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Decellularization of bubaline gallbladder using 0.5% Sodium dodecyl sulfate and seeding of stem cells over decellularized matrices

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Abstract
Decellularization is a process which removes of all the cellular and nuclear material while minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining extra cellular matrix. Various methods of decellularization are used to decellularize the tissues like specific physical, chemical, and enzymatic methods. Sodium dodecyl sulfate (SDS) is an ionic biological detergent with a long-standing record in tissue engineering for decellularization of tissues. Stem cells are the pluripotent cells, capable of differentiation into numerous cells types**.** Decellularized extracellular matrices provide a native framework for cell adhesion at the site of a tissue deficit, therefore allowing local cells to migrate into the matrix, adhere and undergo differentiation. Thus prepared seeded matrices have wide range of uses in regenerative medicine. In the present study, we developed a biomaterial by decellularizing the bubaline gallbladder with 0.5% SDS and seeding the stem cells over it.

Keywords: Decellularization, bubaline gallbladder, 0.5% sodium dodecyl sulfate and stem cells

1. Introduction
The cells in every tissue have major histocompatibility I and II antigens eliciting rejection reactions. If cells with these antigens are eliminated from tissues, immune ejection can be avoided. Decellularization process removes these antigen epitopes, DNA, and damage associated molecular pattern (DAMP) molecules (Gilbert *et al.,* 2006; Lotze, 2007) [2, 6]. An optimum decellularization protocol efficiently removes of all the cellular and nuclear material while minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining extra cellular matrix (Gilbert *et al.,* 2006) [2]. Decellularization of the tissues or organs can be brought about by specific physical, chemical, and enzymatic methods which leave a material composed essentially of decellularized *i.e.* extra cellular matrix (ECM) components. Sodium dodecyl sulfate (SDS) is an ionic biological detergent with a long-standing record in tissue engineering for decellularization of tissues (Hudson *et al.,* 2004; Rieder *et al.,* 2004) [3, 7]. The extracellular matrix (ECM) is a complex structure that helps to support cells and comprises key components of the basement membrane that anchor and replenish the cells. Exogenous ECMs provide a native framework for cell adhesion at the site of a tissue deficit, therefore allowing local cells to migrate into the matrix, adhere and undergo differentiation (Badylak, 2007) [1]. Stem cells are the pluripotent cells, capable of differentiation into numerous cells types, including fibroblasts, osteoblasts, chondrocytes, adipocytes, myocardial cells, vascular endothelial cells, neurones, hepatocytes, epithelial cells, and other tissue cells (Jiang *et al.,* 2011, Li and Ikehara, 2013) [4, 5]. Local transplantation of stem cells via a decellularized matrix (scaffold) has advantages over direct transplantation such as local injection or intravenous infusion. Decellularized matrix provides a three-dimensional scaffold into which seeded stem cells could incorporate and help to build the foundation for the integration of local tissue. Thus prepared seeded matrices can be utilized for various purposes in regenerative medicine. But, such biomaterials are not easily available due their high cost and technology involved. In the present study, we prepared a biomaterial which can be availed easily by decellularizing the bovine gallbladder with 0.5% SDS and seeding the stem cells over it.

2. Materials and methods

2.1 Decellularization of bubaline gall bladder with 0.5% Sodium dodecyl sulfate

The decellularization of gall bladder was done as per the protocol developed by Shakya (2014) [8]. The gall bladder of bovine was collected from local abattoir (Fig. 1). Immediately after collection, the gall bladder was kept in cool normal saline solution containing 0.02% EDTA and antibiotic (amikacin 1mg/ml). The tissue was rinsed with normal saline before and start of the protocol. The maximum time period between retrieval and initiation of protocol was less than 4 h. The neck and fundus of the gall bladder were trimmed, followed by a longitudinal incision to obtain a flat sheet of tissue. The inner mucosal layer of gall bladder was peeled off and outer serosal layer was removed by mechanical delamination with a blunt edge. Tissue was cut into 2x2 cm^2 size pieces. The gallbladder tissue was decellularized using 0.5% ionic biological detergent sodium dodecyl sulfate (SDS) having concentration 10 mM tris buffer solution for 48 h (Fig. 2). The acellularity and collagen fiber arrangement were assessed at 48 h by histological examination. The tissue was thoroughly washed in phosphate buffered saline (PBS) solution after completion of protocol. The prepared acellular matrices derived from cholecyst were stored at 4 ^{0}C in PBS solution containing 0.1% amikacine till further use.

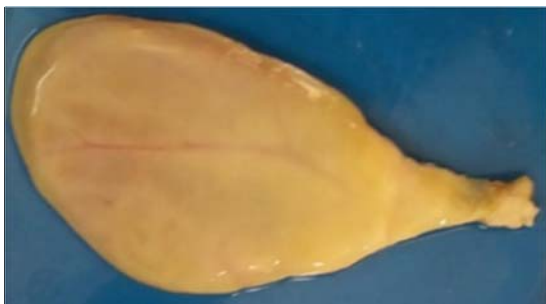

Fig 1: Bovine gallbladder.

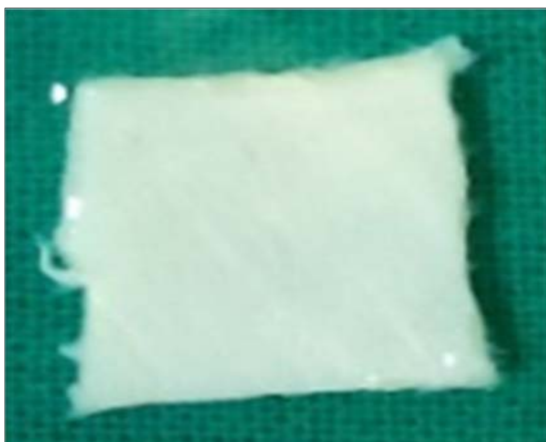

Fig 2: 0.5% SDS treated delaminated gallbladder.

2.2 Isolation and culture of stem cells

The bone marrow was collected from femur and tibia after euthanizing the rat. The area between hip and hock joint all around its circumference was clipped and shaved. A skin incision was made all along the length starting from coxo-femoral and hock joints followed by separating the muscles to visualize the bones. The coxo-femoral and hock joints were disarticulated. The muscles and attaching tissues were carefully removed from the bones. The proximal most part of the femur near neck and distal most part of femur near stifle joint were cut, using a bone cutter. The bone marrow cavity was flushed from one side using sterile phosphate buffered saline (PBS) into petridish and the same PBS was used to reflush the marrow cavity. In a similar manner, tibia bone was also cut both at proximal and distal ends and repeatedly flushed from one side with PBS. The collected bone marrow was transferred into a sterile vial for culturing.

Three milliliter of collected bone marrow aspirate was taken in a sterile vial and a equal amount of Rosewell Park Memorial Institute-1640 (RPMI) having broad spectrum antibiotic. It was mixed gently and centrifuged at 1800 rpm for 15 minutes. The supernatant was discarded and remaining volume was carefully layered over to double the amount of lymphocyte separation media (Histopaque 1077). Again it was centrifuged at 1800 rpm for 30 min. The buffy coat was collected carefully in a sterile 15 ml tube and RPMI along with a broad spectrum antibiotic and centrifuged for 10 minutes. After discarding the supernatant, the obtained pellet was washed twice and resuspended in growth media *i.e.* RPMI containing 10% fetal bovine serum (FBS) and antibiotic-antimycotic. Later it was seeded in culture flask. The flask was maintained at 37℃ in a humified atmosphere of 5% CO_2 and 95% air in a CO_2 incubator.

2.3 Seeding of stem cells on decellularized gall bladder matrices

The third passed bone marrow derived mesenchymal stem cells were used for seeding on decellularized matrices. After 80-90% confluency they were passaged into new culture flasks. The decellularized matrices were washed 4-5 times with antibiotic containing RPMI and then placed in wells of the cultre plate. The cells were trypsinized to detach the monolayer of cells from the flask. The growth medium *i.e.* RPMI containing 10% fetal bovine serum was added to stop the activity of trypsin, and mixed properly to get single cell suspension. The cells were statically seeded on the matrices at the rate of 10^6 cells/cm^2. It was maintained at 37 ^{0}C in a humidified atmosphere of 5% CO_2 and 95% air in a CO_2 incubator.

3. Results and discussion

The bovine gallbladder was collected from local abattoir. After cleaning the mucosal layer was peeled off easily. The serosal layer was separated mechanically with the help of forceps. The delaminated gallbladder was treated with 0.5% SDS for 48 h. Macroscopically, the bovine gallbladder matrix after treatment with 0.5% SDS appeared soft, whiter and slightly spongy in consistency than native tissue. Microscopically, native bovine gall bladder showed cellularity, mucosal and serosal layer, fatty tissue and some liver portion. Native bovine gall bladder and delaminated bovine gall bladder showed dense compact arrangement of collagen fibers. The microscopic observations of the acellular bovine CEM prepared by this protocol showed complete loss of cellularity. The sub mucosal layer was completely acellular. The CEM showed thin, heavily loose arranged collagen fibers with very high porosity. No debris was evident. The bovine gallbladder specimens treated with 0.5% SDS for 48 h were adequately decellularized and found suitable for the further use as scaffold. The histological observations of native and acellular gallbladder are presented in figure 3-4.

The flushing of medullary canal of femur and tibia of young rats resulted in good amount of bone marrow collection for

culturing of bone marrow derived mesenchymal stem cells. The cell separation technique as per histopaque protocol isolated the mononuclear fraction of bone marrow very clearly. The cells seeded on culture plates were observed daily under the inverted phase contrast microscope (Fig.5) to assess the viability and proliferation of cells. The attachment of rat bone marrow derived mesenchymal stem like cells (r-BMSCs) was observed on 6th-8th day post seeding. The cells showed characteristic growth and adherence pattern *in-vitro* and proliferated rapidly to complete the monolayer in about 12-15 days. The morphology of *in-vitro* cultured cells clearly indicated the presence of mesenchymal stem cells. After 2-3 subcultures, when adherent cells reached a sub confluency, they were detached with 0.25% trypsin-EDTA solution and then seeded on acellular gallbladder matrices. The r-BMC cells were seeded in a drop-wise fashion at a $2x10^4$ cells/cm^2 on 2x2 cm^2 size acellular gallbladder matrices (Fig. 6) kept in each well of 6 well cell culture plate. Morphological assessment was done at different time intervals by phase contrast microscopy.

The protocol for decellularization of bovine gall bladder optimized by Shakya (2014) [8] was used for making acellular CEM. The tissue was subjected to ionic biological detergent (SDS) treatment at 0.5% concentration for 48 h. At 48 h, complete acellularity with no cellular debris was observed. No nuclear bodies were seen and the tissue was primarily composed of extracellular matrix. Desired results were achieved after 48 h of treatment with 0.5% SDS detergent. The submucosal layer was completely acellular. The collagen fibers were loosely arranged as compared to the native tissue. SDS in 0.5% concentration is very effective for removal of cellular components from gallbladder tissue. The cell extraction was effectively achieved without significant disturbances in extracellular matrix morphology and strength. SDS is very effective in removing the cellular components from tissue. Compared to other detergents, SDS yields more complete removal of nuclear remnants and cytoplasmic proteins, such as vimentin (Woods and Gratzer, 2005) [9]. SDS trends to disrupt the native tissue structure and causes a decrease in the GAG concentration and loss of collagen integrity. However, it does not appear that SDS removes collagen from the tissue.

Decellularized bubaline gall-bladder matrices were used as scaffolds for the culture of r-BMSC. The rat bone marrow mononuclear fraction was expended to isolate and propagate the mesenchymal stem cells fraction out of it. The technique of static seeding of mesenchymal stem cells over decellularized matrices of bubaline gallbladder origin was found successful as we could obtain cell attachement to decellularized matrices by day 7.

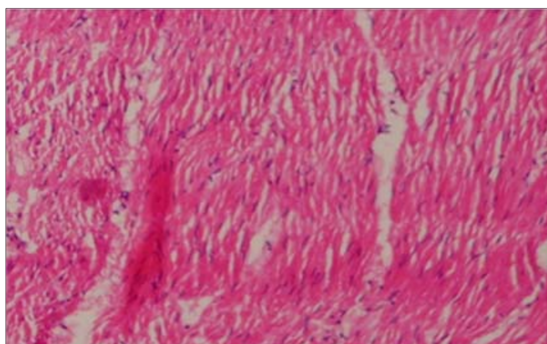

Fig 3: Microphotograph of bovine gallbladder without treatment (H&E, 200X).

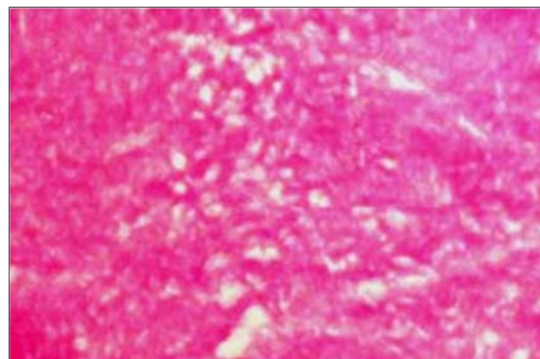

Fig 4: Microphotograph of bovine gallbladder treated 0.5% SDS at 48 hours (H&E, 200X).

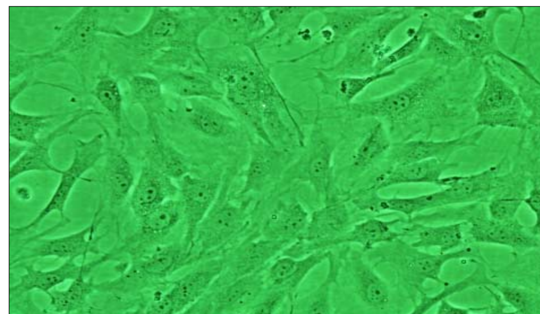

Fig 5: Photograph showing confluent of r-BMSCs culture.

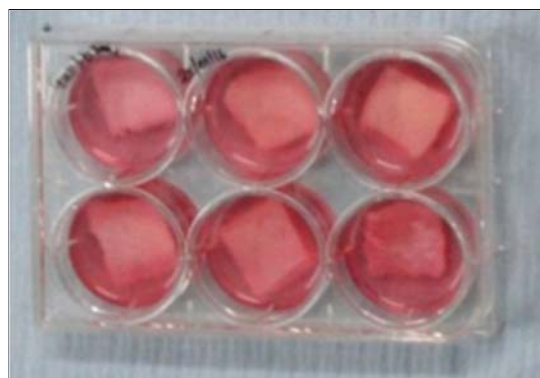

Fig 6: Gallbladder matrices seeded with r-BMSCs.

4. Conclusion

The bovine gallbladder was decellularized with ionic biological detergent SDS at 0.5% concentration for 48 h. Then third passage stem cells (r-BMSCs) were seeded on acellular gallbladder matrices. The entire surface of the bubaline gallbladder matrix was covered by the on growing r-BMSCs. Thus a bioengineered scaffold was developed which can be utilized for wide range of purposes in regenerative medicine.

5. Acknowledgements

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6. Conflict of interest

The authors declare that they have no conflict of interest.

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