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Research Article

Development of Acellular Dermal Matrix from Skin of Different Species of Animals Using Biological Detergents and Enzymes Combinations

Sanjay Purohit, Naveen Kumar*, Ashok Kumar Sharma, and Anil Kumar Sharma

Division of Surgery, Indian Veterinary Research Institute, India

***Corresponding author**

Naveen Kumar, Division of Surgery, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, Pin: 243122, India, Email: naveen.ivri1961@gmail.com

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Abstract

Decellularized tissues have been successfully used in a variety of tissue engineering/ regenerative medicine applications, and the decellularization methods vary as widely as the tissues of interest. The efficiency of cell removal from a tissue is dependent on the origin of the tissue and the specific physical, chemical, and enzymatic methods that are used. Each of these treatments affect the biochemical composition, tissue ultrastructure, and mechanical behavior of the remaining extracellular matrix (ECM) scaffold, which in turn, affect the host response to the material. We have optimized the protocols for making acellular dermal matrix from rabbit, pig, goat, and sheep and buffalo skin using different combinations of ionic and non-ionic biological detergents.

INTRODUCTION

Biologic scaffolds derived from decellularized tissues and organs have been successfully used in both pre-clinical animal studies and in human clinical applications [1-4]. Removal of cells from a tissue leaves the complex mixture of structural and functional proteins that constitute the extracellular matrix (ECM). The tissues from which the ECM is harvested, the species of origin and the decellularization methods for these biologic scaffolds vary widely. Each of these variables affects the composition and ultrastructure of the ECM and accordingly, affects the host tissue response to the ECM scaffold following implantation. Xenogeneic and allogeneic cellular antigens are recognized as foreign by the host and therefore induce an inflammatory response or an immune-mediated rejection of the tissue. However, components of the ECM are generally conserved among species and are tolerated well even by xenogeneic recipients. ECM from a variety of tissues, including heart valves [5,6], blood vessels [7-8], nerves [9-10], skeletal muscle [11], tendons [12], ligaments [13], small intestinal submucosa (SIS) [14-15],urinary bladder [16-17] have been studied for tissue engineering and regenerative medicine applications. The goal of a decellularization protocol is to efficiently remove all cellular and nuclear material while minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM.

Any processing step intended to remove cells will alter the native three-dimensional architecture of the ECM. The most commonly utilized methods for decellularization of tissues involve a combination of physical and chemical treatments. The physical treatments include agitation or sonication, mechanical massage or pressure, or freezing and thawing. These methods disrupt the cell membrane and release cell contents. The physical treatments are generally insufficient to achieve complete decellularization and must be combined with a chemical treatment. Enzymatic treatments, such as trypsin, and chemical treatment, such as ionic and non-ionic biological detergents, disrupt cell membranes and the bonds responsible for intercellular and extracellular connections. Tissues are composed of both cellular material and ECM arranged in variable degrees of compactness depending on the source of the tissue. The ECM must be adequately disrupted during the decellularization process to allow for adequate exposure of all cells to the chaotropic agents and to provide a path for cellular material to be removed from the tissue. The intent of most decellularization processes is to minimize the disruption and thus retain native mechanical properties and biologic properties.

In the present study we used continous agitation as physical treatment combined with chemical treatment using different combinations of ionic and non-ionic biological detergents for

making acellular dermal matrix from rabbit, pig, goat, and sheep and buffalo skin.

MATERIALS AND METHODS

Fresh ventral abdominal skin of pig, goat, sheep and buffalo was procured from the local abattoir. The skin of rabbit was collected from the animal euthanized for some other reasons. The skin was preserved in cold physiological saline just after the collection and later on rinsed with normal saline to remove the adhered blood. The maximum time period between the retrieval and the initiation of protocols was less than 4 h.

The tissue samples were cut into 2×2 cm^2 size pieces. The epidermis of the bovine, porcine, caprine and rabbit skin was removed using trypsin and sodium chloride in different concentration and time intervals and was subjected to different protocols for the preparation of acellular dermal matrix. The optimization of de-epidermis protocol was done on the basis of gross, histopathological studies. The optimized protocol was used to separate the epidermis and remaining cellular dermis matrix was further used for optimization of acellular protocols for each species.

Four protocols for the acellularity of dermal graft were tested. Trypsin enzyme and biological detergents combination were used. Two anionic and two non-ionic detergents with trypsin were used for making acellular dermis matrix. All the solutions were filter sterilized and each procedure was performed aseptically. The time intervals, concentration and pH of the chemicals were modified to obtain the desirable result. The biomaterials were preserved for histopathological and SEM examination. The dermal tissue in each solution was continuously agitated at 180 RPM at 37°C in orbital shaker to provide better contact of tissue with treatments solution in each protocol.

Protocol-I (trypsin-anionic biological detergent no.1)

In this protocol the biomaterial was treated with trypsin (HiMedia Laboratories Pvt. Ltd., India, RM 6216_0) solution containing sodium salt of EDTA (Qualigens Fine Chemicals, Bombay, India, No. 18454) and sodium azide (Sigma Aldrich Co., St Louis, MO, USA, S2002) for 24-48 h. This was followed by treatment with anionic biological detergent no. 1 at room temperature (37°C) for 12-24 h. It was followed by trypsin for 12-24 h. Again the biomaterials were treated with anionic biological detergent solution no. 1. Finally the tissue was thoroughly rinsed twice with distilled water for 15-30 min each.

Protocol- II (trypsin-anionic biological detergent no.2)

In this protocol the treatment procedure was similar to protocol I except anionic biological detergent no. 2 was used in place of anionic biological detergent no.1.

Protocol- III (trypsin-non-ionic biological detergent no.1)

In this protocol the treatment procedure was similar to protocol I except non-ionic biological detergent no. 1 was used in place of anionic biological detergent no.1.

Protocol- IV (trypsin-non-ionic biological detergent no.2)

In this protocol the treatment procedure was similar to protocol I except non-ionic biological detergent no. 2 was used in place of anionic biological detergent no.1.

The acellular dermal grafts prepared by different protocols were evaluated on the bases of the histological score card which include three parameters with different grading:

Cellularity: +++=90-100%, ++=80-90%, +=70-80%, -=<70%

Compactness of collagen fibers: +++=compact, ++=mildly loose, +=moderately loose, -=heavy loose;

Collagen fiber morphology: +++=Normal to the skin dermis, ++=mildly thick, +=thin, -=very thin.

RESULTS

Optimization of protocols for rabbit skin

This phase was further divided into two parts. In part A the protocols for de-epithelization of the skin were optimized. In part B optimization of protocols to prepare acellular dermal matrix was done.

For de-epithelization, the skin of different species was subjected to 0.25%, 0.5%, 1%, 2% trypsin (pH 6) and 1M, 2M NaCl (pH 6) treatment to separate the epidermis. The observations were recorded from 48h to 108h of post treatment.

Part A. Optimization of protocols for de-epithelization: The details of the gross and microscopic examination are presented in the (Table 1). The native rabbit skin was presented in (Figure 1a). The observations were recorded at 6 h intervals upto 48 h.On the basis of the gross and microscopic observations 0.5% trypsin concentration and 24 h time interval was optimized and adopted for further application in next phase (Figure 1b).

Part B. Optimization of protocols for preparation of acellular dermal matrix: The de-epithelized skin of rabbit Figure (1c) was subjected to anionic and non-ionic biological detergents with trypsin to extract out the cellular contents from the dermis.

- **Protocol-I:** The de-epithelized skin was subjected to 0.5% trypsin treatment for 24 h. It was further treated with 0.5% and 1% anionic biological detergent no. 1 for 12 h. Then it was again subjected to 0.5% trypsin treatment for 12 h and later on with same biological detergent for 12 h.
- **Protocol-II:** In this protocol the treatment procedure was similar to protocol I except anionic biological detergent no. 2 was used instead of anionic biological detergent no.1.
- **Protocol-III:** The treatment procedure was similar to protocol I except non-ionic biological detergent no. 1 was used in place of anionic biological detergent no. 1.
- **Protocol-IV:** In this protocol the non-ionic biological detergent no. 2 with the concentration of 0.5% and 1% was used for 12 h.

Table 1: Optimization of the protocol de-epithelization of rabbit skin.

Time Interval (h)	Gross observations					Microscopic observations																			
						De-epithelization					Basement membrane					Cellularity of dermis					Collagen fibers				
	Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl	
	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M
0	-	-	-	-	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
6	-	++	+++	-	++	-	++	+++	+	++	+++	++	++	++	++	+++	++	++	++	++	+++	++	++	++	++
12	-	+++	++++	+	++	-	++	+++	++	+++	+++	++	++	++	+	+++	++	+	++	+	+++	++	++	++	++
18	-	+++	++++	++	++	+	+++	+++	++	+++	+++	++	++	++	+	+++	++	++	++	+	+++	++	++	++	++
24	+	+++	++++	++	++++	+	+++	+++	++	+++	+++	++	++	++	-	+++	++	+	++	+	++	++	++	++	+
30	+	+++	++++	++	++++	+	+++	+++	++	+++	+++	++	++	++	-	+++	+	+	++	+	++	++	++	++	+
36	+	+++	++++	++	++++	+	+++	+++	+++	+++	++	++	++	++	+	++	++	+	+	+	++	++	++	++	+
42	+	+++	++++	++	++++	+	+++	+++	+++	+++	++	+	++	+	+	++	+	-	+	-	+	+	++	++	+
48	++	+++	++++	++	++++	++	+++	+++	+++	+++	++		+	+	-	++	-	-	+	-	+	+	++	++	+

Gross observations: - =no separation of epidermis, +=separation in broken pieces, ++=separation of epidermis in broken layer, +++= separation of epidermis in a single layer, ++++= partially digested epidermis separation; **De-epithelization:** +++=complete de-epithelization, ++=epidermis comes away in patches but is incomplete, += epidermis comes away in from few patches only, -=epidermis remain fully attached; **Basement membrane:** +++=thick and continuous staining of BM, ++=less intense continuous staining of BM, += discontinuous/patchy staining of BM, -=no BM staining; **Cellularity of dermis:** +++=normal cellularity, ++=moderate no of cells, += mild no of cells, -=no cellular materials; **Collagen fibers:** +++=compact, ++=mildly loose, += moderately losse, -=heavily losse.

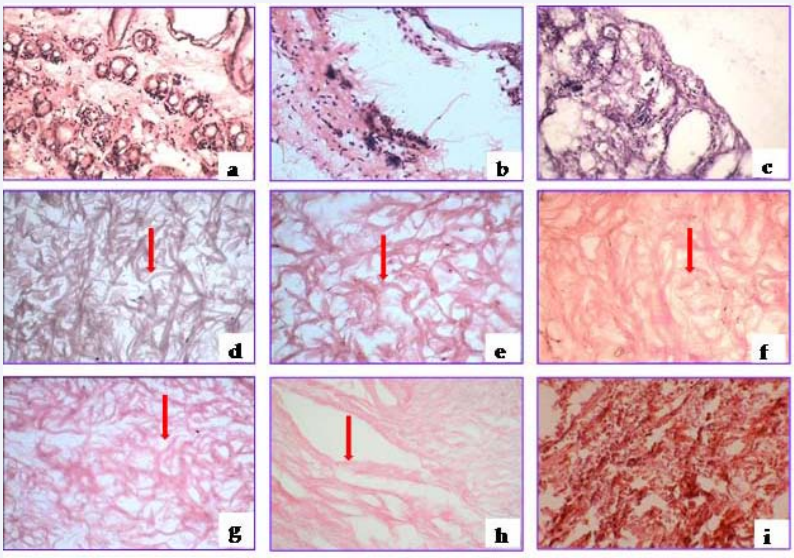

Figure 1 (a-i): Optimization of acellular protocols for rabbit skin.

The protocols were further modified to obtain the desirable results. The reaction time of 0.5% trypsin treatment after the biological detergents was increased to 24 h used instead of 12 h. The concentration of solutions and their reaction time intervals in remaining steps were same as previous protocols.

Microscopic observations: In 0.5% anionic biological detergent no.1 the scaffolds showed 70 to 80% decrease in the cellular contents with mildly thin and moderately loose collagen fibers. However, in 1% concentration the scaffolds were almost acellular with mildly thin and loose collagen fibers (Figure 1d). In 0.5% and 1% anionic biological detergent no. 2 the scaffolds were almost acellular with mildly thin and loose collagen fibers (Figure 1e). However, moderate loosening of the collagen fibers were observed in 1% concentration of the detergent (Figure 1f). In 0.5% and 1% non-ionic biological detergent no.1 the scaffolds showed 70 to 80% decrease in the cellular contents with mildly thin and moderately loose collagen fibers. However, the morphology of the collagen deteriorates in 1% concentration of the detergent.

When time interval of second trypsin treatment was increased from 12 h to 24 h in these protocols, the 0.5% anionic biological detergent no.1 showed 80 to 90% decrease in the

cellular contents with mildly thin and moderately loose collagen fibers. However, in 1% concentration the scaffolds were almost 100% acellular with moderately thin and loose collagen fibers (Figure 1g). In 0.5% and 1% anionic biological detergent no. 2 the scaffolds were almost acellular with mildly thin and loose collagen fibers (Figure 1h). The compactness and morphology of collagen scaffold deteriorate in the higher concentration of the detergents (Figure 1i).

Optimization of protocols for pig skin

Part A. Optimization of protocols for de-epithelization: The pig skin was subjected to 2% trypsin (pH 6) treatment. The details of the gross and microscopic examination are presented in the (Table 2). The results of the gross and microscopic observations revealed that the 2% trypsin at 48 h time interval epidermis was easily separated with dermis (Figure 2a). The de-epithelized dermis Figure (2b) in histological section showed cellularity with retained basement membrane (Figure 2c). This concentration and time interval of trypsin treatment were optimized and used in next phase.

Part B. Optimization of protocols for preparation of acellular dermal matrix: The deepithelialized pig skin was subjected to following protocols for preperation of acellular porcine dermal matrix.

- **Protocol-I:** The de-epithelized skin was subjected to 2% trypsin concentration for 24 h. The 2% and 3% concentrations of the anionic biological detergent no. 1 were used for 12 h. The 2% trypsin was again used for 12 h.
- **Protocol-II:** The treatment procedure was similar to protocol I except anionic biological detergent no. 2 was used instead of anionic biological detergent no.1.
- **Protocol-III:** The treatment procedure was similar to protocol I except non-ionic biological detergent no. 1 was used in place of anionic biological detergent no. 1.
- **Protocol-IV:** The treatment procedure was similar to protocol I except non-ionic biological detergent no. 2 was used in place of anionic biological detergent no. 1.

Microscopic observations: In 2% anionic biological detergent no.1 the scaffold showed about 70% decrease in the cellular contents with mildly thick and loose of the collagen fibers (Figure 2d). In 3% concentration the scaffolds showed almost similar cellular contents as 2% but, the collagen fibers became thin and moderately loose. In 2% anionic biological detergent no. 2 the scaffolds showed more than 70% decrease in the cellular contents with moderately loose and mildly thick collagen fibers (Figure 2e). In 3% concentration the scaffolds became 100% acellular with mildly thick and loose collagen fibers (Figure 2f). In 2% and 3% non-ionic biological detergent no. 1 the scaffolds showed 90 to 100% decrease in the cellular contents with mildly thin and moderately loose collagen fibers (Figure 2g). However, the morphology of collagen deteriorates in 3% of the detergents. In 2% and 3% non-ionic biological detergent no. 2 the scaffolds showed the cellular contents below 70% (Figure 2h) but the morphology and compactness of the collagen fibers were deteriorated in 3% concentration of detergents (Figure 2i).

The concentration of the biological detergent was increased from 2% and 3% to 4% and 5% in the modified protocols. The results of the modified protocols showed more or less similar cellular contents as observed in previous protocols. However, the

Table 2: Optimization of the protocol de-epithelization of porcine skin.

Time interval (h)	Gross observations					Microscopic observations																			
						De-epithelization					Basement membrane					Cellularity of dermis					Collagen fibers				
	Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl	
	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M
0	-	-	-	-	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
12	-	-	-	-	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
24	-	-	-	-	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
36	-	+	+	+	+	-	-	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
48	-	++	++	++	++	-	+	+	++	++	+++	+++	+++	+++	++	+++	+++	+++	+++	++	+++	+++	++	+++	++
60	+	++	+++	++	++	-	++	++	+++	+++	+++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	++	++	++
72	++	++	+++	++	++	+	+++	+++	+++	+++	+++	++	++	++	++	+++	+++	+++	++	+++	+++	++	++	++	++
84	++	+++	+++	++	++	++	+++	+++	+++	+++	++	++	++	++	+	++	+++	++	++	+++	++	++	++	++	++
96	+++	+++	+++	++	++	++	+++	+++	+++	+++	++	++	++	+	+	++	++	++	++	+++	++	++	++	++	++
108	+++	+++	+++	++	+++	++	+++	+++	+++	+++	++	++	++	+	+	++	++	++	++	+++	++	++	++	++	++

Gross observations: - =no separation of epidermis, +=separation in broken pieces, ++=separation of epidermis in broken layer, +++= separation of epidermis in a single layer, ++++= partially digested epidermis separation; **De-epithelization:** +++=complete de-epithelization, ++=epidermis comes away in patches but is incomplete, += epidermis comes away in from few patches only, -=epidermis remain fully attached; **Basement membrane:** +++=thick and continuous staining of BM, ++=less intense continuous staining of BM, += discontinuous/patchy staining of BM, -=no BM staining; **Cellularity of dermis:** +++=normal cellularity, ++=moderate no of cells, += mild no of cells, -=no cellular materials; **Collagen fibers:** +++=compact, ++=mildly loose, += moderately losse, -=heavily losse.

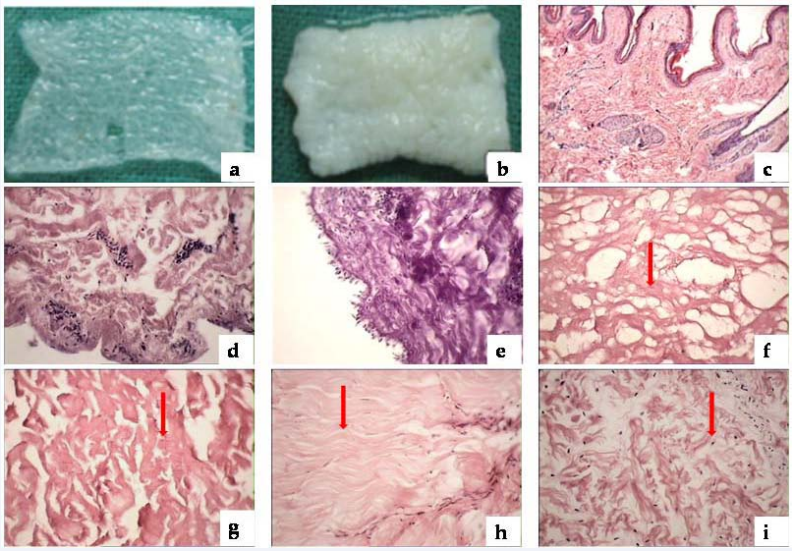

Figure 2 (a-i): Optimization of acellular protocols for pig skin.

compactness and morphological changes revealed deterioration of the graft quality in their respective detergent groups.

Optimization of protocols for goat skin

Part A. Optimization of protocols for de-epithelization: The details of the gross and microscopic examination are presented in the Table (3). The results of the gross and microscopic observations revealed that the 1% trypsin at 36 h time interval epidermis was easily separated with dermis (Figure 3,a). The de-epithelized dermis (Figure 3b) in histological section showed cellularity with retained basement membrane (Figure 3c) cellularity and mild looseness of the collagen fibers. This concentration and time interval of trypsin treatment were optimized and used in next phase.

Part B. Optimization of protocols for preparation of acellular dermal matrix: The deepithelialized goat skin was subjected to following protocols for preperation of acellular dermal matrix.

- **Protocol-I:** The de-epithelized skin Figure (3b) of goat was subjected to 1% trypsin concentration for 24 h. The 1% and 2% concentration of the anionic biological detergent no.1 was used for 12 h. The 1% trypsin was again used for 12 h.
- **Protocol-II:** In this protocol the treatment procedure was similar to protocol I except anionic biological detergent no. 2 was used in place of anionic biological detergent no.1.
- **Protocol-III:** The treatment procedure was similar to protocol I except non-ionic biological detergent no. 1 was used in place of anionic biological detergent no. 1.
- **Protocol-IV:** In this protocol also the treatment procedure was similar to protocol I except non-ionic biological detergent no. 2 was used in place of anionic biological detergent no. 1. Time interval with 1% trypsin treatment was increased from 12 h to 24 h in modified protocols.

Microscopic observations: In 1% and 2% anionic biological detergent no.1 the scaffolds showed 90 to 100% decrease in the cellular contents with mildly thick and loose collagen fibers (Figure 3d). However, moderate loosening of the collagen fibers was observed in 2% concentration. In 1%) and 2% anionic biological detergent no. 2 the scaffolds were almost acellular with mildly thin and loose collagen fibers (Figure 3e). However, in 2% of the detergent treatment thin collagen fibers were observed. In 1% and 2% non-ionic biological detergent no. 1 the scaffolds showed 80 to 90% decreased in the cellular contents with mildly thick and loose collagen fibers (Figure 3f and g). In 1% and 2% non-ionic biological detergent no. 2 the scaffold showed 90 to 100% decrease in the cellular contents with mild to moderate thin and moderately loose collagen fibers (Figure 3h). In further modified protocols in 1% and 2% anionic biological detergent no. 2 the scaffolds were almost acellular (Figure 3i).

Optimization of protocols for sheep skin: Part A. Optimization of protocols for de-epithelization: The details of the gross and microscopic examination are presented in the (Table 4). On the basis of the gross and microscopic observation 1% trypsin concentration and 36 h time interval was optimized (Figure 4b,c) and used in next phase.

Part B. Optimization of protocols for preparation of acellular dermal matrix: The deepithelialized sheep skin was subjected to protocols similar to that of goat.

Microscopic observations: In 1% anionic biological detergent no. 1 the scaffold showed 70% to 80% decrease in the cellular contents with mildly thick and mildly loose collagen fibers which increases 80% to 90% as concentration increased to 2%. In 1% and 2% anionic biological detergent no. 2 the scaffold showed 80% to 90% decreased in the cellular contents with mildly thin and loose collagen fibers. The 90% to 100% decrease in cellular

Table 3: Optimization of the protocol de-epithelization of caprine skin.

Time interval (h)	Gross observations					Microscopic observations																				
						De-epithelization					Basement membrane					Cellularity of dermis					Collagen fibers				
	Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl	
	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M
0	-	-	-	-	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
12	-	-	++	-	++	-	-	+	-	+	+++	+++	++	+++	++	+++	+++	++	+++	++	+++	+++	++	+++	++
24	-	-	+++	-	++	-	-	++	-	++	+++	+++	++	+++	++	+++	+++	+	+++	+	+++	+++	++	+++	++
36	+	+	+++	+	++	-	-	+++	-	++	+++	+++	+	+++	++	+++	++	+	+++	+	++	++	++	++	++
48	+	++	+++	++	+++	-	+	+++	+	+++	+++	++	++	+++	++	+++	++	+	++	+	++	++	++	++	++
60	+	++	+++	++	++++	-	+	+++	+	+++	+++	++	++	++	++	++	++	+	++	+	++	++	++	++	++
72	++	++	++++	++	++++	-	++	+++	++	+++	+++	++	++	++	++	++	++	+	++	+	++	++	++	+	++
84	++	++	++++	++	++++	+	++	+++	++	+++	+++	++	++	++	++	++	+	+	+	+	++	+	++	+	++
96	++	+++	++++	++	++++	+	++	+++	+++	+++	+++	++	+	++	++	+	+	-	+	+	++	+	+	+	+
108	++	++++	++++	++	++++	+	+++	+++	+++	+++	+++	+	++	+	++	+	+	-	+	-	++	+	+	+	+

Gross observations: - =no separation of epidermis, +=separation in broken pieces, ++=separation of epidermis in broken layer, +++= separation of epidermis in a single layer, ++++= partially digested epidermis separation; **De-epithelization:** +++=complete de-epithelization, ++=epidermis comes away in patches but is incomplete, += epidermis comes away in from few patches only, -=epidermis remain fully attached; **Basement membrane:** +++=thick and continuous staining of BM, ++=less intense continuous staining of BM, += discontinuous/patchy staining of BM, -=no BM staining; **Cellularity of dermis:** +++=normal cellularity, ++=moderate no of cells, += mild no of cells, -=no cellular materials; **Collagen fibers:** +++=compact, ++=mildly loose, += moderately losse, -=heavily losse.

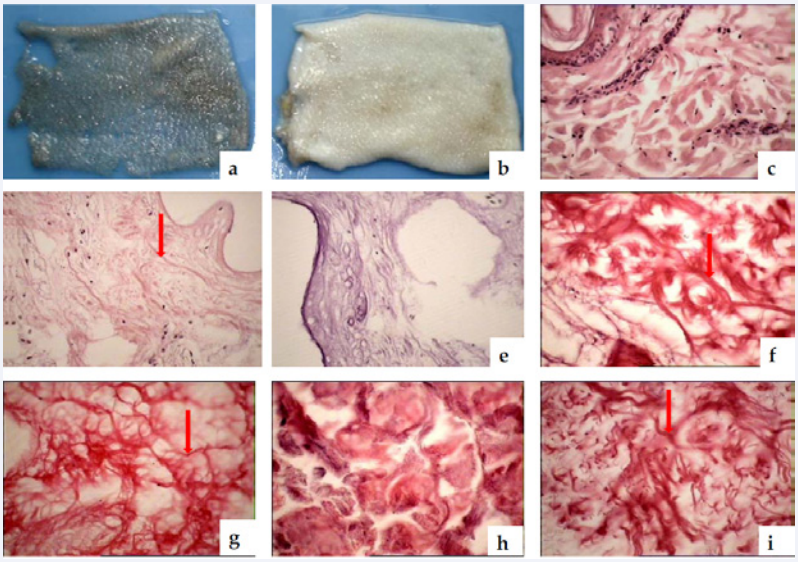

Figure 3 (a-i): Optimization of acellular protocols for goat skin.

contents was observed when the concentration increased from 1 to 2% (Figure 4d). In 1% and 2% non-ionic biological detergent no. 2 the scaffold showed 90 to 100% decrease in the cellular contents with mild thick and loose collagen fibers (Figure 4e). In further modified protocols the time interval for second trypsin treatment was incresed from 12 h to 24 h. In 2% concentration the scaffold showed the 90 to 100% decrease in cellular contents with mildly thick and mildly loose collagen fibers (Figure 4f).

Optimization of protocols for buffalo skin

Part A. Optimization of protocols for de-epithelization: On the basis of the gross observations the desirable removal of epidermis in a single layer was not possible with trypsin and NaCl solutions. The separation of epidermis was done with the help of dermatome (Figure 5 a,b). Histological observations also showed that removal of epidermis was not possible with trypsin and NaCl solutions (Figure 5c). Figure (5d) shows removal of epidermis with dermatome.

Part B. Optimization of protocols for preparation of acellular dermal matrix: The deepithelialized buffalo skin was subjected to following protocols for preperation of acellular dermal matrix.

Table 4: Optimization of the protocol de-epithelization of ovine skin.

Time interval (h)	Gross observations					Microscopic observations																				
						De-epithelization					Basement membrane					Cellularity of dermis					Collagen fibers				
	Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl		Trypsin			NaCl	
	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M	0.25%	0.5%	1.0%	1M	2 M
0	-	-	-	-	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
12	-	-	-	-	-	-	-	++	-	-	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
24	-	+	+	-	-	-	++	+++	-	+	+++	+++	++	++	++	+++	++	++	+++	++	+++	+++	++	+++	++
36	+	++	+++	-	-	++	+++	+++	+	++	+++	++	++	++	++	++	++	+	++	++	++	++	++	++	++
48	++	+++	+++	+	+	++	+++	+++	+++	+++	++	++	++	++	++	++	++	+	++	++	++	++	++	++	++
60	++	+++	+++	+	+	+++	+++	+++	+++	+++	++	+	++	++	++	++	++	++	++	++	++	++	++	++	++
72	++	+++	+++	+	+	+++	+++	+++	+++	+++	+	+	++	++	++	+	+++	+	++	++	++	++	+	++	++
84	+++	+++	++++	++	++	+++	+++	+++	+++	+++	++	+	++	++	+	++	+	+	++	++	++	++	++	++	++
96	+++	+++	++++	++	++	+++	+++	+++	+++	+++	++	+	++	++	+	++	+	++	++	++	++	++	++	++	++
108	+++	+++	++++	++	++	+++	+++	+++	+++	+++	++	+	++	++	+	+	+	++	++	++	++	++	++	++	++

Gross observations: - =no separation of epidermis, +=separation in broken pieces, ++=separation of epidermis in broken layer, +++= separation of epidermis in a single layer, ++++= partially digested epidermis separation; **De-epithelization:** +++=complete de-epithelization, ++=epidermis comes away in patches but is incomplete, += epidermis comes away in from few patches only, -=epidermis remain fully attached; **Basement membrane:** +++=thick and continuous staining of BM, ++=less intense continuous staining of BM, += discontinuous/patchy staining of BM, -=no BM staining; **Cellularity of dermis:** +++=normal cellularity, ++=moderate no of cells, += mild no of cells, -=no cellular materials; **Collagen fibers:** +++=compact, ++=mildly loose, += moderately losse, -=heavily losse.

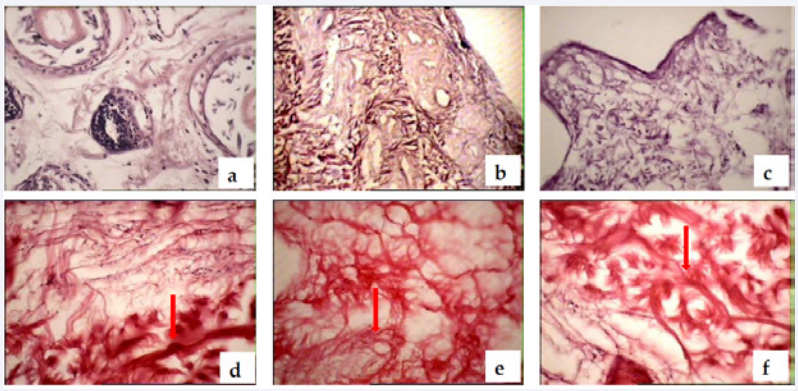

Figure 4 (a-f): Optimization of acellular protocols for sheep skin.

- **Protocol-I:** The de-epithelized skin of bovine was subjected to 2% trypsin concentration for 24 h. The 2%, 3%, 4% and 5% concentration of the anionic biological detergent no.1 was used for 12 h. Then the samples were again treated with 2% trypsin for 12 h. In next step the samples were subjected to same time and concentration of detergents treatment. The samples were washed thrice at every step to rinse the residuals of previous steps.
- **Protocol-II:** In this protocol the treatment procedure was similar to protocol I except anionic biological detergent no. 2 was used in place of anionic biological detergent no.1.
- **Protocol-III:** In this protocol the treatment procedure was similar to protocol I except non-ionic biological detergent no. 1 was used in place of anionic biological detergent no.1.
- **Protocol-IV:** In this protocol the treatment procedure was similar to protocol I except non-ionic biological detergent no. 2 was used in place of anionic biological detergent no.1.

Microscopic observations: In 2% and 3% anionic biological detergent no.1 and 2 the scaffolds showed 80 to 90% decrease in the cellular contents. The collagen fibres were mildly thin in 2%, became mildly thick and loose in 3% solution. In 2% of non-ionic biological detergent no.1 the scaffolds showed 80 to 90% decrease in the cellular contents (Figure 5e) and in 3% solution scaffolds showed 90 to 100% decrease in the cellular contents (Figure 5f). The collagen fibres were mildly thick and mildly loose in architecture. In 2% non-ionic biological detergent no. 2 the scaffold showed 80 to 90% decrease in the cellular contents (Figure 5g) and 90 to 100% decrease in 3% solution (Figure 5h). In 4% and 5% concentration of anionic biological detergent no.1 the scaffolds showed 90% to 100% decrease in the cellular

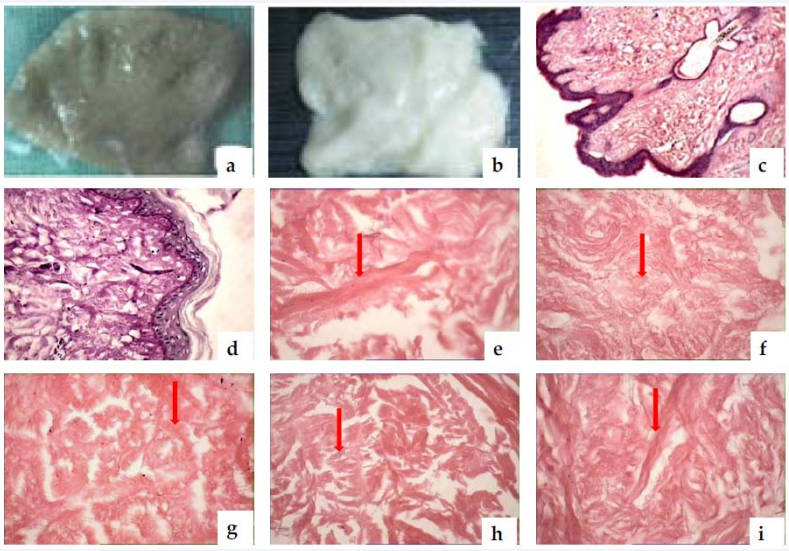

Figure 5 (a-i): Optimization of acellular protocols for buffalo skin.

contents. In 4% and 5% concentration of anionic biological detergent no. 2 the scaffolds also showed 90 to 100% decrease in the cellular contents. Collagen fibres were mildly thin and loose in 4% and mildly thick (Figure 5i) and moderately loose in 5% concentration.

DISCUSSION

The primary constituent of the skin is collagen which is essentially polymers of amino acids. The collagen molecule consisted of 3 chains of poly amino acids or polypeptides arranged in a trihelical configurations ending in a non-helical carboxyl and amino terminals one at each end. These non-helical ends are believed to contribute to most of the antigenic properties of collagen. In natural state the collagen trihelical configurations are held in place by direct chemical bonds, hydrogen bonds and water-bridged cross-links [18].

The collagen material is a ready to use, mechanically stable, in-vivo non-contractible and primarily free of any non-biologic and synthetic components. It is nonpyrogenic, biologically and immunologically neutral and long-term preservable. Collagen is generally treated as a self tissue by recipients into whom it is placed and is subjected to the fundamental biological processes of tissue degradation and integration into adjacent host tissues when left in its native ultrastructure [19]. Collagen is a natural substrate for cellular attachment, growth and differentiation in its native state. Certain sequences of the collagen fibrils are chemotactic and promote cellular proliferation and differentiation [20].

Wainwiright [21] produced acellular dermal matrix chiefly consisted of collagen by a controlled process that removes the epidermis and the cells from the dermis without altering the structure of the extracellular matrix and the basement membrane complex. Decellularization techniques resulted in the removal of nucleus and cytoplasmic cellular components, lipids and its membranes along with soluble proteins and basement membrane components of cellular material [22]. In the present study we successfully developed acellular dermal matrices from different species of animals by removing cells without altering the structure of ECM using biological detergents and trypsin enzyme combinations. Prasertsung et al. [23] developed a new method for de-epithelization of porcine skin using sodium sulphide to dehaired the skin, followed by removal of epidermis using glycerol.

Dispase treatment of skin followed by cryopreservation [24] or extended incubation in culture medium. Sesamoto et al. [25], has been reported to remove epidermis and intact cells from the dermis effectively. However, varying amounts of cell debris remained interspersed throughout the dermis. Livesey et al. [26], treated human cadaver skin with prolonged incubations in I M NaCl to remove epithelium. Chakrabarty et al. [27], optimizated the protocol for de-epithelization of the human skin using 1 mol/L of sodium chloride at 37°C for 8 h. The de-epithelization of the porcine skin was done with 0.25% trypsin for 18 h and subsequently with 0.1% sodium dodecyl sulphate for 12 h at room temperature [28].

In the present study the optimization of protocols for de-epithelization was done using trypsin. For rabbit 0.5% trypsin for 24 h, porcine 2% for 48 h, caprine and ovine 1% for 36 h was optimized. The sodium chloride was not found suitable for de-epithelization the skin samples in these species, except rabbit. The skin of the buffalo did not de-epithelized by the trypsin and sodium chloride treatment. The epidermis of buffalo skin was separated with the dermatome. Trypsin attacks the desmosome complex between the cells thus remove the epidermis in a single layer. Livesey et al. [26], but on increased time intervals the partially digested epidermis came out due to the partial digestion of the cells. However, NaCl was not found suitable to separate the epidermis in these species which was effective in human skin. In these study anionic biological detergents gives better results than non-ionic biological detergents. Sodium dodecyl sulphate (SDS) was found very effective for removal of cellular components from tissue. Compared to other detergents SDS yields complete

removal of nuclear remnants and cytoplasmic proteins as also reported by Woods and Gratzer [23]. Ionic detergents are effective for solubilizing both cytoplasmic and nuclear cellular membranes, but tend to denature poteins by disrupting protein-protein interactions [29]. In general anionic detergents are used extensively in decellularization protocols due to their mild effects on tissue structure. Non-ionic biological detergents (TritronX-100) do not usually denature proteins. They are suited to investigate the subunits structure of membrane proteins. It inhibits protein-protein and protein-lipid bonding [30].

Allogenic dermis is antigenic because donor cellular components evoke an immune reaction following transplantation [31,32]. Grillo and McKhann [33] grafted allogenic mouse skin treated with repeated freeze-thaw cycles and found that these allografts became vascularized but were rapidly absorbed. The epithelium of fresh humane skin [34] or cryopreserved human skin [35] could be removed following incubation of the skin in buffer or culture medium for several days with the resulting dermal matrix retaining a dense lamina and cellular debris. However, long incubation of these samples resulted in increased antigenicity by altering native collagen [25]. Ben-Bassat et al. [36], reported that brief, mild trypsin treatment (2 h, 37°C) of cryopreserved skin removed all intact cells but, the basement membrane structure was preserved. Cellular antigens are predominantly responsible for the immunological reaction associated with allografts [32]. Remnants of cell components in xenografts may contribute to calcification and/or immunogenic reaction [22].

However, Booth et al. [37], documented that sodium dodecyl sulphate (0.03-1 per cent) or sodium deoxycholate (0.5-2.5 per cent) alone resulted in total decellularization at 24 h incubation of porcine aortic wall among the Triton X-100, sodium dodecyl sulphate, sodium deoxycholatee, MEGA 10, TnBP, CHAPS and Tween 20. In the present study, optimization of the protocol was done to make skin acellular using the combinations of trypsin with biological detergents in different concentrations. Several workers prepared the acellular dermal matrix of porcine with trypsin and Triton X-100 combination [38-40]. Samouillan et al. [41], observed that the collagen triple helix was destabilized and the elastin network was swollen after sodium dodecyl sulphate treatment, whereas, Triton X-100 and sodium deoxycholate treatments did not affect the structural integrity of either collagen or elastin.

CONCLUSION

In the present study protocols for making acellular dermal matrix from rabbit, pig, goat, sheep and buffalo skin were optimized and were summarised below.

Rabbit skin

Complete de-epithelialization was achieved by treatment with 0.5% trypsin (pH 6.0) for 24 h. The de-epithelialized dermis was treated with 0.5% trypsin (24h), followed by 1% ionic biological detergent no.1 (12h) then again 0.5% trypsin (24h) and lastly with ionic biological detergent no.1 (12h) resulted in complete acellular dermal matrix.

Pig skin

Complete de-epithelialization was achieved by treatment with 2% trypsin (pH 6.0) for 48 h. The de-epithelialized dermis was treated with 2% trypsin (24h), followed by 3% ionic biological detergent no.1 (12h) then again 2% trypsin (12h) and lastly with 3% ionic biological detergent no.1 (12h) resulted in complete acellular dermal matrix.

Goat skin

Complete de-epithelialization was achieved by treatment with 1% trypsin (pH 6.0) for 36 h. The de-epithelialized dermis was treated with 1% trypsin (24h), followed by 1% ionic biological detergent no.1 or 2 (12h) then again 1% trypsin (24h) and lastly with 1% ionic biological detergent no.1or 2 (12h) resulted in complete acellular dermal matrix.

Sheep skin

Complete de-epithelialization was achieved by treatment with 1% trypsin (pH 6.0) for 36 h. The de-epithelialized dermis was treated with 1% trypsin (24h), followed by 1% ionic biological detergent no.1 or 2 (12h) then again 1% trypsin (24h) and lastly with 1% ionic biological detergent no.1 or 2 (12h) resulted in complete acellular dermal matrix.

Buffalo skin

De-epithelialization was achieved by dermatome. The de-epithelialized dermis was treated with 2% trypsin (24h), followed by 3% ionic biological detergent no.1 or 2 (12h) then again 2% trypsin (24h) and lastly with 3%ionic biological detergent no.1or 2 (12h) resulted in complete acellular dermal matrix.

[**Anionic biological detergent no. 1**: Sodium Deoxycholate, Desoxycholic acid sodium salt, **Anionic biological detergent no. 2:** Sodium Dodecyl Sulphate / Sodium Lauryl Sulphate, **Non-ionic biological detergent no. 1:** Tritron-X100, **Non-ionic biological detergent no. 2:** Tween-20, Polyoxyethylene sorbitan monolaurate]

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