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Development and Evaluation of Stem Cells Laden Bioscaffold using Buffalo Rib Periosteum

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ABSTRACT

The present research work was done to evaluate and compare decellularized periosteum and decellularized periosteum seeded with rabbit amniotic membrane-derived mesenchymal stem cells (rAM-MSCs) (recellularized periosteum) to prepare it as ready to use bio-scaffold. Periosteum was harvested from buffalo ribs procured from local slaughterhouses. Decellularization of the periosteum was done by the chemical method using 2% triton X-100 & 1% SDS detergents. After decellularization of the periosteum, it was evaluated for the absence of any periosteal cells and integrity of the extracellular matrix of the periosteum with hematoxylin and eosin (H & E) staining and scanning electron microscopy (SEM), respectively. After confirming successful decellularization, 1×10^6 cells/cm^2 rAM-MSCs were seeded over decellularized periosteum. After successful recellularization, it was evaluated for the attachment of seeded rAM-MSCs over the surface of decellularized periosteum and confirmed by using H & E staining, and SEM, respectively. Decellularized periosteum provided a compatible and supportive extracellular matrix for attachment of rAM-MSCs and can be used as a bioscaffold. Recellularized periosteum can deliver rAM-MSCs for various clinical purposes like fracture healing and bone regeneration in critical-sized bone defects. Also, decellularized periosteum proved to be a better scaffold for rAM-MSCs growth and attachment. Thus, decellularized periosteum after stem cell recellularization proves to be a good bioscaffold for therapeutic application in veterinary clinical practices.

HIGHLIGHTS

- Stem cell laden bioscaffold was prepared from buffalo rib periosteum.
- Rabbit amniotic membrane-derived mesenchymal stem cells (rAM-MSCs) model was used to evaluate.
- Developed bioscaffold could be used in veterinary regenerative medicine.

Keywords: Decellularization, Periosteum, Bioscaffold, Amniotic Membrane, Mesenchymal Stem Cells, Scanning Electron Microscopy

Regenerative medicine and tissue engineering are interdisciplinary topics that aim to develop innovative approaches for repairing, replacing, or regenerating damaged or diseased tissues and organs. These fields combine biological science, bioengineering, and clinical medicine principles to address the pressing need for effective therapies in various clinical applications. Tissue engineering involves the fabrication of artificial tissue constructs in the laboratory by combining cells, biomaterials, and bioactive factors. The process typically starts with isolating stem cells from the donor source. These stem cells are then seeded onto a scaffold made of biocompatible materials, which provides structural support and guides the growth and organization of cells. Bioactive factors such as growth factors or cytokines may be added

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to promote cell proliferation and differentiation. Over time, the cells within the scaffold develop into functional tissue, which can be transplanted into the recipient.

Tissue engineering and regenerative medicine promise revolutionizing healthcare by providing innovative tissue repair and regeneration solutions. As research continues and technologies advance, these fields can significantly enhance results and quality of life. Periosteum is a highly specialized connective tissue surrounding the bones' outer surface. The periosteum serves several crucial functions in the growth, maintenance, and repair of bones in the human body.

The periosteum is a dense, fibrous membrane composed of an outer fibrous layer and an inner cambium layer. The outer layer comprises collagen fibers, providing strength and protection to the bone, while the inner layer contains osteoprogenitor cells responsible for bone formation and repair. During fetal development, the periosteum plays a vital role in the growth and shaping of bones. It helps facilitate bone formation by providing a framework for the deposition of minerals and the differentiation of osteoblasts, which are responsible for bone synthesis. The periosteum is richly supplied with blood vessels, including arteries, veins, and capillaries. These blood vessels carry oxygen, nutrients, and other essential substances to the underlying bone tissue, ensuring nourishment and vitality. When a bone is fractured or damaged, the periosteum is crucial in healing. Immediately after an injury, blood vessels within the periosteum rupture, forming a hematoma. This clot serves as a scaffold for migrating cells involved in bone repair, such as osteoblasts and fibroblasts. The periosteum also provides a source of new osteoblasts, which contribute to forming new bone tissue during the healing process.

The periosteum is a critical component of the skeletal system. It provides structural support and protection to bones and is a dynamic interface between the bone and surrounding tissues. The periosteum's functions in bone development, repair, and attachment contribute to the skeletal system's overall strength, integrity, and functionality (Le *et al*., 2017). Considering the potential of the periosteum, it was selected to prepare a bioscaffold that is further used in different regenerative treatments.

MATERIALS AND METHODS

This research was done at the Reproductive Physiology/ Stem Cell Laboratory at the Physiology and Climatology Department of the ICAR-IVRI, Izatnagar, India.

Collection of sample

Fresh ribs of the buffalo were collected from a local slaughterhouse. All the musculature around the ribs was removed using a sterile BP blade. Then ribs were washed using a normal saline solution containing an antibiotic-antimycotic solution. After a thorough cleaning, the periosteum was removed from the ribs by the periosteal elevator.

Decellularization of periosteum

The collected periosteum tissue was immediately cut into several pieces of about 2×2 cm^2. The periosteum pieces were cleaned by using antibiotic-antimycotic supplemented 1X PBS. The periosteum pieces were washed with agitation (120 rpm) in ultrapure water overnight at RT in a shaker incubator. Decellularization was done by sequential treatment of periosteum with 2% Triton X-100 for 12 hrs and 1% SDS for 4 hrs, with continuous agitation in a shaker incubator. Finally, the obtained periosteum was rinsed for 48 hrs (3 exchanges/day) in milli-Q water to remove all reagents used for decellularization. Finally, it was stored at 4^oC in 1X PBS containing an antibiotic-antimycotic solution.

Evaluation of the decellularized periosteum

The decellularized periosteum was evaluated by Hematoxylin and Eosin (H & E) staining and subsequently subjected for scanning electron microscopy (SEM).

Recellularization of periosteum

For 24 h, D-periosteum was immersed in 10% of DMEM. P3 rAM-MSCs were seeded onto the D-periosteum in 06 well plate at 1×10^6 cells/cm^2 density. On every 4th day, the culture medium has been changed.

Evaluation of the recellularized periosteum

The recellularized periosteum was also evaluated by H & E staining & SEM.

Hematoxylin and Eosin Staining

Histopathological examination and evaluation of the decellularized & recellularized periosteum section were performed. Samples were fixed in 10% formaldehyde. The samples were briefly kept under running tap water overnight and transferred to an automatic tissue processor for further processing. In the ascending percentage of ethanol, samples were dehydrated, xylene used for clearing and embedded with melted paraffin wax (melting point 58ºC to 60ºC), and tissue paraffin blocks were made. Using a microtome, these blocks were sectioned and cut into 5µm thickness, picked on glass slides, and H & E used to stain to notice the successful decellularization & recellularization.

Scanning Electron Microscopy

The SEM examination is used to study surface structural aspects & status of decellularization & rAM-MSCs attachment to the decellularized periosteum. Fixing respective samples was done using the chilled 2.5% glutaraldehyde overnight, followed by washing with 1X PBS to remove all fixative solution. Afterward, samples were completely dehydrated by using an ascending concentration of ethanol. Ultra-dehydration was done by hexamethyldisilane (HMDS) before mounting. Later samples were coated with a gold sputter. The processed samples were analyzed in 20 kV accelerating voltage by scanning electron microscope.

RESULTS AND DISCUSSION

Decellularization successfully eliminated immunogenic cells while preserving a healthy extracellular matrix. Decellularization by ionic detergent proved effective for producing bioscaffolds that required collagen to be preserved in the extracellular matrix. The decellularized periosteum had no cell nuclei, as H&E staining and SEM revealed. Fig. 2 illustrates that the periosteum's two layers, cambium and fibrous, were well-ordered. Fig. 3a and 3b illustrate the ultra-microscopic structure of decellularized periosteum as seen by SEM, where no cells can be found on the decellularized periosteal surface. The microarchitecture of the cambium and fibrous layers of the decellularized periosteum was visible in the SEM pictures. The surface of the decellularized periosteum was uneven and extremely porous, comprised of long bundles of fibrils resembling a collagen network. Chen *et al*. (2015) reported similar outcomes.

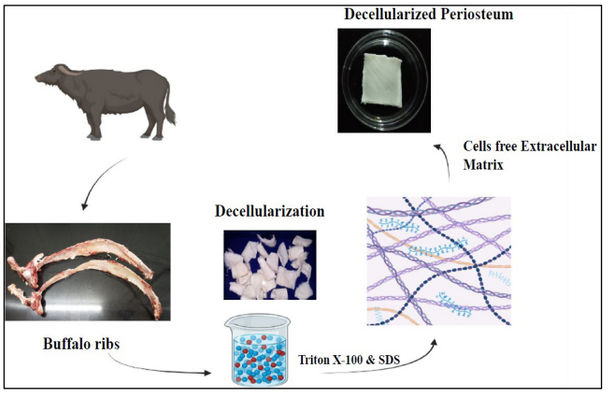

Fig. 1: Schematic Presentation of Decellularization Procedure of Periosteum

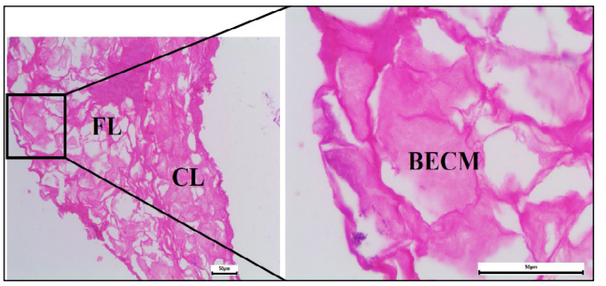

Fig. 2: H & E Staining of Decellularized Buffalo Rib Periosteum at 10x & 40x magnification. (BECM-Bone Extracellular Matrix; CL-Cambial layer; FL-Fibrous layer)

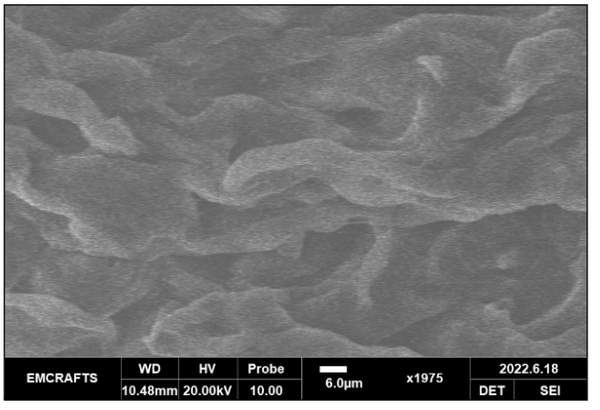

Fig. (3a): SEM Images of Decellularized Periosteum at 2000x

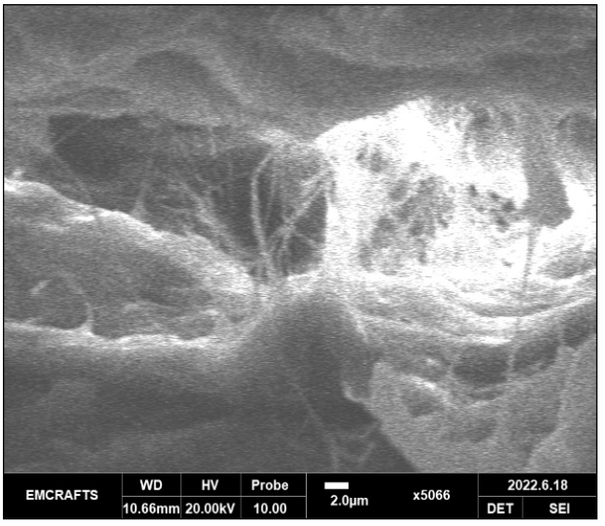

Fig. (3b): SEM Images of Decellularized Periosteum at 5000x

The ability of rAM-MSCs to multiply and adhere to decellularized periosteum demonstrates that it is biocompatible. Fig. 4 displays the recellularized periosteum after H & E staining. This depicts cell attachment on the periosteum that has lost its cells. As demonstrated in Fig. 5, simultaneous SEM imaging provides an ultra-microscopic view of the recellularized periosteum that reveals rAM-MSC attachments to the decellularized periosteal surface.

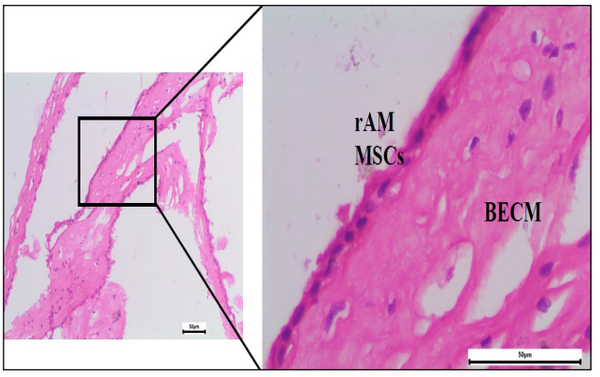

Fig. 4: H & E Staining of Recellularized Buffalo Rib Periosteum at 10x & 40x magnification. (rAM MSCs-Rabbit Amniotic Membrane derived Mesenchymal Stem Cells; BECM-Bone Extracellular Matrix)

H&E staining examination of decellularized periosteum revealed collagen fibers in the extracellular matrix that retained their pink colouration. Integrity and continuity of collagen fibers were preserved in the decellularized periosteum of the tibia (Zhang *et al*., 2017). There was no marked variation in the structure and distribution of ECM collagen between fresh periosteum and acellular periosteum, showing that decellularization does not significantly alter the collagen structure (He *et al*., 2020).

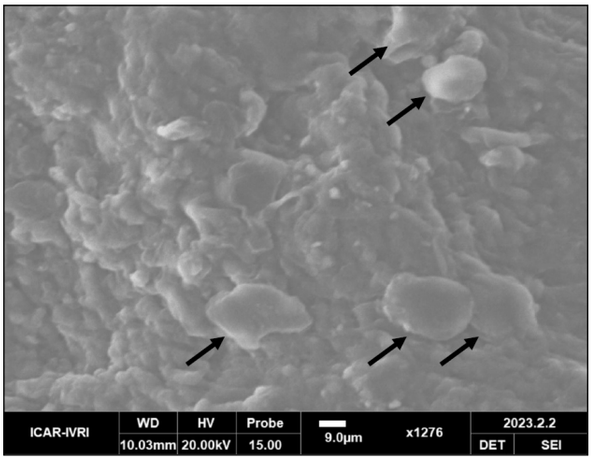

Fig. 5: SEM Images of Recellularized Periosteum at 1200x. (Black arrow showing attached rAM-MSCs)

Similar to the findings of He *et al*. (2020), the decellularized layer of the periosteum showed increased porosity as the spaces between the collagenous fiber bundles widened in the fibrous layer of the periosteum. The increased porosity was caused by removing most cell components from their compartment, leaving only collagen. The appropriate decellularization procedure should not compromise the three-dimensional ultrastructure and composition of the bioscaffold (Chalikias and Tziakas, 2015). After decellularization, cells were eliminated, leaving behind a loose, porous collagen scaffold.

Chen *et al*. (2015) investigated the decellularized rabbit periosteum's ultrastructure. The surface of the decellularized periosteum was irregular and very porous. It comprises lengthy bundles of fibrils connected by a collagen-containing network. Decellularized periosteum possessed the same uneven fibrous surface and three-dimensional architecture as the natural periosteum, and collagen integrity was unaffected. Following decellularization, the collagen bundles became somewhat less compact (Zhang *et al*., 2017).

This study used decellularized periosteum and recellularized periosteum to create bioscaffolds. Widely employed to fix non-homologous anatomical sites, such as intestinal submucosa, dermis, amnion, and pericardium, etc., products obtained from site-specific similar tissue have been significantly more effective for active tissue remodelling than non-site-specific tissue sources (Zhang *et al*., 2008). The extracellular matrix is always in dynamic equilibrium with its functional and structural cells, and it fluctuates constantly in response to cell, tissue, and organ demand (Bissell *et al*., 1986). The characteristics of the extracellular matrix vary depending on the kind of tissue (Frantz *et al*., 2010). In addition to being a source of osteogenic cells, the periosteum also induces early revascularization (Knize, 1974). The fibrous layer of the periosteum expresses PTHrP, while the inner cambial layer expresses the PTH/PTHrP type I receptor (PTHR1). PTHrP may promote osteoblastic activity and fracture healing. MSCs have a lifetime source in the periosteum (Moore *et al*., 2014). Schonmeyr *et al*. (2009) created a material resembling periosteum from acellular dermis and MSCs.

A naturally occurring 3D scaffold with a tissue-specific structure of extracellular matrix molecules that can be formed by decellularized tissue explant does not appear to be easily synthesized in the laboratory. Recent interest has increased in producing biological scaffolds from decellularized tissues or organs. Decellularization is crucial for removing cellular and antigenic factors from tissue explants to prevent infectious infections, reduce inflammatory reactions against the periosteum, and reduce the rejection rate following implantation. The key benefit of decellularized periosteum is that it retains the characteristics of the natural cell environment. With adequate and optimal decellularization, the extracellular matrix's dynamic biomolecular and physical cues are retained and can stimulate cell proliferation (Frantz *et al*., 2010). *In vitro* and *in vivo*, the D-periosteum is biocompatible (Chen *et al*., 2015).

Due to the elimination of cellular DNA, decellularized extracellular matrix has a relatively low risk of eliciting an immunological response (Crapo *et al*., 2011). Chemical or enzymatic treatments are necessary for decellularization procedures (Crapo *et al*., 2011). The native periosteum is collected and decellularized from buffalo ribs. In chemical decellularization, the cell membrane was sequentially dissolved by non-ionic (2% Triton-X100) and ionic (1% SDS) detergents for 12 and 4 hours, respectively (Chen *et al*., 2015). Bioactive factors were present after decellularization and communicated with reseeded cells (Xue *et al*., 2012).

Our data indicate that the procedure employed to decellularize the periosteum was successful. H & E staining of the D-periosteum revealed the absence of cells, revealing empty gaps in the cambial layer. The two-layer periosteum structure was largely preserved (cambium and fibrous layers). The fibers of collagen were arranged in a crisscross manner. Complete removal of cells left collagen with a largely unaltered structure.

The results demonstrate that the recellularized periosteum is a suitable and supporting scaffold. The augmentation of rAM-MSCs was successful, and these cells proliferated significantly on decellularized periosteum for one week, demonstrating exceptional biocompatibility *in vitro*. The adhesion of rAM-MSCs to the decellularized periosteum supports the notion that it preserved its native properties, most of which are required for recellularization. On the decellularized periosteum, rAM-MSCs were successfully proliferated. However, recellularized periosteum was discovered to be an effective biomaterial for MSCs administration.

CONCLUSION

Histology and scanning electron microscopy demonstrate that the decellularized periosteum provides a suitable and supportive matrix for the attachment and delivery of MSCs for various clinical applications. With the assistance of MSCs, recellularization enhances the potential of bioscaffold during various therapeutic procedures. Due to its biological origin, periosteum used in veterinary clinical practices is non-toxic and biocompatible with various tissues. Moreover, decellularization will aid in cutting the chance of graft rejection. Consequently, effective decellularization and successful recellularization will improve the quality of regenerative therapy.

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