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Preparation of Porcine Acellular Diaphragm Matrix (ADM) using sodium deoxycholate as a biological detergent for application in surgical reconstruction

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Abstract
Acellular Tissue Matrix is now been considered suitable alternatives to synthetic materials in reconstructive surgery. In the present study, fresh porcine diaphragm was subjected to biological detergent treatment under physical agitation in orbital shaker to make it cell free. To standardize the decellularization protocol, different concentrations of Sodium Deoxycholate (1%, 1.5%, 2% and 2.5%) solutions were used and the cut pieces of diaphragm were kept under physical agitation in an orbital shaker (180 rpm) at 35 ^{0}C till 36 h. The detergent solution in each concentration was changed in every 6 h and representative samples were kept in 10 % neutral formalin in every 12 h. The histological analysis from each representative tissue sample with different concentration and duration combinations were carried out to standardize the optimum decellularization protocol.
Fresh Porcine Diaphragm treated with 2% sodium deoxycholate under physical agitation in orbital shaker at 35 ^{0}C for 24 h was found to be efficient enough for complete removal of cells as well as maintaining the normal collagen fibre pattern when compare to other combinations.

Keywords: Decellularization, Porcine, Diaphragm, Sodium Deoxycholate

1. Introduction
The synthetic material used in reconstructive surgery are now being replaced by biological materials like acellular dermal graft (Gangwar, 2002) [1], acellular diaphragm (Kumar *et al.,* 2015) [5], small intestine (Kumar *et al.,* 2010) [4] to minimize its graft related complications. The biological biomaterials are good alternative option to deal with the surgical reconstruction procedure; but cellular graft may induce immunological reactions due to the presence of histocompatible antigens. These complications can be prevented by making the tissue cell free with standard decellularization protocol before being use as implanting material. The goal of decellularization is to ameliorate the antigenicity of the biological graft by efficiently removing all its cellular and nuclear material while minimizing any adverse effect of the composition, biological activity and mechanical integrity of the remaining extracellular matrix. Sodium deoxycholate, a chemical detergent can be used as decellularizing agent under physical agitation in preparation of acellular diaphragm matrix. In the present study, sodium deoxycholate salt was used to standardize the decellularization protocol for Porcine Diaphragm in preparation of Porcine Acellular Diaphragm Matrix (ADM).

2. Materials and Methods
Decellularization protocol was followed as per the method described by Kumar *et al.,* (2015) [5]. Fresh porcine was collected from a local abattoir and immediately preserved in chilled (4 °C) sterile 1X phosphate buffered saline (PBS, pH 7.4) containing 0.1% Amikacin (Mikacin, Aristo Pharmaceuticals Private Limited, Mumbai, India), and 0.20% EDTA. After collection of the whole diaphragm the tendinous portion was excised and washed thoroughly with sterile PBS to remove all the adherent blood; thereafter the diaphragm was cut into the desired size and then placed in different concentrations of sodium deoxycholate solution (1%, 1.5%, 2% and 2.5%) at a constant temperature (35 °C) on an orbital shaker under physical agitation (180 rotations/minute). Representative tissue samples collected after different periods of detergent treatments (12, 24, and 36 h) were fixed in 10% neutral buffered formalin, serially dehydrated

with ethanol, cleared in xylene and embedded in paraffin wax. Sections (5µm) were cut and stained with hematoxylin-eosin for histological analysis, to optimize the decellularization protocols. The chemical detergent solution was changed in every 6 h in each concentration. The resulting ADM was rinsed with PBS extensively under constant agitation (180 rotations per minute) and temperature (35 ^{0}C) on an orbital shaker to remove the residual chemicals. Further, the prepared matrices were stored in a sterile PBS solution, containing 0.1% Amikacin and 0.1% Sodium Azide at -20 °C until use. The present study was in full compliance with the Institutional Animal Ethics Committee, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India.

3. Result and Discussion

A histological photograph of the tendinous portion of diaphragm without physical agitation in orbital shaker in Sodium Deoxycholate solution showed normal cellular pattern (Fig 4.1). Treatment of diaphragm at different concentration of sodium deoxycholate for shorter incubation period (12 h) did not damaged the normal architectural fibre pattern of the tissue, but resulted in retention of whole cells (Fig 4.2); while a higher concentration (2.5% sodium deoxycholate) with longer duration resulted in complete removal of cells, but lead to disintegration of natural three-dimensional collagen structure within the matrix (Fig 4.3). In comparison to the aforementioned incubation periods and temperature combination; cellularization protocol carried out in 2% sodium deoxycholate solution, incubated for duration of 24 h at 35 ^{0}C under physical agitation in orbital shaker was found to best in preserving the distinctive, natural, three-dimensional collagen structure within the prepared matrix. The histological examination of the acellular matrix revealed that the cells had been completely removed and the collagen fibres were orderly arranged (Fig 4.4). Gilbert *et al.,* (2006) [2] reported that ionic biological detergent like sodium deoxycholate initiate decellularization with disintegration of cell membrane and productively eliminate cellular residues; which accelerate by mechanical stirring. Schmidt and Baier (2000) [7] explained that decellularization can be brought about by specific physical, chemical, and enzymatic methods which leave a material composed of essentially of extra cellular matrix (ECM) components and resultant acellular tissues retained their natural mechanical properties and promoted remodeling of the prosthesis by neovascularization and recellularization by the host tissue. In contrary to author's findings Kumar *et al.,* (2013 and 2015) [6, 5] standardized decellularization protocol for small intestine and diaphragm of bubaline origin with application of 2% sodium deoxycholate for a period of 36 h and 48 h at 37 ^{0}C under physical agitation respectively. Kumar *et al.,* (2014) [3] effectively carried out de-epithelization of rabbit skin with 0.25% enzymes and 2M sodium chloride solution for 8 hours, followed by decellularization was done 1% biological detergent for 48 hours under continuous physical agitation at 180 RPM in orbital shaker.

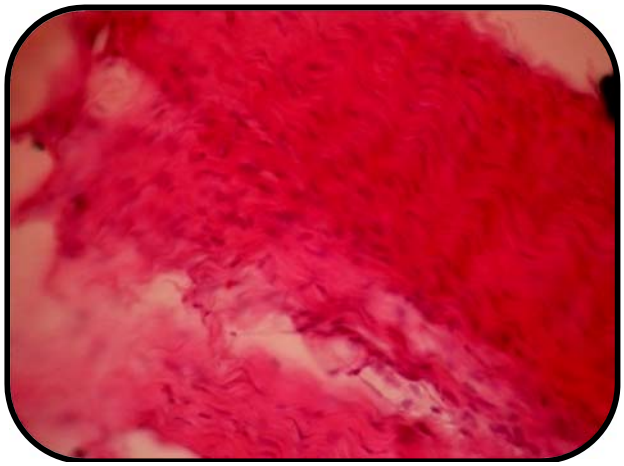

Fig 4.1: Showing Normal Cellular Pattern of Unprocessed Porcine Diaphragm

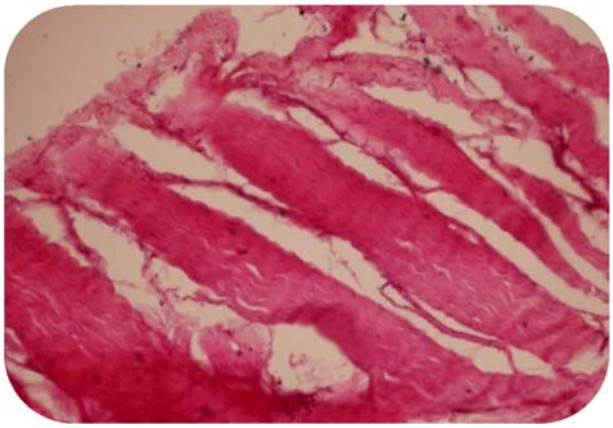

Fig 4.2: Showing numbers of whole cells treated diaphragm for 12 h.

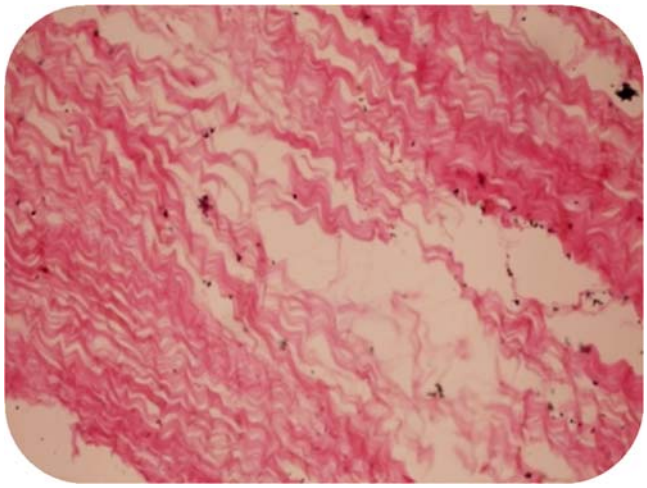

Fig 4.3: Showing disintegration of fibre pattern in treated diaphragm for 36 h.

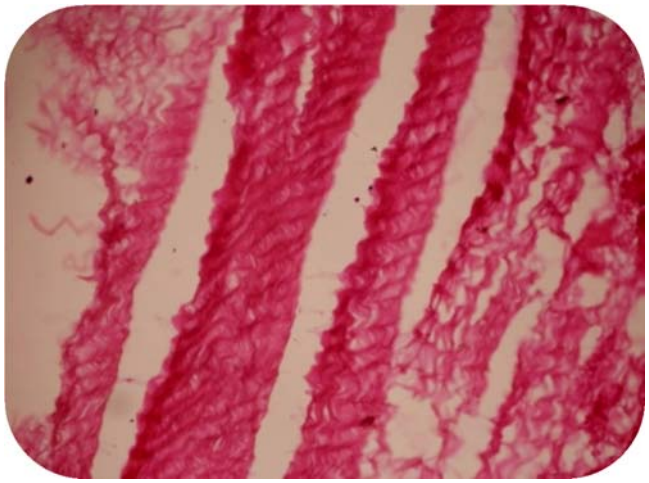

Fig 4.4: Histogram Showing good pattern of collagen fibres along with complete removal of cells

4. Conclusion

Use of biological detergent like sodium deoxycholate is an efficient decellularization agent for compete removal of cells in porcine diaphragm with physical agitation in orbital shaker under control temperature and speed. The concentration of the detergent solution and its time duration might vary with type tissue, its organ source and species involved. In the present study, porcine Acellular Diaphragm Matrix (ADM) could be prepared by treating with 2% sodium deoxycholate solution under physical agitation in orbital shaker at 180 rpm and 35 ^{0}C for duration of 24 hours.

5. References

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