Tissue Engineered Human Ear Pinna Derived from Decellularized Goat Ear Cartilage: Clinically Useful and Biocompatible auricle construct

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Abstract

Surgery of the entire ear pinna even today presents a challenge to reconstructive surgeons, in the absence of a universally acceptable, quality construct for clinical use. In this article, the authors present a technique to generate a flexible, human-size ear with the aim to meet this limitation for ear reconstructive surgeries. The construct was engineered by using a decellularized goat ear cartilage. This was characterized by hematoxylin-eosin (H/E), diamidino-2-phenylindole (DAPI), Masson's trichrome (MT), Alcian Blue (AB) staining and Scanning Electron Microscopy (SEM) for extracellular matrix (ECM) analysis. The decellularization protocol followed yielded complete removal of all cellular components without changing the properties of the ECM. In vivo biocompatibility of the ear, pinna showed demonstrable recellularization. Recellularization was tracked using HE, DAPI, MT, AB staining, toluidine staining, SEM, vascular-associated protein (VAP), and CD90+ expressing cells. VAP expression revealed specific vasculogenic pattern (angiogenesis). CD90+ expression reflected the presence of the stromal cell. The graft maintained the properties of ECM and displayed chondrocyte recruitment. In summary, the decellularized goat ear pinna (cartilage) exhibited xenograft biocompatibility, stable mechanical properties, and in vivo chondrocyte recruitment. Subsequently developed tissue-engineered ear pinna offer potential for cartilage flexibility and individualization of ear shape and size for clinical application.

Abstract

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Keywords: Ear pinna, Cartilage, Reconstructive surgery, decellularized scaffold, Biocompatibility, recellularization

Introduction:

Congenital deformities of the external ear (pinna or auricle and external auditory canal [EAC]) are collectively termed microtia. Congenital auricular malformations range from smaller than normal pinna (Grade I), partial ear with closed-off external ear canal (Grade II), small peanut shaped cartilage with relatively well-formed ear lobe (Grade III) and total absence of ear (Grade IV). Higher grades are accompanied by different levels of hearing loss and EAC abnormalities too (Arora, Sahoo, Gopi, & Saini, 2016)(Barron & Pandit, 2003). Occurrence reported varies from 0.83 to 17.4 per 10,000 births with higher incidence reported in Hispanics, Asians, and native Americans and associated consequences such as lack of confidence, poor interpersonal skills and antagonism increasing with age have been reported (Duisit et al., 2018).

Current therapies use the autologous intercostal cartilage hand-carved to serve as grafts for ear reconstruction. Remodeling of cartilage to the appropriate shape falls to the operating surgeon. However, this autologous graft is associated with several unwanted ramifications such as inflammation, lack of neo-cartilage maturation, donor site morbidity, risk of pneumothorax and costochondritis (Hasan et al., 2014)(Kim & Evans, 2005)(Jeevitaa Kshersagar, Kshirsagar, Desai, & Bohara, 2018)(Reighard, Hollister, & Zopf, 2017). A xenogeneic graft would demonstrate enhanced compliance but is accompanied with the higher chance of immune rejection with reduced patency (Assaw, 2018). Allografts, though promising, also have their own limitations such as host versus graft reaction, chances of opportunistic infectious diseases or premature degradation of the implants. Non-biological source implants or alloplastic/prosthetics made of silicone, hydroxyapatite, nonporous materials, polyethylene implants do not appear natural but can be biometric. The synthetic implants have a higher chance of secondary tissue infection at the site of implantation, framework fracture and extrusion (Tardalkar, Desai, Adnaik, Bohara, & Joshi, 2017)(Ten Koppel, Van Osch, Verwoerd, & Verwoerd-Verhoef, 2001) Recent trends in reconstructive surgery involve use of preformed implants to construct an external ear or custom source implants at the time of surgical implantation. These are plagued with the challenge of ensuring mechanical stability of the implant (Arora et al., 2016). The present status of cell-based therapies, with the dangers of dedifferentiation, preclude their application in creation of an intact and complete functioning model (Youngstrom, Barrett, Jose, & Kaplan, 2013).

Tissue engineering [TE] provides a viable alternative for reconstructive surgeries of the external ear (Borrelli, Hu, Longaker, & Lorenz, 2020). TE involves combining living cells with a natural/synthetic support or scaffold to build a three-dimensional living construct that is functionally, structurally and mechanically equal to or better than the tissue that is to be replaced (Hasan et al., 2014; Kim & Evans, 2005). Tissue Engineered technologies, where the scaffold is generated through a decellularization process followed by cell seeding, could meet scientific and clinical needs faster than other methods. (Duisit et al., 2018).

Cartilage tissue engineering is an area that warrants greater attention in this respect. A nascent area of investigation, it faces the dilemmas of scaffold maintenance, unstable biomechanical properties, preservation of ECM and biocompatibility(Barron & Pandit, 2003). The decellularized ECM scaffolds serve as a natural template for organ regeneration without immunogenic reaction (Díaz-Moreno et al., 2018; J. Kshersagar, Kshirsagar, Desai, Bohara, & Joshi, 2018; Youngstrom et al., 2013). They have the ability to support cell adherence and proliferation by allowing nutrition and gas exchange resulting in new tissue generation in the requisite shape. The efficiency of the TE ear pinna scaffold has been evaluated in in-vivo experiments mainly at subcutaneous implantation sites (Ten Koppel et al., 2001).

In this study, a chemical and enzymatic method of decellularization of goat ear cartilage to generate a scaffold, was used. The processes did not alter the cartilage-specific ECM. The derived construct was evaluated in xeno transplant model in vivo with reference to biocompatibility, functional recellularization and tensile strength using histology, SEM and mechanical testing.

1. **Material and Methods:**
2. **Sample collection:**

Goat ear pinna (n=30) samples were harvested at the slaughterhouse. The entire dermis and epidermis were removed carefully. All cartilages were rinsed in 70% alcohol followed by normal saline containing antibiotics solution of 100 ug/ml Streptomycin (Abbott Healthcare, India),100 U/ml Penicillin (Alembic Pharmaceuticals Ltd, India), and 2.5 ug/ml Gentamycin (Cadilla Pharmaceuticals Ltd, India). The rinsed and dried cartilages were stored at -40degC until further use.

Decellularization of goat ear pinna:

Goat ear cartilage samples were decellularized using protocol developed in-house to remove all cellular components from ear pinna to make it nonimmunogenic. Decellularization comprised of cycles carried out by five methods (Figure 1). For the five methods, the goat ear cartilages were treated using a sterile box in different solutions on shaker (REMI RX-12R-DX, India) at 180 rpm. (Figure 1) Method 1 (M1) used a solution of 2% sodium dodecyl sulphate (SDS) for 12 hours followed by distilled water for 12 hours. Method 2 (M2) used 2% sodium deoxycholate for 6 hours followed by 2% SDS treatment for 6 hours. Method 3 (M3) used 5% Dimethyl sulfoxide (DMSO) (v/v) for 6 hours followed by 2% SDS treatment for 6 hours. Method 4 (M4) used distilled water treatment for 2 hours followed by freeze and thaw and overnight treatment with 5% DMSO thereafter. Method 5 (M5) used 1% trypsin (v/v) treatment for 12 hours followed by distilled water for 12 hours. At completion of the treatments as specified, the ear cartilages were stored at -40°C in the deep freezer overnight. All samples were thawed the next morning at room temperature and the cycle repeated till complete decellularization was achieved. After decellularization, prepared scaffolds (Figure 2A) were stored in distilled water containing antibiotics at - 40degC.

Testing of decellularized ear pinna scaffolds:

The decellularized ear pinna scaffolds were tested as follows:

1. Histological analysis
2. DNA quantification
3. Mechanical testing
4. Scanning Electron Microscope
5. **Histological analysis:**

Native ear pinna of goat serve as a Control and decellularized ear pinna were investigated at every 10^{th} cycle by histology. Specimens were fixed with 10% neutral-buffered formalin, dehydrated through alcohol grades and embedded in paraffin wax. The sections were stained for chondrocyte nuclear structures using haematoxyline and Eosin (HE). Sections were stained with DAPI (Invitrogen, CA, USA) to stain adenine–thymine rich regions in DNA. Demonstration of collagen was made by Masson's trichrome (MT) stain (Suvik & Effendy, 2012). Glycosaminoglcans (GAG) content of samples was determined using Alcian blue (AB pH-2.5) (Sigma, A5268) staining.

DNA quantification:

DNA was quantified spectrophotometrically with optical densities at 260nm and 280 nm to yield purity of nucleic acid. Residual DNA was quantified in control and decellularized ear pinna using UV Spectrophotometer (UV-1800 UV-VIS Spectrophotometer) at 260 nm as elaborated here. Samples were collected at concentration of 1 mg/ml and allowed to freeze at - 20degC. After 15 minutes all samples were crushed with mortar pestle. 40 µl of DNA solution was transferred to 3.96 ml of DW in 4 ml cuvette. Utmost care was taken to ensure that solution was air bubble free. Solution was kept for 10 minutes to ensure the complete diffusion of DNA throughout the solution. This represented a 1: 100 dilution of the standards and DNA samples. The spectrometer was set at 260 nm and reading was noted down (Tardalkar et al., 2017).

Mechanical testing:

Mechanical testing was performed by using displacement controlled setup. Control and decellularized scaffold were cut into required cylindrical-shaped 4 cm strips to obtain specimens for mechanical analysis. Burst pressure of scaffold was derived using a modified syringe pump with gradual increase in applied pressure. The values were digitally recorded at each pressure. This was used to find out mechanical strain, stress, strength, deformation and elasticity of scaffold (Konig et al., 2009).

Scanning electron microscopy (SEM):

SEM was performed on cross sections of goat ear pinna cartilage scaffolds to investigate cellular architecture and its porosity. After dehydration in oven at 60°C, the decellularized scaffold samples were fixed to the stage using double sided tape. Images of scaffolds in varying magnification from 80X to 1700 X were taken using JEOL JSM 6360 SEM model at Department of Physics, Shivaji University, Kolhapur.

Fabrication of human ear pinna mould:

The auricle mold for the creation of human ear-like cartilage construct was prepared by a Poly Vinyl Chloride (PVC) which is a non-toxic and chemically inert material. PVC sheets were converted into proper anatomical 3D human ear pinna mold in different sizes such as 55mm, 65mm, and 75mm using a patented method (Rahman, Griffin, Naik, Szarko, & Butler, 2018).

Biocompatibility evaluation of the scaffold

The animal study and all experiments were approved by Institutional Animal Ethical Committee (IAEC) (Ref. - 6/IAEC/2017) of D Y Patil Education Society Deemed University, Kolhapur.

Twenty-four six-month old male mice (*Mus Musculus*) weighing approximately 150 to 160 g were used for study. The animals were divided into control and experimental groups. The experimental mice were kept according to principles of laboratory animal care at control room temperature condition and humidity (˜52%). After 4 weeks, animals were sacrificed by cervical dislocation.

Post implantation assessment of scaffolds.

This was done by histological assessment, mechanical testing, SEM and Immunohistochemistry

Histological assessment

The control and samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with HE, DAPI, MT stain, AB pH 2.5, Toludine blue staining, to demonstrate cellular recruitment. Immunohistochemistry was performed for VAP and $CD90^{+}$expression. Sections (4µ) were taken on the positively charged slides (Pathnsitu biotech, India). Before processing, slides were washed with D/W containing 0.05% tween 20 followed by antigen retrieval using sodium citrate pH 7.4. Serum blocking was done by goat serum and slides were incubated with VAP (Invitrogen, India) and CD90 (Invitrogen, India) overnight at 4^{0}C followed by wash with D/W containing 0.05% tween 20. Staining was done using anti mouse secondary antibody conjugate with Alexa 488 (Molecular Probes, USA) and counterstained using DAPI. Slides were mounted with DAKO mounting media. Stained sections were viewed under fluorescent (Nikon eclipse Ti Japan)

Mechanical testing

Mechanical testing was performed by tensometer, while measuring under tensile loader at room temperature The tensometer applied tensile force to strips of biocompatible ear pinna scaffolds treated with M2, M3 and M5 successively.

SEM analysis:

SEM was performed on cross sections of recellularized M2 (Magnification X 3500), M3 (Magnification X 3000) and M5 (Magnification X 1400) scaffold to investigate cellular architecture and its porosity.

Statistical Analysis Data are reported as the mean ± SD. Differences between three or more groups were assessed using one-way ANOVA. Significance level $p = 0.05$ was set for all the tests. In the figures, statistical significance is denoted as * for p -value [?] 0.05, ** for p -value [?] 0.001, *** for p -value [?] 0.0001.

1. **Results**
2. **Macroscopic appearance of scaffold**

The auricular-shaped scaffold produced by decellularizing goat ear pinna cartilage had a very similar anatomical shape to human auricle with an almost identical size. The engineered auricle was resilient and smooth and had maintained its structural integrity and texture (Figure 2B).

Histology

Histological examinations of the control and decellularized scaffolds were done at every 10^{th} cycle with HE, AB pH-2.5, DAPI, and MT to ascertain whether the cellular components were totally removed and histoarchitecture was preserved.

HE staining of the scaffold showed that complete decellularization was achieved at 40^{th} cycle for M5 and for M1-M4 80^{th} cycle were needed for complete decellularization as shown in Figure 3A with progressive reduction in sequential cycles. Methods M2, M3 and M5 showed negligible nuclear material compared to M1 and M4. Control cartilage showed differentiated chondrocyte with lacunae and nucleus was better organized (Figure E-A)

DAPI staining used to crosscheck the decellularization process revealed complete removal of cellular material in all methods (Figure 3B). Control revealed abundant bluish stained nucleus (Figure 3E-B)

MT staining showed intact appearance of collagen and ECM in decellularized goat ear pinna cartilage scaffolds (Figure 3C) with no obvious disruptions to histoarchitecture. Scaffolds treated with methods 2 and 3 showed better preservation of collagen and ECM compared to other methods. Control showed normal bundle of collagen and ECM (Figure 3E-C).

AB pH-2.5 staining demonstrated that the major structural components via GAGs showed no disruption following treatment with preserved architecture. (Figure 3D). Control showed well organized ECM and GAGs (Figure 3E-D).

DNA quantification:

The DNA content of samples in experimental group showed reduction with each subsequent cycle compared to control (Figure 4A). Scaffolds processed with Method 2 showed the most reduction while Methods 1 and 4 yielded the least reductions in DNA content.

Mechanical testing:

Elasticity, stress rupture and ultimate stress limit of control and decellularized scaffold were confirmed by mechanical testing (Table 1). The mechanical testing analysis showed that the ultimate stress, strain and elasticity for control and decellularized ear scaffold were not significantly different. Ultimate stress and tensile strength in samples treated by M2 and M5 were similarly good. Significant elasticity was seen in samples treated by M2 and M5. Overall M2 and M3 showed better retention of mechanical properties compared to M1, M4 and M5.

Decellularized scaffold prepared by method 2, 3 and 5 showed the significantly biophysical stable, elastic and load-bearing object. Elasticity, stress rupture and ultimate stress limit of control and decellularized scaffold were confirmed by mechanical testing and summarized in Table 1. The mechanical testing analysis showed that the ultimate stress, strain and elasticity for control and decellularized ear scaffolds was not significantly different. Strain of decellularized scaffold was 0.32+-0.015 mm, 0.55+-0.018 mm, 0.27+-0.011 mm, 0.35+-0.014 mm, 0.25+-0.010 mm and 0.34+-0.011 mm in control, method 1, method 2, method 3, method 4 and method 5 respectively. There was no significant difference ($p<0.0001$) in control and control and M3, M4. Ultimate stress (N/mm2) of decellularized scaffold was 60.17+-1.20, 20.33+- 1.80, 98.00+- 2.00, 18.21+-

1.00, 13.01+- 1.00 and 20+- 2.50 N/mm2 in control, method 1, method 2, method 3, method 4 and method 5 respectively. There was significant difference ($p<0.0001$) between control and M2, M4, M5 Ultimate stress and tensile strength in samples treated by M2 and M5 were good and similar. Significant elasticity was seen in samples treated by M2 and M5. Overall M2 and M3 showed better retention of mechanical properties compared to M1, M4 and M5. Deformation (mm) study of scaffold showed 6.5+- 0.5, 5.5+- 1, 5.5+- 0.5 , 7.0+- 1.5, 5.5+- 0.5, and 8.5+- 0.8 in control, method 1, method 2, method 3, method 4 and method 5 respectively (Graph 3). Elasticity (N/mm2) of decellularized scaffold was 21.90+-17.12, 71.21+- 18.000, 45.94+- 20, 55.57+- 11, 95.56+- 18, and 72.17+- 25 in control and all methods 1, 2, 3, 4, 5 respectively ($p<0.05$). Method 2 and method 5 showed no significant difference in elasticity. Overall method 2 and 3 maintained mechanical properties as compared to method 1, 4, 5. Decellularized scaffold prepared by method 2, 3 and 5 showed the significantly biophysical stable, elastic and load-bearing object. Moreover, scaffolds prepared by these methods have more stability, elasticity and flexibility to attain human shaped ear pinna. Hence scaffolds prepared by 2, 3 and 5 were selected for in vivo experiment.

Scanning electron microscopy (SEM) analysis:

Scanning electron microscopy of decellularized ear pinna scaffold (Figure 4B) by M2 showed 3D network of ECM in overall morphology (Magnification, 1000X), M3 sample (Magnification, 1700X) and M5 sample (Magnification, 1000X). SEM results proved that M2 and M3 showed an excellent result to maintain 3D network of ECM and overall morphological porosity as compared to M5.

Biocompatibility evaluation

Selection: Scaffolds achieved by M2, M3 and M5 showed well preserved ECM (collagen and GAG), porous microstructure, superior mechanical stability and elasticity compared to the decellularized ear pinna generated via M1 and M4. These could harbor potential to attain shape desired for the human ear pinna. Hence ear pinna generated by these methods was used for biocompatibility studies.

Method: The scaffolds (n=3) developed from M1 and M4 were transplanted into the peritoneal cavity of mice (Figure 5A) for four week.

HE staining used to monitor the recellularization process showed that the ear pinna supported the chondrocyte growth as manifest by visible nuclei (Figure 7A). Complete recellularization was achieved at 4 weeks after grafting, when the scaffolds showed well organized 3D ECM and collagen in MT staining (Figure 7B), presence of GAGs on AB pH2.5 staining (Figure 7C) and recruitment of live chondrocyte cell (Figure 7D) on toludine blue staining. IHC staining revealed expression of VAP representing vasculogenesis (Figure 7B) and CD 90^{+}(Figure 7C) representing stromal markers and recruitment of chondrocyte specific progenitor cells.

Mechanical testing after biocompatibility study:

Grafted scaffolds showed good mechanical strain, stress, deformation and elasticity as compared to control. The values of mechanical properties of recellularized pinna was summarized in (Table 3 and Figure 6). The Graph 1 showed strain (mm) of recellurized pinna as 0.20+-0.10 mm, 0.32+-0.02 mm and 0.17+-0.005 for method 2, method 3 and method 5 respectively. Ultimate stress (N/mm2) of control and In vivo decellularized ear pinna were similar. M5 decellularization showed highest ultimate stress bearing capacity which was decreased significantly after recellularization (Graph 2). Deformation (mm) did not show much alteration during decellularization and recellularization process in M2 and M5 (Graph 3). In vivo decellularized ear pinna generated by M3 showed highest deformation. The Graph 4 showed elasticity (N/mm2) of biocompatible scaffold was 25.01+-23.40 and 20.90+-12.35 as compared to control (21.90+-17.12 N/mm2). M5 decellularization showed significant increase in elasticity, which was decreased to (20.9 ± 5.35) after in vivo recellularization.

Scanning electron microscopy (SEM) analysis:

Scanning electron microscopy of scaffold showed the 3D network of ECM cell structure in transplanted M2 (Magnification X 3500), M3 (Magnification X 3000) and M5 (Magnification X 1400) scaffold (Figure 5B).

SEM analysis revealed that transplanted scaffolds maintained ECM collagen, elastic fibers and significantly showed bunch of chondrocyte cell recruitment. It showed very excellent results of regeneration of and recellularization with chondrocytic cells.

Discussion:

This study assessed the tissue engineered scaffold of ear pinna cartilage by decellularization using different chemical treatments methods. The study also extended to biocompatibility and recellularization of the scaffold on implantation into an animal model. Chemical and enzymatic detergents solubilize cell membranes and remove DNA thus proving to be effective agents to remove immunogenic tissue material.

These different decellularization methods proved efficacy to generate ideal ear pinna scaffold. Current decellularization protocols are designed to overcome the previous limitations (Utomo, Pleumeekers, & Nimeskern, 2015)(Cervantes et al., 2013). Rahman S et al presented a protocol to decellularize cartilage using trypsin followed by freeze and thaw cycles (Rahman et al., 2018). The present study found that antioxidant treatment followed by freeze and thaw cycles can efficiently remove the DNA content and also preserve the ECM and mechanical properties. This protocol does require more cycles (80 cycles) for decellularization compared to the enzymatic method. However, it has been found to generate stable human ear pinna shaped cartilage with better mechanical properties. It also provides high specificity for removal of antigenic material. On the other hand, enzymatic decellularization protocol was quick and was able to achieve complete decellularization at the 40^{th} cycle. However, this rapid decellularization was at the cost of not maintaining the mechanical properties. The resultant scaffold was more elastic, thin and more time was needed to generate the desired human ear pinna.

The present study shows that scaffold prepared by Methods 2, 3 and 5 were ideal scaffolds with excellent porosity and mechanical properties that retain excellent anatomical ear pinna shape molded in different size and shapes- 55, 65, and 75mm.

The ECM was characterized by histochemistry in this study. HE staining revealed a well-maintained structure of the scaffold with preserved ECM. The residual DNA content after decellularization was greatly reduced compared to that found in native tissue. M5 showed faster decellularization compared to other methods. This is significant as DNA is a sensitive indicator of cell debris due to its high stability and as a marker strongly correlates with adverse host immunogenicity. MT and AB staining demonstrated no obvious disruption to the overall histo-architecture following treatment and were found to maintain collagen and GAGs structure receptively. The results of decellularization methods were promising and appreciable as the amount of collagen remain unchanged. This outcome is desirable as collagen molecules play a vital role in maintaining ECM and also determining tissue functions. SEM analysis reported a preserved ECM of ear pinna cartilage without evidence of any damaged area. Biocompatible testing conducted on animal models (albino mice) found satisfactory vasculogenesis and angiogenesis in graft (Figure. 5A). Biocompatibility and recellularization detected through MT and AB staining pH 2.5, revealed maintenance of ECM architecture in vivo. Scaffolds prepared by Methods 2, 3 and 5 subjected to surface topography confirmed good elasticity. Biocompatibility Biocompatible testing of grafted human ear pinna found fully vascularized. IHC staining with VAP revealed that the transplanted ear pinna express vasculogenesis and angiogenesis marker VAP, while CD90 + marker expression revealed that chondrogenic differentiation as shown in Figure.7 B and C. Expression of CD90+ is important with regard to enhanced collagen type II and chondrogenic differentiation. IHC staining revealed chondrogenic differentiation (Figure. 7B and C) with the graft showing cells in most places amidst abundant collagen matrix and proteoglycan content after four weeks. This was confirmed by Toluidine blue staining in (Figure 7A). Thus, this study provided a novel approach to generate ear pinna xenograft for clinical applications of auricle cartilage to overcome the present limitations. The decellularized scaffold is biodegradable, biocompatible, preserves ECM and it can reduce post-transplant management problem.

Conclusion:

Current reconstructive surgery of the entire ear pinna remains one of the biggest challenges for the plastic

surgeons. Through this study, the authors propose generation of tissue-engineered ear pinna as an alternative to overcome the present limitations in clinical usage of auricle cartilage grafts such as post-transplant management ear pinna scaffold, graft biocompatibility, donor limitation, and preservation of extracellular matrix (ECM), mechanical properties and limitation of cartilage recellularization.

The present was designed to yield an ideal decellularized biocompatible scaffold whose post-transplantation recellularization was satisfactorily characterized for stem cell recruitment and mechanical properties. The decellularized scaffold that has been obtained is biodegradable, biocompatible, preserves ECM and thus can reduce post-transplant management problem.

To summarize, this study highlights a novel approach to generate xenogenic three-dimensional Tissue Engineered human ear pinna scaffold for clinical applications.

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Author Contributions M.J. designed the project and N.B., K.T., J.K., S.S., M.J. wrote the manuscript. N.B., K.T., J.K., S.D. conducted experiments. S.S. conducted statistics and revised the manuscript.. M. J. designed the experiments, and wrote, edited, and corresponded this manuscript. All authors reviewed the manuscript.

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Tables

Table No 1: Mechanical testing of decellularized scaffolds by different decellularization methods

Decellularized Scaffold	**Control goat Ear Pinna**	**M1**	**M2**	**M3**	**M4**	**M5**
Strain ±SD	**0.32±0.01**	**0.55±0.01**	**0.27±0.01**	**00.35±0.01**	**0.25±0.01**	**0.34±0.01**
Stress ±SD	**60.17± 1.20**	**20.33± 1.80**	**98.00± 2.0**	**18.21± 1.00**	**13.01± 1.00**	**20.00± 2.5**

Decellularized Scaffold	Control goat Ear Pinna	M1	M2	M3	M4	M5
Deformation ±SD	5.50± 1.0	5.50± 1.0	5.50± 0.5	7.00± 1.50	5.50± 0.50	8.50± 0.8
Elasticity ±SD	21.90± 17.12	71.21± 18	45.94± 2.0	55.57± 4.12	95.56± 18.0	72.17± 5.0

Table No 2: Mechanical testing of recellularized scaffold after implant subcutaneously in rat

Pinna cartilage	Control goat Ear Pinna	M2	M3	M5
Strain ±SD	0.32±0.01	0.20±0.01	0.32±0.02	0.17±0.005
Stress ±SD	60.17± 1.20	59.02± 1.0	34.79± 1.50	57.93 ± 1.30
Deformation ±SD	5.50± 1.0	10.0± 0.5	16.00± 0.5	8.50 ± 0.2
Elasticity ±SD	21.90± 1.12	25.01±3.4	13.28± 1.13	20.90± 2.35

Figure Legends

Figure. 1: Decellularization protocols for goat ear pinna

In this Figure showed five different protocols of decellularization methods were used.

Figure. 2[A]: Decellularized goat ear pinna

After removing of all cellular components goat ear pinna color was changed to white.

Figure.2 [B]: Shape of human ear pinna

Using mold, decellularized goat ear pinna scaffold shaped into human ear pinna.

Figure 3: Histochemical study of decellularization process.

[E-a] HE stained in control showed well-organized blue nucleus chondrocytes resides in lacunae and preserved ECM.

[A] HE stained decellularized ear pinna in [M1-M5] methods showed no cells. M2, M3, M5 method showed fast decellularizaion as compared to other, while at 80th cycle no any nucleus showed.

[E-b] DAPI staining in native ear pinna showed prominent blue fluorescents nucleus.

[B] DAPI staining in decellularized scaffold showed no nucleus. Method 5, M2, and M3 showed complete decellularization earlier than other methods.

[E-c] Massiontrichrome staining in native showed a well-organized form of ECM blue color collagen and elastin.

[C] The organization and mass of collagen fibers remain unaffected after decellularization in all methods.

[E-d]AB pH 2.5 staining in native ear pinna.

[D]AB pH 2.5 staining confirmed that the GAG has been preserved and its organization appeared to remain unaffected after decellularization

Figure 4A: DNA Quantification

Overall decellularized goat ear pinna cartilage such as M2 [0.006±0.001µg/ml], M3, and M5 significantly showed negligible DNA content ($p < 0.0001$) compared to control (0.12 µg/ml), M1 and M4. *Significant difference with $p < 0.05$.

Figure 4B: Scanning electron microscopy of decellularized goat cartilage

SEM analysis of decellularized ear pinna scaffold by M2 (Magnification 1000X), M3 (1700X) and M5 (1000X) methods revealed not many obvious changes in the architecture of ECM and with clear surface porosity, however by method 2 and M3 showed good surface porosity.

Figure 5A: Biocompatibility testing

[A] Peritoneal cavity of mice showed grafted ear pinna scaffold. [B] After 15th days of grafting angiogenesis showed without any immune rejection.

Figure 5B: Scanning electron microscopy of goat cartilage after transplantation:

SEM analysis of grafted ear pinna scaffold by methods M2 (Magnification X 3500), M3 (Magnification X3000) and M5 (Magnification X 1400) revealed well-organized recellularization of chondrocytes and its secreted ECM.

Figure. 6: Mechanical testing transplanted human size ear pinna:

Graph 1.Ultimate strain, Graph 2. Ultimate stress, Graph 3.Deformation, Graph 4.Elasticity.

In graph 1-4, Methods 2 and M 3, M 5 of grafted ear pinna scaffold showed as remarkable stable mechanical properties as compare to native pinna. Overall recellularization M2 and M5 significantly enhanced elasticity as compared to native cartilage. However, re-cellularised scaffold from Method 2, M3 and M5 should maintain elasticproperties and significance one way analysis (ANOVA) (***$p < 0.0001$) in comparison to Control. All the decellularised and re-cellularisedM2, M3 and M5 groups were compared with control for elastic (Young's modulus) properties.

Figure 7: Histochemistry of scaffold after transplantation.

[A] HE stained in grafted ear pinna showed recruited chondrocytes ECM and chondrocytes with its lacunae.

[B] Masson's trichrome staining shows a columnar type of chondrocyte in most places and has an abundant collagen matrix. The red coloration of the hyaline cartilage, reflecting proteoglycan content, is homogenous and the surface of the cartilage is smooth.

[C] AB pH 2.5 staining confirmed that the GAG organization. [D] Toluidine blue staining confirmed in grafted ear pinna chondrogenic differentiation.

Figure. 8: VAP-1 expression in scaffold after transplantation

[A] DAPI staining in grafted ear pinna showed blue fluorescents nucleus.

VAP-1: B) M2, C) M3, D) M5 treatment showed green fluorescent expression of vascular-associated protein marker.

Figure. 9: CD90+ expression in scaffold after transplantation

B) M2, C) M3, D) M5 seen green fluorescent expression as chondrocyte progenitor cell (Mescenchymal stromal cell).

Fig.1: Decellularization protocols for goat ear pinna

Method 1	Method 2	Method 3	Method 4	Method 5
2% SDS (w/v) treatment for 12hrs.	2% sodium deoxycholate (w/v) treatment for 6hrs.	5% DMSO (v/v) treatment for 6 hrs.	Distilled water (DW) treatment for 2hrs, followed by freeze and thaw	1% trypsin (v/v) treatment for 12hrs
All treatment (M1-M5) cycles were carried out in a sterile box on the shaker [REMI RX-12R-DX] at 180 revolutions per minute (rpm).				
Distilled water for 12 hrs	2 % SDS treatment for 6hrs	2 % SDS treatment for 6hrs	Overnight 5% DMSO	Distilled water for 12 hrs
After a specific time point run samples stored - 40 °C in the deep freezer overnight.				
Every treatment sample was thawed the next morning at room temperature and the cycle was repeated.				

Fig.2: (A)Decellularized goat ear pinna

Fig.2: (B) Shape of human ear pinna

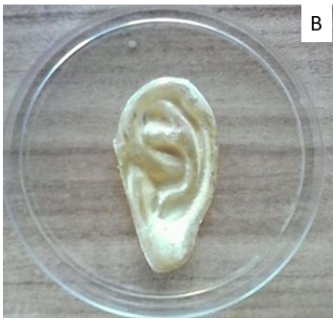


Fig 3 : Histochemical study of decellularization process.

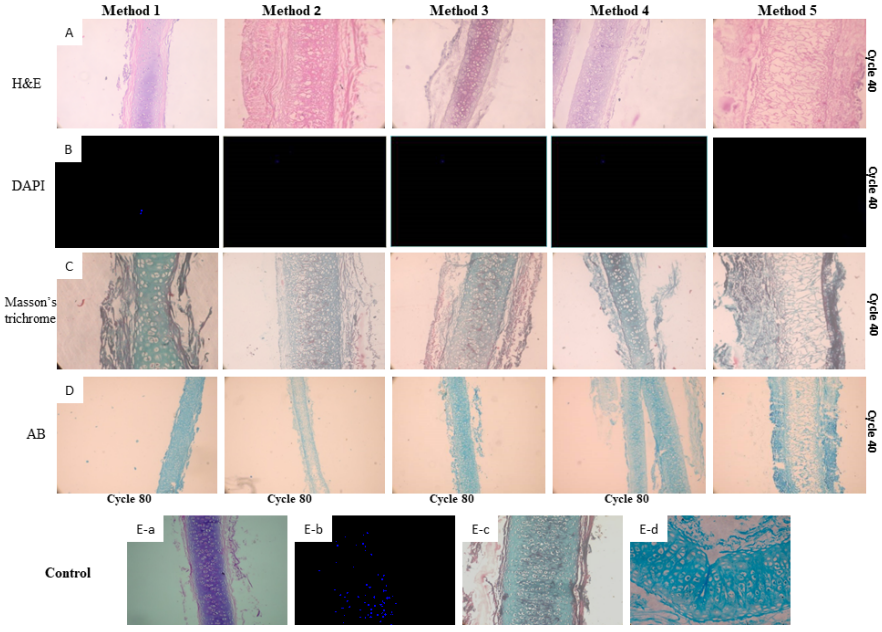


Fig.4A: DNA Quantification

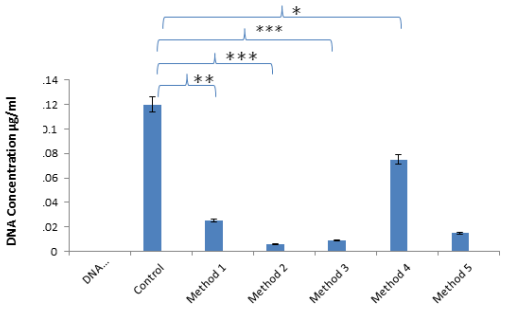


Fig 4B: Scanning electron microscopy of decellularize goat cartilage

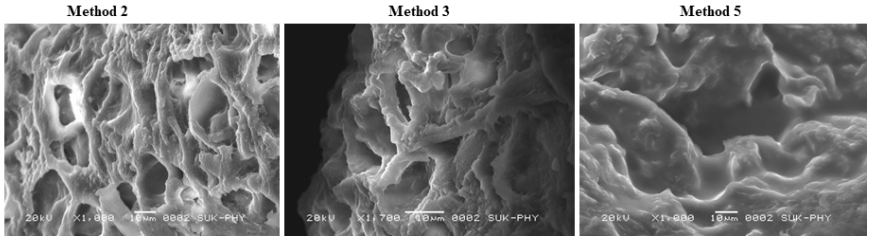


Fig 5A : Biocompatibility testing

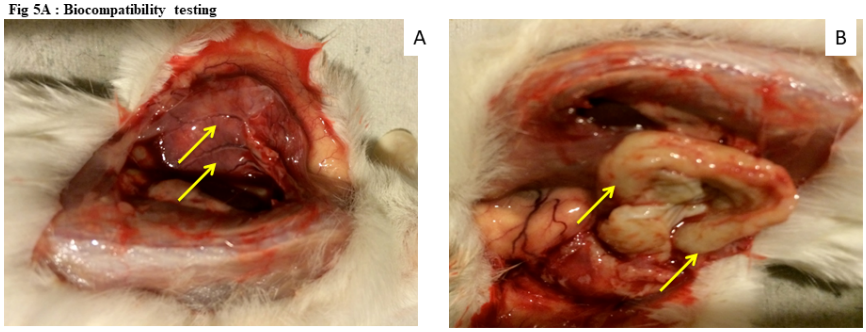


Fig 5B: Scanning electron microscopy of goat cartilage after transplantation

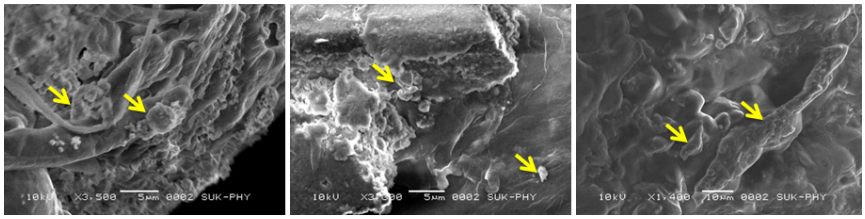


Fig. 6 : Mechanical testing of human size ear pinna done after transplantation

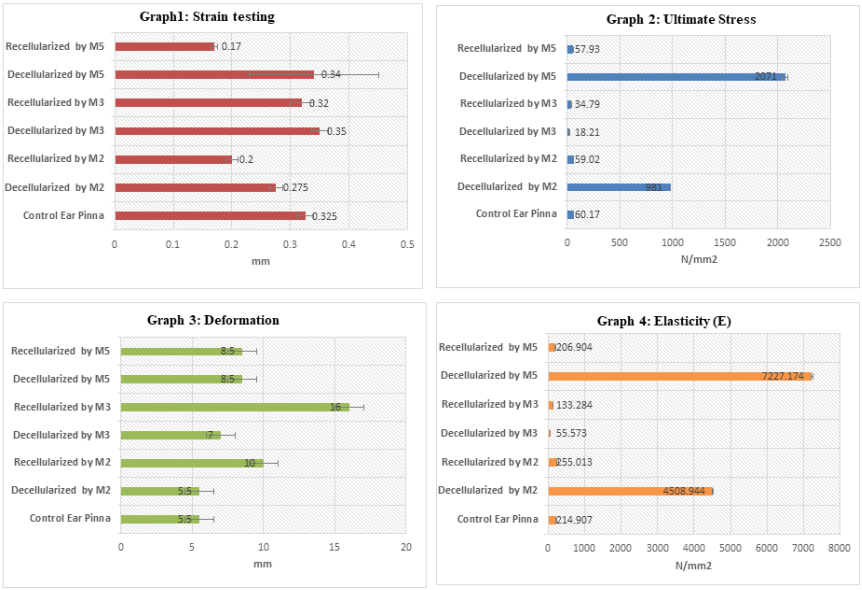


Fig 7: Histochemistry of scaffold after transplantation.

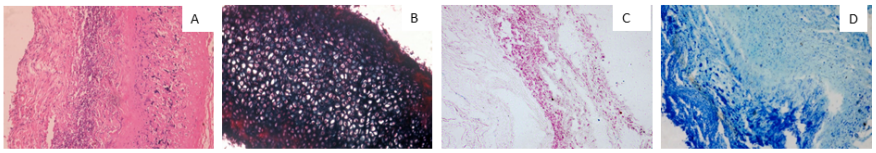
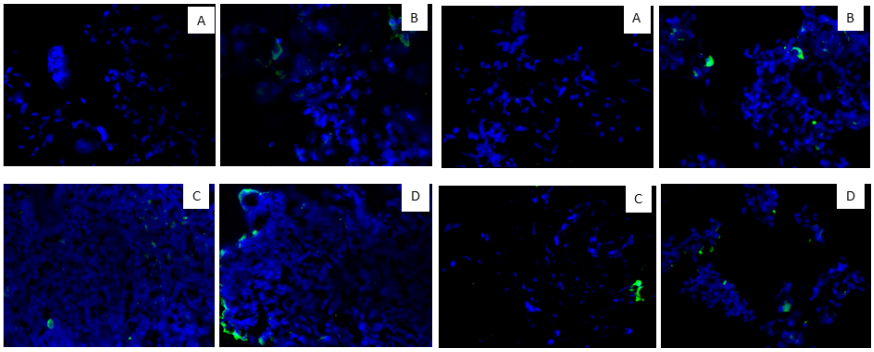


Fig 8A : VAP expression in scaffold after transplantation.

Fig 9B: CD90^{+} expression in scaffold after transplantation.



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