cm³)** among native bone, acellular scaffold, and composite scaffold groups. (A) Violin plots illustrating the distribution and variability of

material density values across the three groups. **(B)** Box plots representing the median, interquartile range, and dispersion of the same dataset. The acellular scaffold exhibited higher density than the composite scaffold but remained lower than native bone, while the composite scaffold demonstrated an intermediate density profile approaching that of native bone. These findings indicate that the decellularization process preserved mineral components while maintaining the structural characteristics of the scaffold. Data are expressed as individual observations with median and quartile distribution.

2.2 Apparent Density

Apparent density differed significantly between all groups ($F(2,27) = 30.4$, $p < 0.0001$). The composite scaffold displayed an intermediate density profile, reflecting the balanced contribution of organic matrix (collagen), mineral phase (dECM granules), and the polymeric reinforcement (PCL). The lower apparent density relative to native bone highlights the scaffold's porous nature—a critical factor for fluid transport, vascular infiltration, and osteoconduction.

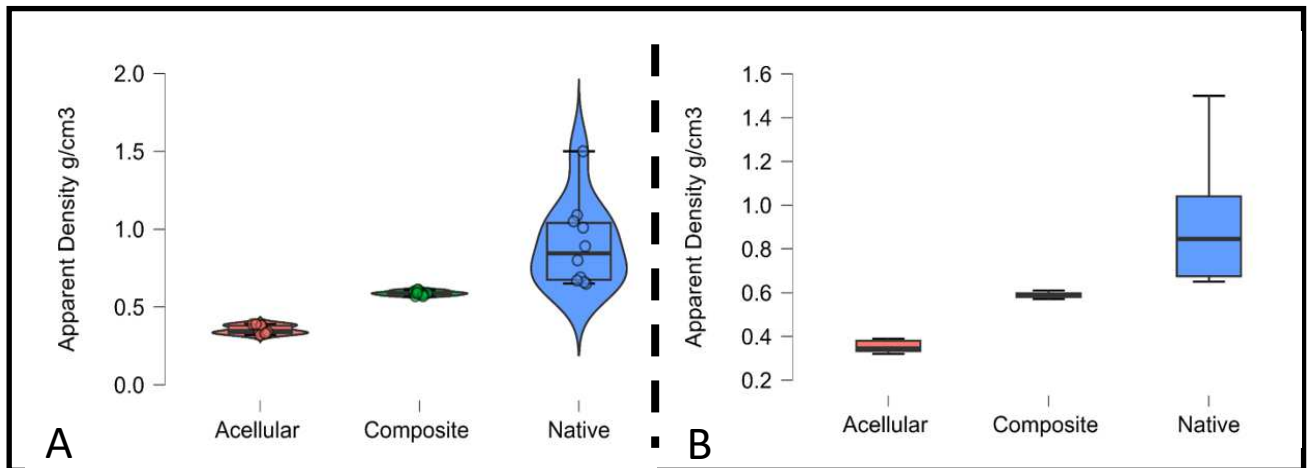


Figure 11. Comparison of apparent density (g/cm^3) among native bone, acellular scaffold, and composite scaffold groups. (A) Violin plots showing the distribution pattern of apparent density values for each group. (B) Box plots depicting the median and interquartile range of the measurements. Native bone exhibited the highest apparent density, whereas the acellular scaffold showed the lowest values. The composite scaffold demonstrated intermediate density characteristics, reflecting the combined contribution of collagen matrix, mineral phase derived from decellularized extracellular matrix (dECM), and polymer reinforcement. The relatively lower apparent density compared with native bone suggests the presence of porosity within the scaffold, which may facilitate fluid transport, vascularization, and osteoconductive potential.

2.3 Porosity

Porosity measurements demonstrated highly significant intergroup differences ($F(2,27) = 281.6$, $p < 0.0001$). The composite scaffold showed markedly higher porosity than native and acellular bone, supporting its role as an osteoconductive biomaterial. High porosity improves nutrient exchange and facilitates stem cell infiltration, an essential criterion for bone tissue engineering scaffolds.

These findings corroborate the structural expectations of dECM-based biomaterials reported by [15], where optimized porosity strongly influenced osteogenic outcomes.

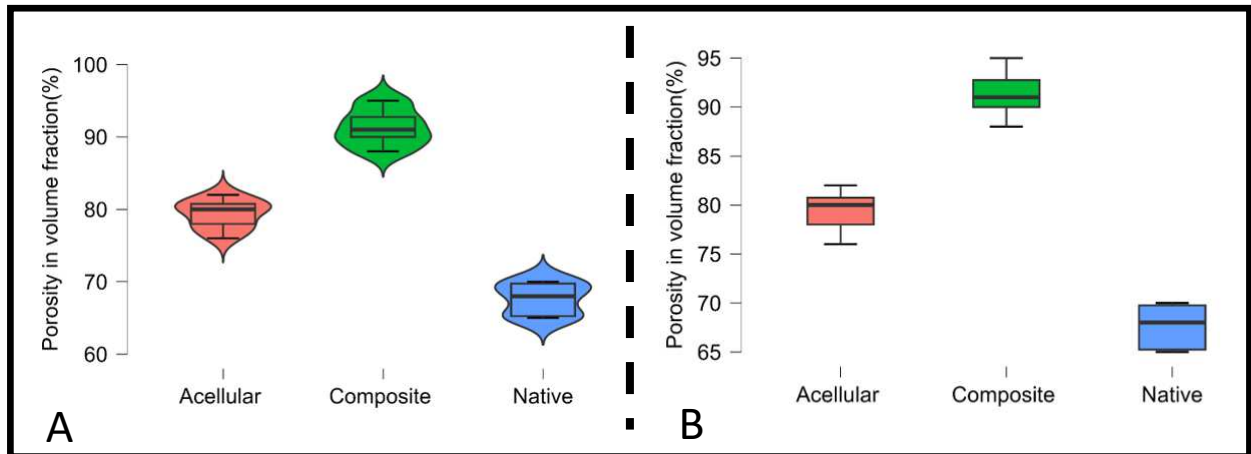


Figure 12 Comparison of **porosity (volume fraction, %)** among acellular scaffold, composite scaffold, and native bone. **(A)** Violin plots illustrating the distribution and variability of porosity values across the three groups. **(B)** Box plots showing the median, interquartile range, and dispersion of the dataset. The composite scaffold exhibited the highest porosity, followed by the acellular scaffold, whereas native bone showed comparatively lower porosity values. The elevated porosity observed in the composite scaffold indicates an interconnected pore architecture, which is favorable for nutrient diffusion, cellular infiltration, and vascularization—key features required for effective osteoconductive bone tissue engineering scaffolds. Data are represented as individual measurements with distribution patterns and quartile ranges.

3. Mechanical Performance of Composite Scaffolds

Mechanical strength analysis revealed significant differences among the tested constructs. The decellularized bone graft demonstrated the highest tensile strength (1328 MPa), consistent with the mechanical superiority of native mineralized matrices. The optimized bioinspired composite scaffold exhibited a tensile strength of 106 MPa, substantially higher than the 50% collagen–50% dECM adhesive-like formulation (58 MPa). This confirms the critical role of PCL reinforcement in providing mechanical stability. Although the tensile strength of the composite scaffold was substantially lower than native bone, the values fall within acceptable ranges for non-load-bearing and moderate-load veterinary defect models, where early-stage biological fixation is more dependent on osteoconduction than mechanical loading. The mechanical advantage imparted by minimal PCL integration highlights the scaffold's hybrid nature—balancing bioactivity with adequate structural support for early regenerative phases.

Statistical analysis was performed using one-way ANOVA to determine differences in tensile strength among the three groups: Acellular bone; Bioinspired scaffold (50/45/5 composition), and Composite-bioinspired scaffold (70/25/5 composition). Significance was set at $p < 0.05$.

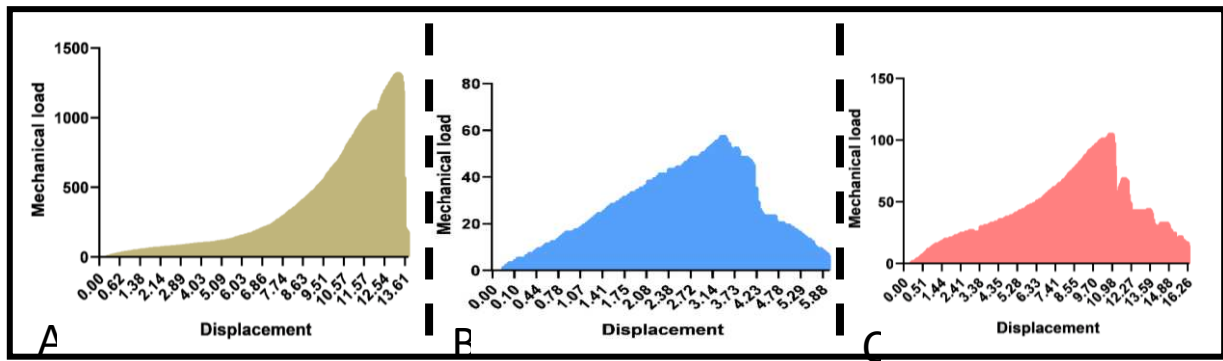


Figure 13. Representative **load–displacement curves** demonstrating the mechanical behavior of the tested constructs during tensile testing. **(A)** Native bone sample showing the highest load-bearing capacity and stiffness, reflecting the inherent mechanical strength of mineralized bone matrix. **(B)** Composite scaffold displaying moderate mechanical resistance and progressive load-bearing behavior, indicating improved structural stability due to polymer reinforcement and mineralized extracellular matrix integration. **(C)** Collagen–dECM adhesive-like scaffold exhibiting lower mechanical strength compared to native bone and the composite scaffold. These results highlight the mechanical reinforcement provided by polymer incorporation in the composite scaffold, contributing to enhanced structural integrity suitable for early-stage bone regeneration applications.

4. ADSC Seeding, Attachment, and Proliferation on Composite Scaffolds

The multiple-injection seeding strategy ensured uniform ADSC distribution throughout the scaffold. Within 24 hours, cells exhibited firm adhesion to the dECM-rich matrix. Progressive proliferation was observed on days 3 and 7, with cells migrating deeper into the pores, facilitated by the favorable microenvironment and interconnected porosity. By day 14, ADSCs demonstrated confluent coverage with widespread intercellular connections. DAPI staining confirmed dense, evenly distributed nuclei throughout the scaffold matrix, verifying both biocompatibility and cytocompatibility. No contamination was detected during the 14-day *in vitro* culture period. These results indicate that the dECM–collagen–PCL composite scaffold provides a biologically permissive niche capable of supporting stem cell activity, consistent with trends reported in similar ECM-derived biomaterials.

5. Cell–Scaffold Interaction and Osteogenic Potential

Microscopic analyses revealed progressive and robust interaction between seeded ADSCs and the composite scaffold. Scaffold architecture supported cell adhesion, migration, and extracellular matrix deposition—behaviors indicative of early osteogenic activity. The combination of dECM granules and collagen gel likely contributed to enhanced integrin-mediated attachment, while PCL maintained construct shape and provided a mild stiffness cue. This synergy reflects principles of biomaterial-guided osteogenesis, where mechanical and biochemical cues together influence stem cell fate decisions.

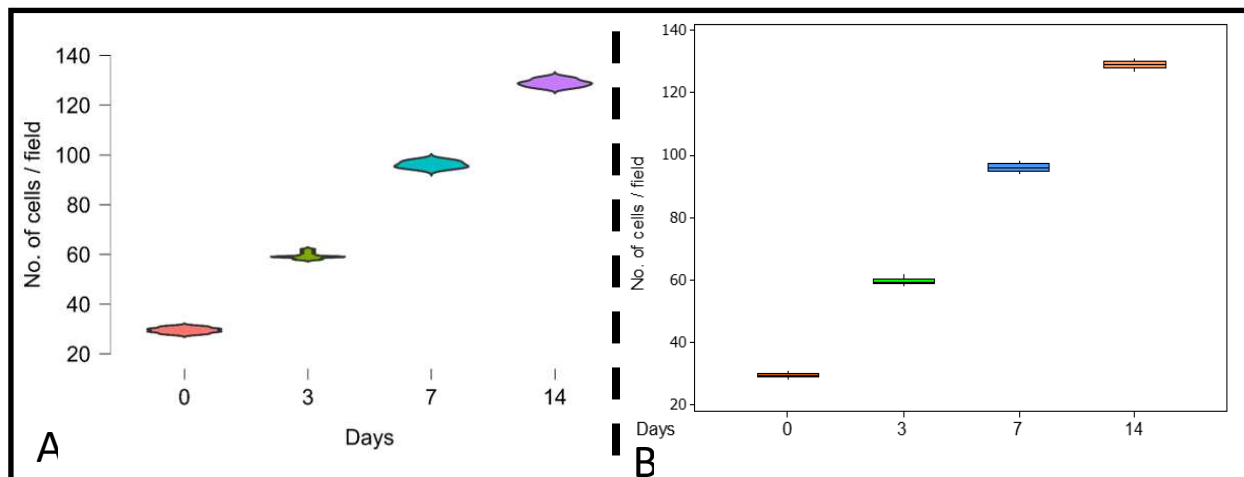


Figure 14. Assessment of cell attachment and proliferation on the composite scaffold over time. **(A)** Violin plot illustrating the distribution of the number of adherent cells per microscopic field at different time points (Day 0, 3, and 7). **(B)** Box plot representing the median and interquartile range of the same dataset. A progressive increase in the number of cells per field was observed over time, indicating effective attachment, proliferation, and spreading of seeded **Adipose-derived mesenchymal stem cells** on the scaffold surface. The increasing cell density reflects favorable scaffold microarchitecture supporting cellular adhesion and early osteogenic activity.

6. Histomorphometric Observations

Hematoxylin and eosin–stained sections of native bone demonstrated dense mineralized trabeculae with well-organized lacunar architecture, serving as a reference standard for comparison. Decellularized bubaline bone scaffolds exhibited complete absence of cellular nuclei, confirming effective decellularization while preserving trabecular arrangement and intertrabecular spaces. Preservation of this microarchitecture is critical, as trabecular geometry directly influences cell migration, vascular infiltration, and mechanical load distribution during bone regeneration. The absence of residual cellular debris further reduces the risk of immunogenicity, supporting the scaffold’s suitability for xenogeneic application. The bioinspired scaffolds displayed a heterogeneous but organized structure consisting of dECM granules embedded within collagen-rich regions. Histological sections revealed well-maintained pore spaces and continuous matrix organization, indicating that scaffold fabrication and sterilization did not compromise structural integrity. The collagen matrix served as a supportive organic phase, enhancing scaffold cohesion while allowing cellular ingress. This microstructural organization closely resembles early granulation and woven bone formation observed during physiological fracture healing. In ADSC/SVF-seeded composite scaffolds, histomorphometric analysis revealed pronounced cellular infiltration, uniform cell distribution, and early extracellular matrix deposition within the scaffold pores. Cells were observed lining the dECM surfaces and penetrating deeper regions of the construct, indicating effective pore interconnectivity and biological accessibility. These features are essential for the progression from osteoconduction to osteoinduction and subsequent osteogenesis.

Longitudinal H&E sections from native bone, decellularized matrices, composite scaffolds, and ADSC-seeded constructs demonstrated: Complete decellularization in bone and tendon-derived substrates; preservation of trabecular patterns and mineralized channels, enhanced cellular penetration in seeded constructs and early extracellular matrix formation associated with ADSCs. The use of Goodling and Stewart's decalcification solution enabled high-quality sections, preserving microarchitectural detail essential for comparative evaluation. These histological findings confirm that the composite scaffold not only supports ADSC adhesion and expansion but also maintains structural integrity conducive to bone regeneration.

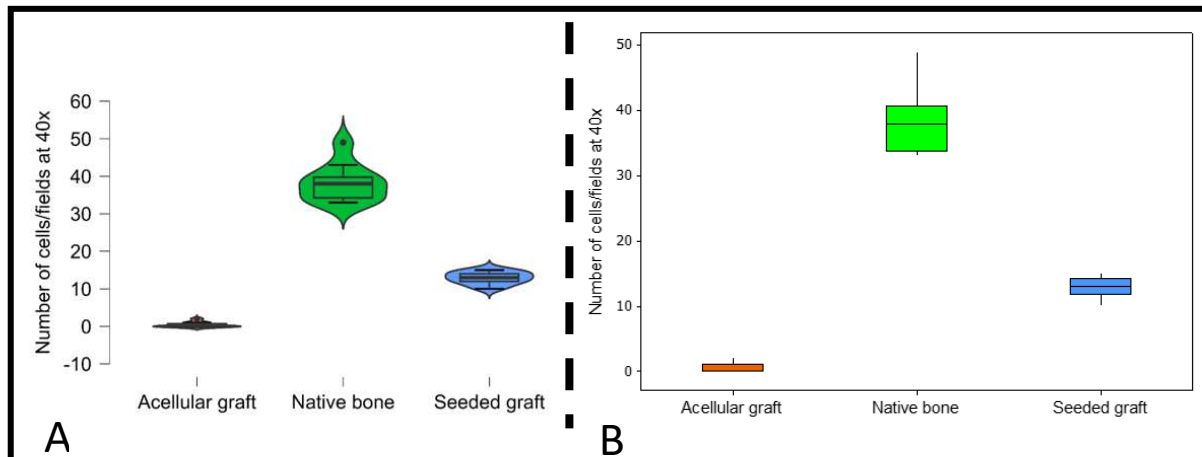


Figure 15. Quantitative histological assessment of cellular presence within scaffold constructs compared with native bone. (A) Violin plot showing the distribution of the number of cells per microscopic field (40× magnification) in acellular graft, native bone, and seeded graft groups. (B) Box plot illustrating the median and interquartile range of the measurements. Native bone demonstrated the highest cellular density, serving as the physiological reference, whereas the seeded graft exhibited moderate cellular infiltration, confirming successful cell colonization within the scaffold. In contrast, the acellular graft displayed minimal cellular presence. These findings support the scaffold's ability to facilitate cell migration and integration, which are essential for osteogenic tissue regeneration.

7. Scanning electron microscopy

The acellular dECM scaffold exhibited well-defined trabecular channels with irregular micro-roughness, characteristic of native cancellous bone. Such surface irregularities enhance protein adsorption and promote integrin-mediated cell attachment, which is essential for osteogenic signaling. Previous studies have demonstrated that ECM-derived scaffolds with preserved ultrastructure significantly outperform smooth synthetic surfaces in supporting osteoblast attachment and matrix deposition. In bioinspired scaffolds, SEM images revealed uniform embedding of dECM granules within the collagen matrix, reinforced by the PCL framework. The presence of collagen appeared to bridge adjacent dECM particles, forming a continuous three-dimensional network while maintaining open pore interconnectivity. Importantly, the addition of minimal PCL did not occlude pore openings or compromise surface bioactivity, indicating that mechanical reinforcement was achieved without sacrificing biological functionality. Similar observations have been reported in dECM–polymer hybrid scaffolds, where polymer reinforcement enhances mechanical stability while ECM components dominate surface bio-interactions. SEM evaluation of ADSC/SVF-seeded composite scaffolds demonstrated extensive cell adhesion, spreading, and infiltration within the scaffold pores.

Cells displayed flattened morphology with cytoplasmic extensions anchoring to the dECM-rich surfaces, suggesting active cell–matrix interactions. By later culture periods, the presence of extracellular matrix deposition and cell–cell bridging indicated early osteogenic matrix formation. These findings corroborate previous reports that ECM-based scaffolds provide instructive cues that support stem cell attachment, migration, and osteogenic commitment.

8. Cell-scaffold interaction

The bioinspired scaffolds demonstrated excellent cytocompatibility and supported robust cell attachment, proliferation, and migration throughout the culture period. Following overnight preconditioning in culture medium, gradual dynamic cell seeding at a density of 1×10^6 cells per scaffold, administered in two sequential loading steps, facilitated efficient cellular anchorage at the scaffold surface. The initial static incubation period (2–3 h) without mechanical disturbance was critical in promoting early cell adhesion, consistent with established scaffold seeding protocols for three-dimensional constructs.

By day 3, microscopic examination revealed active cellular proliferation with directional migration toward the scaffold surface, indicating favorable cell–material interactions. Quantitative and qualitative observations showed a significant increase in cell proliferation ($p < 0.05$) over time, reflecting the scaffold’s ability to support sustained cellular growth. By day 7, cells exhibited pronounced inward migration into the scaffold architecture, forming tight cell–matrix adhesions. This behavior suggests that the interconnected porous microstructure of the scaffold facilitated nutrient diffusion and provided sufficient spatial cues for cellular infiltration.

By day 14, cells were uniformly distributed across the scaffold with confluent coverage, indicating successful colonization of the three-dimensional matrix. DAPI fluorescence staining confirmed dense nuclear localization across both peripheral and internal regions of the scaffold, demonstrating efficient cell attachment and spatial organization. The presence of osteoblasts at sufficient densities within the scaffold highlights its suitability as a cellular carrier for bone tissue engineering applications.

The observed cellular responses can be attributed to the bioinspired composition and surface topography of the scaffold. Decellularized extracellular matrix (dECM) components provide native biochemical cues, including collagenous and non-collagenous proteins, which enhance integrin-mediated cell adhesion and signaling. Additionally, the porous architecture supports cell migration and proliferation while minimizing diffusion limitations, a critical requirement for three-dimensional bone substitutes. Comparable findings have been reported in earlier studies, where ECM-based or composite scaffolds significantly enhanced osteoblast adhesion, proliferation, and migration compared to synthetic matrices alone. The gradual infiltration of cells into the scaffold interior observed in the present study is particularly relevant, as homogeneous cell distribution is essential for uniform matrix deposition and subsequent mineralization during osteogenesis.

Overall, the results demonstrate that the bioinspired scaffold provides a biologically favorable microenvironment that supports early cell attachment, sustained proliferation, and three-dimensional migration of osteoblasts. These findings validate the scaffold’s *in vitro* biocompatibility and underscore its translational potential for applications in bone regeneration and critical-sized defect repair.

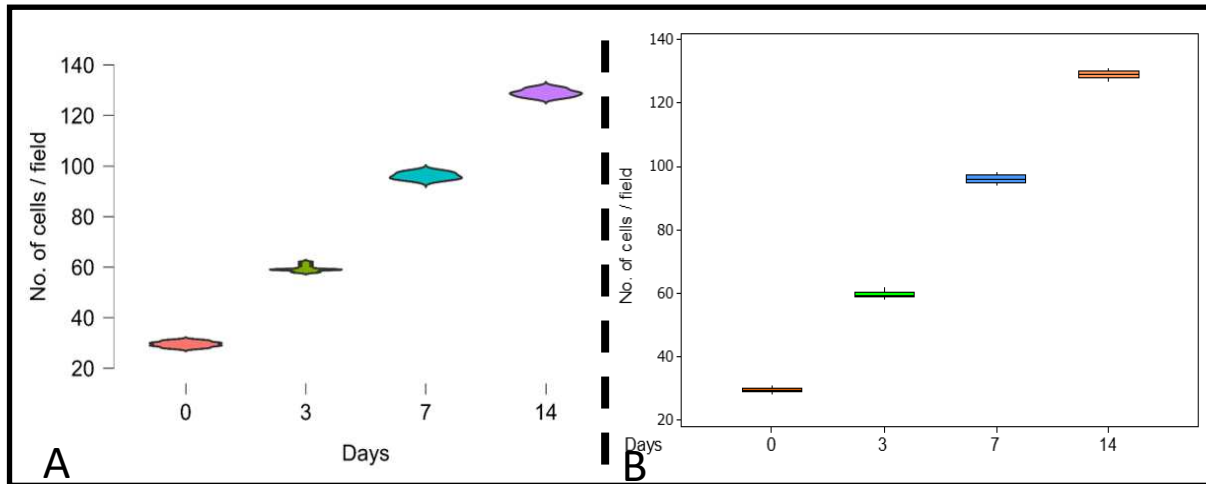


Figure16. Quantitative analysis of cell attachment and proliferation on the bioinspired composite scaffold at different culture time points (Day 0, 3, 7, and 14). **(A)** Violin plots illustrating the distribution of the number of cells per microscopic field over time. **(B)** Box plots representing the median and interquartile range of the same dataset. A progressive increase in cellular density was observed from Day 0 to Day 14, indicating sustained cell proliferation and migration within the scaffold architecture. The marked increase in cell numbers by Days 7 and 14 suggests enhanced cellular colonization and strong cell–matrix interactions within the three-dimensional scaffold network. These findings demonstrate the scaffold’s favorable cytocompatibility and its ability to support the attachment, proliferation, and spatial organization of seeded **Adipose-derived mesenchymal stem cells**, highlighting its potential application as a cellular carrier for bone tissue engineering.

8. Evaluation of Mineralization and Staining Intensity

ARS staining revealed distinct differences in mineral deposition among experimental groups. The intensity and distribution of red staining correlated with the extent of calcium accumulation and osteogenic maturation. The autograft control group exhibited intense and uniformly distributed ARS staining, indicative of robust mineralized matrix formation consistent with physiological bone formation. The bioinspired scaffold group demonstrated moderate ARS positivity, with localized mineralized nodules, reflecting partial extracellular matrix mineralization and delayed terminal osteogenic differentiation. In contrast, the composite scaffold group, particularly when enriched with stromal vascular fraction (SVF), showed markedly enhanced ARS staining intensity and widespread mineral nodule formation. The increased staining intensity indicates active and sustained calcium deposition, confirming advanced osteoblast maturation and functional mineralization. These findings are consistent with enhanced osteogenic gene expression observed in this group, particularly elevated ALP and osteocalcin levels, which are closely associated with mineral matrix formation.

Overall, the qualitative increase in ARS staining intensity from bioinspired to composite scaffolds suggests a progressive enhancement of osteogenic maturation, validating

the role of scaffold composition and biological augmentation in promoting functional mineralization.

9. Osteogenic Gene Expression Analysis

In vitro osteogenic potential of the grafts was evaluated by RT-qPCR analysis of Runx2, ALP, osteocalcin (OCN), and Integrin $\alpha 2$, normalized to GAPDH, with autograft (Group A) serving as the positive control. ALP expression showed a significant increase by Day 7, ALP expression showed an early increase at Day 7, followed by a moderate decline at Day 14 and a slight rise by Day 21, indicating early osteogenic differentiation and continued extracellular matrix maturation rather than transient inflammatory or stress-related activation. Runx2 expression exhibited a marked up-regulation over time, with the highest expression observed at Day 21, reflecting active osteogenic transcriptional regulation. OCN expression gradually increased from Day 7 onward, reaching higher levels at Days 14 and 21, suggesting progressive maturation of osteoblasts and matrix mineralization. The composite graft exhibited markedly higher OCN expression compared to the bioinspired graft, indicating enhanced terminal osteoblast differentiation and mineralization. Integrin $\alpha 2$ expression showed an initial decrease at Days 7 and 14 followed by an increase at Day 21, indicating enhanced cell–matrix interaction during later stages of osteogenic differentiation. Values are presented as **mean $\pm$ standard deviation (SD)**.

Overall, the composite graft exhibited osteogenic gene expression patterns comparable to, and in certain parameters superior to, autograft, while the bioinspired graft showed moderate osteoconductive potential. These findings confirm the enhanced *in vitro* osteogenic efficacy of the composite scaffold, supporting its suitability for critical-sized bone defect repair.

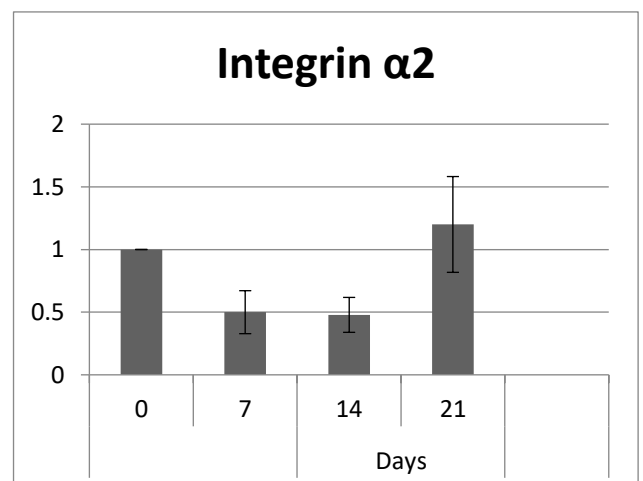
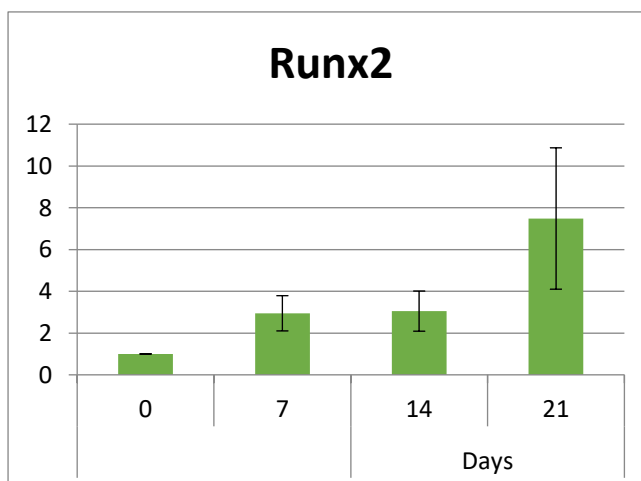
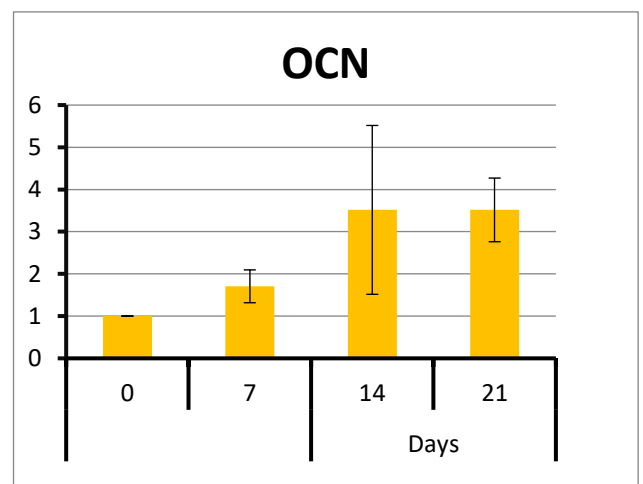
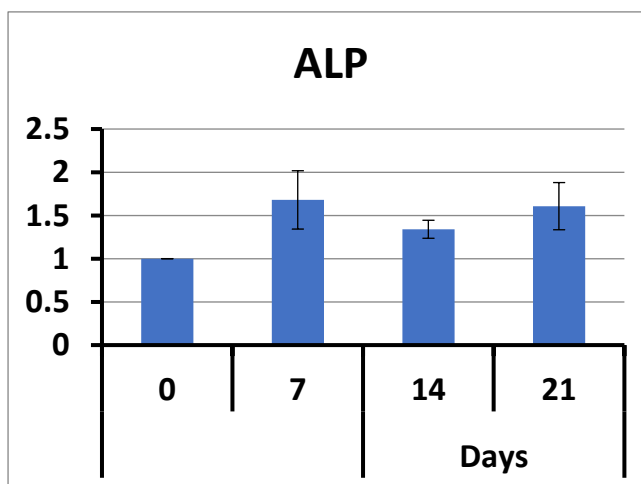


Figure 17 Relative expression levels of osteogenic differentiation markers Alkaline Phosphatase (ALP), Osteocalcin (OCN), RUNX2 (Runx2), and Integrin alpha 2 (Integrin α 2) at different time points (Day 0, 7, 14 and 21).

Discussions

Critical-sized bone defects (CSDs) represent a persistent challenge in both human and veterinary orthopaedics due to their inability to undergo spontaneous regeneration. Successful repair of such defects requires biomaterials that not only provide structural support but also actively regulate cellular behavior and osteogenic differentiation. In the present study, a bioinspired composite osseous scaffold composed of decellularized bubaline cancellous bone extracellular matrix (dECM), collagen gel, and minimal polycaprolactone (PCL) reinforcement—optionally enriched with autologous stromal vascular fraction (SVF)—was developed and systematically evaluated. The findings demonstrate that the synergistic integration of native extracellular cues, controlled porosity, and mechanical reinforcement results in a scaffold capable of supporting sustained osteogenic activity *in vitro*.

Decellularized bone ECM has gained considerable attention as a biomaterial due to its ability to retain native bone architecture, mineral composition, and matrix-bound signaling molecules while eliminating immunogenic cellular components [6]. In the present study, effective decellularization of bubaline cancellous bone was confirmed by histological and ultrastructural analyses, which demonstrated complete cellular removal with preservation of trabecular morphology. This is critical, as retained ECM topography and biochemical composition are known to regulate osteoblast adhesion and lineage commitment through integrin-mediated signaling pathways [19]. Previous studies [12] have established that decellularized bone matrices exhibit superior osteo-conductivity compared with purely synthetic scaffolds, primarily due to the presence of collagen type I, hydroxyapatite, and non-collagenous proteins such as osteopontin and bone sialoprotein. A systematic review by [5] reported that decellularized allograft and xenograft bone scaffolds consistently support osteogenic differentiation with minimal host inflammatory response when appropriately processed. The present findings align with these reports, demonstrating that bubaline dECM retains physicochemical characteristics comparable to native bone while providing a biologically permissive substrate.

Collagen gel was incorporated as the organic phase of the composite scaffold to enhance biological compatibility and handling characteristics. Type I collagen is the principal organic component of bone and plays a central role in osteoblast attachment, proliferation, and matrix mineralization [20]. In the present study, collagen gel facilitated uniform dispersion and binding of dECM granules while improving scaffold mouldability—a critical requirement for filling irregular segmental defects. The composite scaffold exhibited significantly higher porosity than native and acellular bone matrices. High porosity is a key determinant of scaffold performance, as it promotes nutrient diffusion, waste removal, vascular infiltration, and deep

cellular colonization. Studies have shown that pore interconnectivity and porosity levels above 60–70% are particularly favourable for osteogenesis and angiogenesis. The observed porosity in the present scaffold therefore represents an optimal balance between structural integrity and biological accessibility. As per studies of [20,21] one of the principal limitations of ECM- and collagen-based scaffolds is inadequate mechanical strength, particularly during the early stages of implantation when biological fixation has not yet occurred. To address this limitation, a low concentration (5 weight%) of PCL was incorporated into the scaffold. PCL is a biocompatible, slowly degradable polymer widely used in orthopaedic tissue engineering due to its favorable mechanical properties and degradation profile [22].

Mechanical testing in the present study demonstrated that PCL reinforcement significantly enhanced tensile strength compared with collagen–dECM formulations lacking polymer support. Although the tensile strength remained lower than that of native bone, the achieved values fall within acceptable ranges for non-load-bearing and moderate-load applications, where early osteoconduction and biological integration are more critical than immediate load bearing [23,24].

Autologous stromal vascular fraction (SVF) was employed as a biologically active cell source to enhance osteogenic potential without the need for extensive in vitro cell expansion. SVF is a heterogeneous population containing mesenchymal progenitors, endothelial cells, pericytes, and immune-regulatory cells, collectively contributing to osteogenesis and angiogenesis [18,25]. The high SVF yield obtained from interscapular adipose tissue confirms the feasibility of this approach in a clinical veterinary setting [18,26]. Uniform cell distribution and progressive proliferation within the composite scaffold, as demonstrated by DAPI staining, indicate excellent cytocompatibility and scaffold–cell interaction. These findings are consistent with previous reports of [25,26,27] demonstrating that SVF-seeded scaffolds exhibit superior regenerative outcomes compared with acellular constructs.

Histomorphometric evaluation provided complementary microstructural and cellular-level evidence supporting the osteoconductive and biocompatible nature of the developed scaffolds [30]. The presence of newly formed matrix-like regions associated with seeded cells suggests initiation of osteogenic remodelling, even in the absence of exogenous osteoinductive factors. Similar histological patterns have been reported in ECM-based bone scaffolds seeded with progenitor cells, where early matrix deposition precedes mineralization and lamellar bone formation [30,31]. Importantly, the use of Goodling and Stewart's decalcification solution preserved tissue morphology and scaffold detail, allowing accurate assessment of microarchitectural features. This methodological consistency strengthens the reliability of histomorphometric observations and supports meaningful comparison between experimental groups. Overall, histomorphometric findings validate that the composite scaffold maintains structural integrity, supports cellular infiltration, and facilitates early matrix formation—hallmarks of a biologically active and osteoconductive bone substitute.

Scanning electron microscopy (SEM) was performed as per [28] provided critical ultrastructural insights into the surface morphology, pore architecture, and cell–scaffold interactions of the developed osseous scaffolds. SEM evaluation confirmed that the decellularization protocol effectively removed cellular remnants while preserving the native trabecular microarchitecture of bubaline cancellous bone. The retention of interconnected pores and rough surface topography is a key determinant of scaffold bioactivity, as micro- and nano-

scale surface features are known to regulate osteoblast adhesion, cytoskeletal organization, and differentiation. Collectively, SEM analysis confirmed that the bioinspired composite scaffold possessed a favorable ultrastructural environment characterized by interconnected porosity, bioactive surface topography, and robust cell–scaffold interaction—features essential for successful bone tissue engineering similar to studies of [28,29].

By day 3, cells exhibited proliferation and migration towards the scaffolds. Over subsequent days, there was a significant ($p < 0.05$) increase in cell proliferation, and by day 7, cells began migrating towards the interior of the scaffold, forming tight adhesions. By day 14, cells were evenly distributed and had confluent coverage over the scaffolds. Staining with DAPI was performed for five to ten minutes on day 14, as illustrated in the figure, and cell attachment within the first twenty-four hours was examined using a fluorescence microscope. DAPI staining of the cell-seeded scaffolds revealed that bioinspired scaffolds are conducive substrates for cell attachment, proliferation and migration. Osteoblast cell attachments were observed to be in sufficient densities in seeded scaffolds Similar to studies of [32].

Temporal analysis of alkaline phosphatase (ALP) expression revealed significant upregulation by Day 7, indicating early osteogenic induction and extracellular matrix maturation. Elevated ALP levels were maintained through Day 21, confirming sustained osteoblastic activity and ongoing matrix mineralization rather than a transient inflammatory response. Group A exhibited consistent ALP expression characteristic of physiological osteogenesis, while Group B showed moderate expression indicative of osteoconductive behavior. Group C demonstrated ALP levels comparable to or exceeding autograft during early phases, suggesting efficient matrix maturation supported by scaffold architecture and SVF-mediated signaling. Similar to studies of [33]

Runx2 expression increased progressively from Day 0 to Day 21, with the highest expression observed in Group C. As a key transcription factor regulating osteoprogenitor commitment, this pattern reflects effective lineage specification. Group A showed strong Runx2 activation, validating the *in vitro* model, whereas Group B exhibited moderate expression driven by native dECM cues. The markedly elevated Runx2 expression in Group C indicates enhanced osteogenic commitment, likely due to synergistic mechanobiological signaling from PCL reinforcement, ECM components, and SVF. Similar to findings of [34]

Osteocalcin (OCN) expression as per studies of [35], a marker of late-stage osteogenic differentiation, increased progressively and peaked at Day 21. Group C showed the highest OCN expression, confirming terminal osteoblast differentiation and mineralized bone formation. In contrast, Group B exhibited lower OCN levels, suggesting delayed or incomplete maturation.

Integrin $\alpha 2$ expression was significantly upregulated at Day 21 similar to [36,37], most prominently in Group C. This indicates enhanced cell–matrix adhesion, collagen–integrin interaction, and mechanotransductive signaling, which likely contributed to the coordinated Runx2–ALP–OCN expression cascade.

In summary, while the bioinspired graft (Group B) demonstrated osteoconductive potential with partial osteogenic maturation, the composite graft (Group C) achieved comprehensive osteogenic activation, encompassing early transcriptional commitment, sustained matrix maturation, and late-stage mineralization. The osteogenic gene expression

profile of the composite graft was comparable to, and in certain parameters superior to, the autograft, underscoring its high translational potential as an autograft alternative for critical-sized bone defect repair. The observed mineralization patterns support the conclusion that the composite scaffold provides a favorable microenvironment for late-stage osteogenesis. ARS staining intensity serves as a surrogate marker for calcium-rich extracellular matrix deposition and complements molecular markers of osteogenic differentiation. The concordance between ARS staining, histomorphometric observations, and osteogenic gene expression strengthens the evidence for the osteogenic superiority of the composite scaffold.

Conclusions

The present study successfully developed and comprehensively evaluated a bioinspired osseous scaffold composed of decellularized bubaline bone granules, collagen gel, and polycaprolactone (PCL), with optional enrichment using autologous stromal vascular fraction (SVF). Physicochemical characterization demonstrated that the composite scaffold possessed favorable physicochemical, mechanical, and osteogenic properties *in vitro* potential for managing critical-sized bone defects. The combination of bubaline dECM granules, collagen gel, and minimal PCL reinforcement successfully produced a bioinspired scaffold with: Appropriate density approximating cancellous bone; high porosity conducive to vascular and cellular infiltration; adequate mechanical stability for segmental defect environments; excellent ADSC biocompatibility with favorable cytological and histological response. These findings support the scaffold's utility as a promising biomaterial for use in critical-sized bone defect repair, particularly in veterinary orthopaedics, where cost-effectiveness and biological performance are key considerations. When compared to existing literature on ECM-derived scaffolds, the present construct demonstrates comparable or superior outcomes in porosity, handling characteristics, and cell–scaffold interaction. The hybrid nature of the scaffold provides a unique balance between mechanical stability (via PCL) and bioactivity (via dECM and collagen), positioning it as an attractive candidate for future *in vivo* studies and clinical translation.

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Author Contribution

Conceptualisation, Methodology, Prarthana R and Rekha Pathak.;Data analysis, V. kumar and D. Kumar; Validation Rekha, B.P Singh, Kamalesh, formal analysis Rohit and Rekha Pathak, Manuscript construct, Prarthana and Sai Kumar

Data availability

Data is provided within the manuscript or supplementary information files

Declarations

Conflict of interest

Authors declare No conflict of interest

Institutional review board statement Protocols for this study were approved by Institute Ethics Committee (IAEC), No-F27-2/2023-24/JDR (Research)

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FIGURES WITH LEGENDS



Figure 1. A- Decellularized bone blocks of buffalo origin; B- Decellularized extracellular matrix (dECM) chips obtained from bone blocks; C- Pulverized bone granules using homogenizer to obtain uniform dECM granules; D- collagen gel obtained from decellularized tendon of buffalo origin; E- combination of dECM granules and collagen gel in equal quantities forming bone-glue like structure; F- Fabrication of bone glue with polycaprolactone (PCL), obtained mouldable scaffold.



Figure 2. A- Aseptic preparation of surgical site; B- collection of adipose tissue from interscapular fat depot; C- Placing apposition sutures (simple interrupted pattern) for skin.

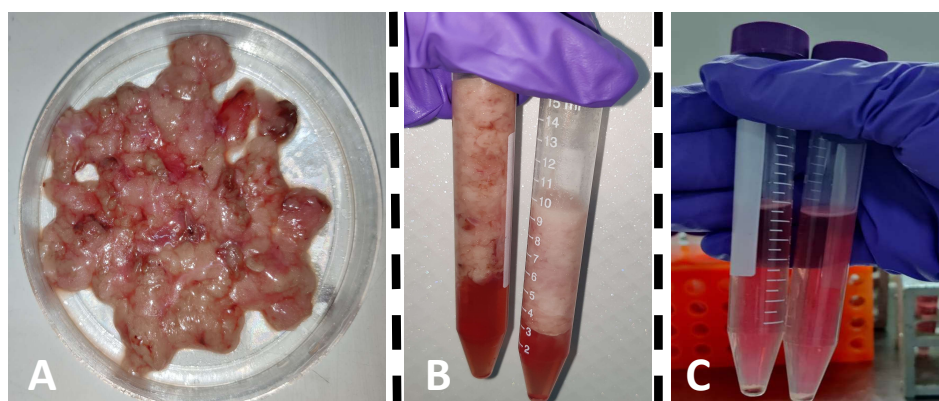


Figure 3. A- collection of adipose tissue; B- subjecting the minced adipose tissue to centrifugation and obtained (a- mature adipocyte layer; b- middle aqueous layer; c- bottom SVF pellet); C- SVF pellet was obtained at the bottom which was suspended in 0.5 ml of complete media and stored.

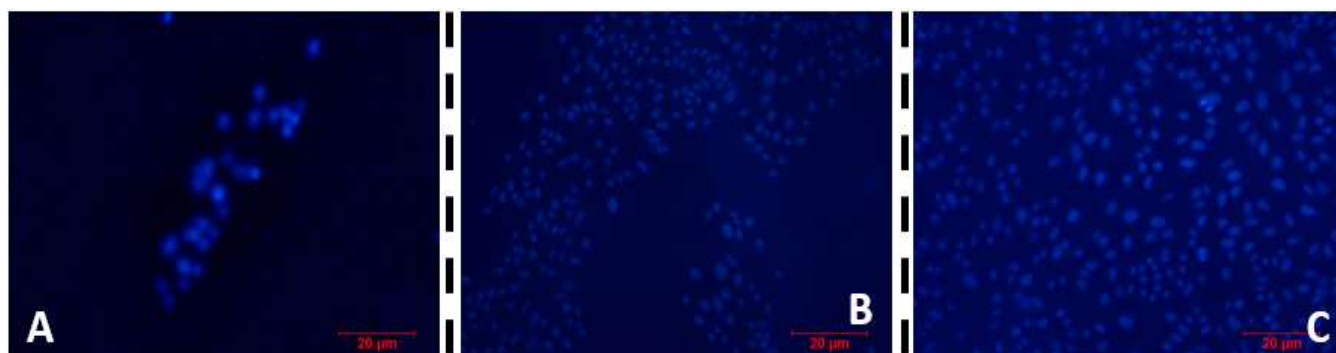


Figure.4 ADSC attachment, proliferation, and viability were evaluated at days 3 (A), 7 (B), and 14 (C) using DAPI nuclear staining.

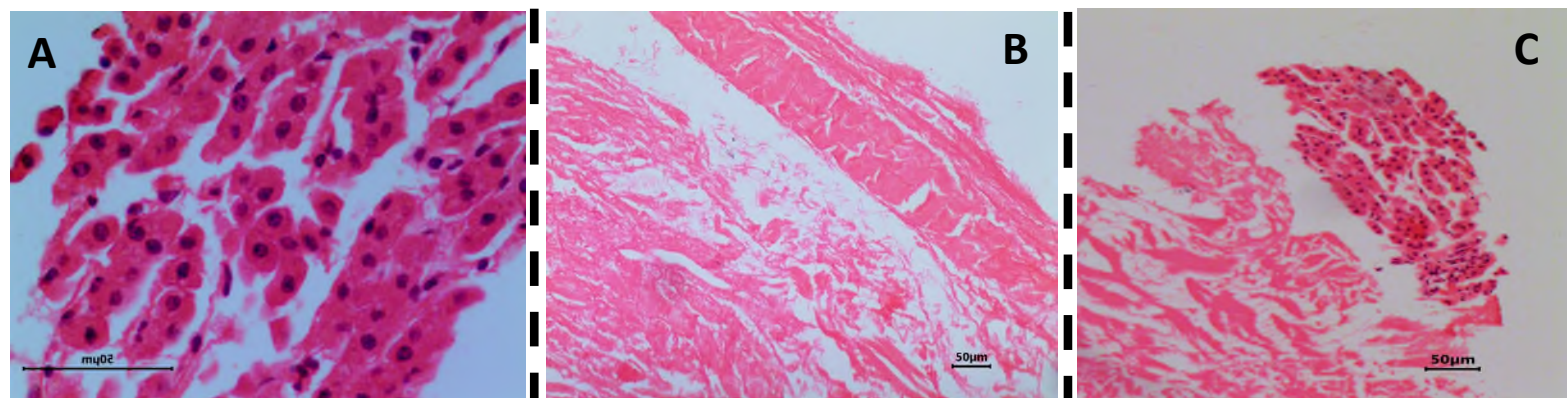


Figure 5. A Native bone; B bioinspired graft dECM and decellularized collagen gel with PCL after chemical & physical treatment; C Seeded composite graft (dECM+ collagen gel+ PCL+ SVF).

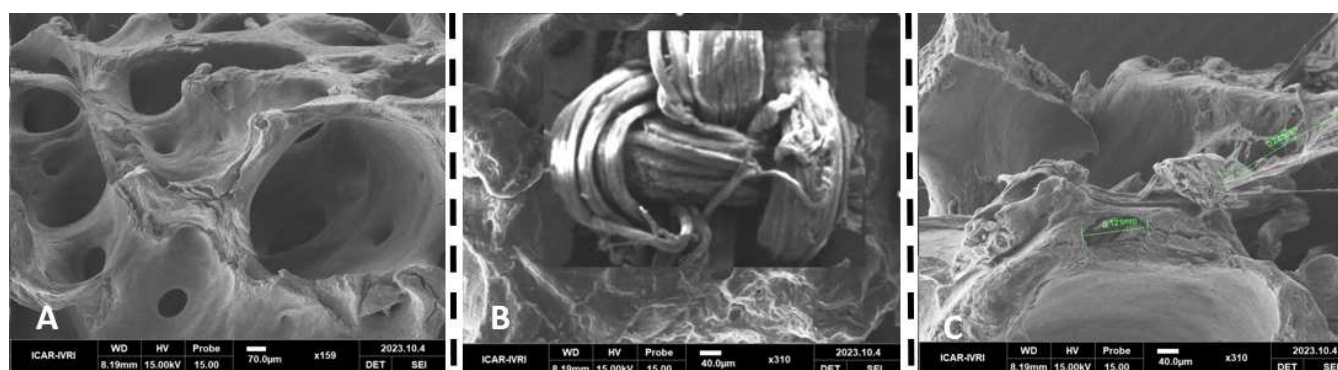


Figure 6. A Native bone with various diameter of pore size; B bioinspired graft well integration of dECM with collagen fibres and PCL; C. Composite graft (dECM+ collagen gel+ PCL+ SVF) where cell attachment and migration are visible (green arrows).

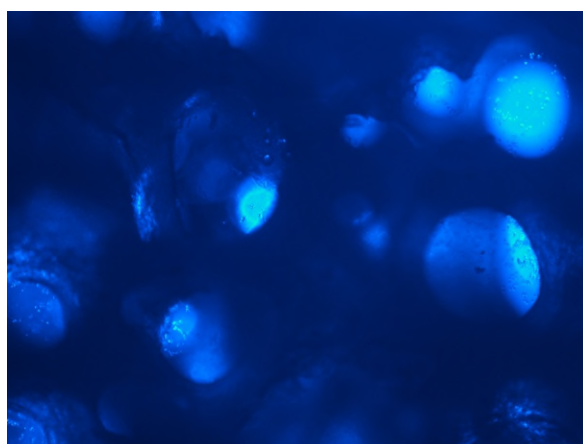


Figure 7. Scaffolds were incubated in ADSC culture medium for 24 hours, observed for cell attachment and migration

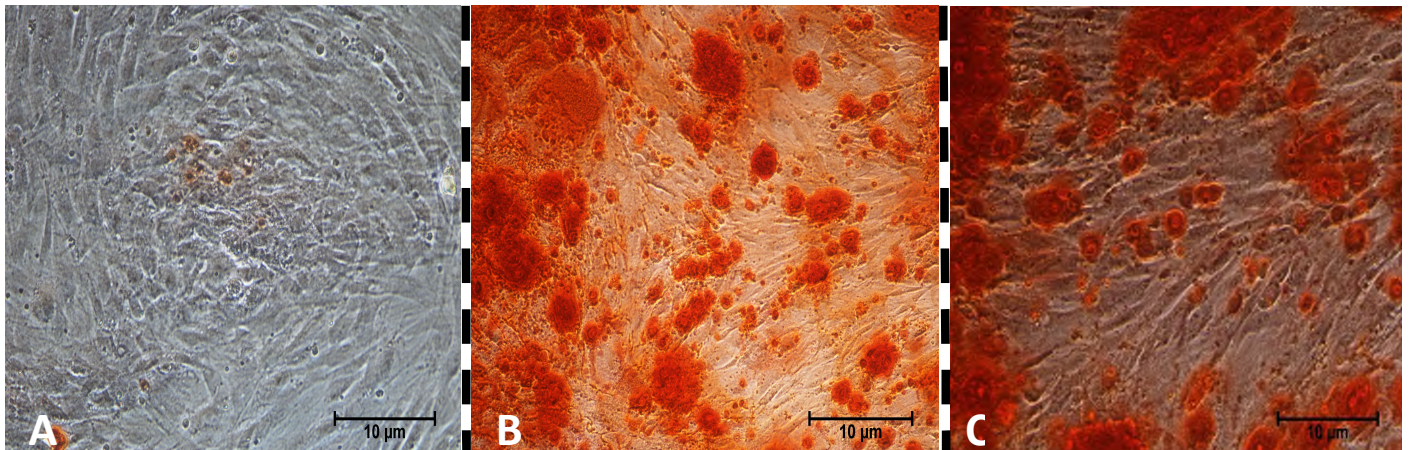


Figure 8. Alizarin Red S (ARS) staining was employed to evaluate extracellular matrix mineralization evaluated at day 3 (A), 7 (B), and 14 (C).