Veterinary World, EISSN: 2231-0916
Available at www.veterinaryworld.org/Vol.7/November-2014/22.pdf

RESEARCH ARTICLE
Open Access

Bone marrow derived cell-seeded extracellular matrix: A novel biomaterial in the field of wound management

V. Remya[1], Naveen Kumar[1], A. K. Sharma[1], Dayamon D. Mathew[1], Mamta Negi[1], S. K. Maiti[1], Sameer Shrivastava[2], Sonal[2] and N. P. Kurade[3]

1. Division of Surgery, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India; 2. Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India; 3. Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India.

Corresponding author: V. Remya: e-mail: remyamukund04vet@gmail.com, NK: naveen.ivri1961@gmail.com, AKS: ashok.sharma1010@gmail.com, DDM: dayamon@gmail.com, MN: mamtaanegi@gmailcom, SKM: maiti_62@rediffmail.com, SS: sameer_vet@rediffmail.com, SS: sonalvet@gmail.com, NPK: npkurade@yahoo.co.in

Received: 04-06-2014, **Revised:** 19-10-2014, **Accepted:** 24-10-2014, **Published online:** 30-11-2014

doi: 10.14202/vetworld.2014.1019-1025. **How to cite this article:** Remya V, Kumar N, Sharma AK, Mathew DD, Negi M, Maiti SK, Shrivastava S, Sonal, Kurade NP (2014) Bone marrow derived cell-seeded extracellular matrix: A novel biomaterial in the field of wound management, *Veterinary World, 7*(11): 1019-1025.

Abstract

Aim: Extensive or irreversible damage to the skin often requires additional skin substitutes for reconstruction. Biomaterials have become critical components in the development of effective new medical therapies for wound care.

Materials and Methods: In the present study, a cell matrix construct (bone marrow-derived cells (BMdc) seeded extracellular matrix [ECM]) was used as a biological substitute for the repair of full-thickness skin wound. ECM was developed by decellularizing fish swim bladder (FSB). Goat bone marrow-derived cells (G-BMdc) were seeded over this decellularized matrix. Efficacy of this cell matrix construct in wound repair was tested by implanting it over 20 mm^2 × 20 mm^2 size full-thickness skin wound created over the dorsum of rat. The study was conducted in 16 clinically healthy adult rats of either sex. The animals were randomly divided into 2 equal groups of 8 animals each. In Group I, animal's wounds were repaired with a cellular FSB matrix. In Group II, wounds were repaired with G-BMdc seeded a cellular FSB matrix. Immune response and efficacy of healing were analyzed.

Results: Quality of healing and immuno tolerance to the biological substitute was significantly better in Group II than Group I.

Conclusion: Seeding with BMdc increases the wound healing potency and modulates the immune response to a significantly negligible level. The BMdc seeded acellular FSB matrix was found to be a novel biomaterial for wound management.

Keywords: biomaterial, decellular, extra cellular matrix, wound.

Introduction

Many natural skin substitutes such as xenografts, allografts, and autografts have been used for repair of dermal defects. However, these naturally derived skin substitutes cannot accomplish skin regeneration due to limited donor sites, risk of infection, slow healing, and association with the formation of scar [1]. In this context, biomaterials have become critical components in the development of effective new medical therapies for wound care. Extracellular matrix (ECM) provided as biomaterials/bio-scaffold to replicate the biological and mechanical function of the native ECM found in tissues. Common features of ECM-associated tissue remodeling includes extensive angiogenesis, recruitment of circulating progenitor cells, rapid scaffold degradation, and constructive remodeling of damaged or missing tissues and is distinctly different phenomenon from that of scar tissue formation [2]. The advantage of using these ECM based biomaterial is that the repair mechanisms approach optimal conditions, i.e., it can not only repair but can also regenerate new tissue that is similar to that of the recipient [3]. However, there may also be inconveniences like graft rejection [4]. One of the areas for improvement and research is the control of graft rejection [5]. The histocompatibility antigens present on the transplanting cells is responsible for graft rejection in cellular grafts [6]. Removal of cells (decellularization) from a tissue or an organ leaves the complex mixture of structural and functional proteins that constitute the ECM. Various kinds of extraction techniques are reported for decellularization [7]. 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 on the composition, biological activity, and mechanical integrity of the remaining ECM [8]. The primary structural framework for ECM is provided by collagen. Collagen itself is excellent in its biocompatibility, biodegradability, and is weak in antigenicity [9]. Therefore, ECM has less immunogenicity and better tolerance as a biological graft [10]. Decellularized matrices have been widely used for engineering functional tissues and organs such as cartilage, skin, bone, bladder, blood vessels, heart, liver, and lung [11-16] and have achieved impressive results. According to

the literature, different types of ECM were used for repair of dermal wounds [17,18].

Bovine Type I collagen is perhaps the most widely used biologic scaffold for therapeutic applications due to its abundant source and its history of successful use. Outbreak of bovine spongiform encephalopathy and foot and mouth disease has caused restrictions on use of collagen from bovine or porcine origin. Collagens extracted from fish swim bladders (FSB) may be good substitutes. The swim bladder is a rich source of Type I collagen [19].

The role of bone marrow-derived cells (BMdc) in regenerative therapy can be applied to enhance the regenerative process. Moreover, immunomodulatory property of these cells can be exploited to ameliorate graft rejection hence to facilitate graft take [20].

The present study was undertaken to develop novel cell matrix construct-based biomaterial for dermal wound management. Matrix of biomaterial was developed from FSB after decellularization process. BMdc isolates of goat origin were seeded over this ECM to prepare cell matrix construct. Reparative and regenerative potential of this newly developed biomaterial with emphasis on the immuno modulatory potential of seeded BMdc against biological graft rejection was evaluated on the full-thickness cutaneous wound created in the rat model.

Materials and Methods

Ethical approval

This study was in full compliance with Institutional Animal Ethics Committee of the Indian Veterinary Research Institute.

The study was carried out in two stages. In the first stage, cell matrix construct was developed. FSB ECM was prepared by decellularizing the native swim bladder and, thereafter, this ECM was seeded with BMdc of goat origin (G-BMdc). In the second stage, efficacy of this cell matrix construct-based biomaterial was tested in full-thickness cutaneous wound in the rat model.

Preparation of acellular FSB ECM

Swim bladders of the fresh water fish (*Labeo rohita*) were decellularized as per the standardized protocol with some modifications [21]. In brief, fresh FSB was collected and immediately preserved in ice-cold sterile phosphate buffered saline (PBS, pH7.4) containing a broad spectrum antibiotic (amikacin-1 mg/ml), and a proteolytic inhibitor (0.02% ethylenediaminetetraacetic acid). In the laboratory the samples were decellularized using 0.5% sodium deoxycholate (SDC), an ionic detergent for 24 h. The FSBs were subjected to continuous agitation in a horizontal orbital shaker maintained at 37°C, at the rate of 180 revolutions/min during decellularization process to provide better contact of tissue with chemicals. After decellularization, the prepared ECM was washed 3 times (2 h each) with sterile PBS to remove the residual chemicals. To confirm the acellularity and collagen fiber's architecture of the treated matrix light microscopic and scanning electron microscopic examination was done. The tissues were stored at −20°C in PBS solution containing 0.1% amikacin till use.

Collection and isolation of BMdc

Bone marrow was aspirated from the iliac crest of a 6-month-old goat using bone marrow aspiration needle under sterile condition (Figure-1). During the entire procedure, the animal was maintained under high epidural anesthesia. Isolation and *in-vitro* culture of G-BMdc were done with cell culture media (Roswell Park Memorial Institute medium 1640 [RPMI]) inside biosafety cabinet as per standard protocol [22]. The cell isolates were purified and maintained by subculturing up to a third passage.

Preparation of cell matrix construct

Third passage cell isolates were used for preparing cell matrix construct. The cells were counted using a light microscope and neubauer counting chamber. Decellularized FSB matrix was maintained in RPMI 1640 culture media in six-well culture plates. The cells were seeded at a concentration of 5×10^5 cells/10 ul. After 10 min, scaffolds were flipped over to the other side, and the same quantity of cells was seeded. Six well plates were precoated with 2% agarose to prevent cells from attaching or migrating to the culture dish. This cell matrix construct was kept in a CO_2 incubator maintained at 37°C in a humidified atmosphere of 5% CO_2. Morphological assessment of cells seeded matrix was done by scanning electron microscopy (SEM) at day 7.

Evaluation of healing potential of the cell matrix construct

The *in-vivo* biocompatibility and healing potential of cell matrix construct were determined by implantation cell-seeded acellular matrix over the surgically created 20 mm^2 ×20 mm^2 size full-thickness skin wound on the dorsal thoracic area of the rats and compared with acellular matrix (without G-BMdc seeding) implanted wounds of the same size and site. Experiment was conducted in two different groups

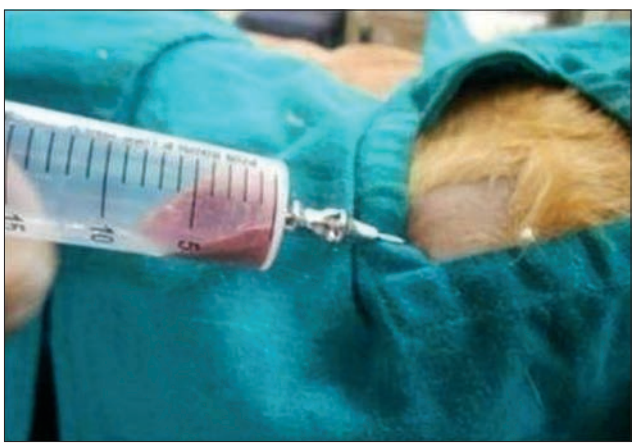

Figure-1: Aspiration of bone marrow from the iliac crest of goat.

of 8 animals each. Implant was protected from desiccation by double layer implantation technique. An additional upper layer of acellular matrix was applied in both the groups. Evaluation was done on the basis of general behavioral changes, warmth of the graft, gross, immunological and histopathological observations at different time intervals.

Gross appearance of healing was analyzed by the digital photographs of the repair site taken from a fixed distance. Analysis of shape, size, irregularity, and color of the lesions determined. The parameters were recorded on days 0, 3, 7, 14, 21, and 28 post-operatively.

The ability of bioengineered implant (cell-seeded acellular matrix in comparison with simple acellular matrix) to stimulate the immune system of the host was assessed by performing enzyme-linked immunosorbent assay (ELISA) and lymphocyte stimulation test (LST). Evaluation of the humoral immune response was done by indirect ELISA. The free protein contents of native FSB was estimated and expressed in mg/ml using bovine serum albumin (BSA) as a standard [23]. Serum samples were collected at days 0, 21, 28, and 35 post-operatively. The hyper immune serum (HIS) was raised in adult rat to check the host immune response against the antigen prepared from native FSB (without decellularization). The immunization dose at the rate of 200 µg/rat was given on day 0, 14, 21, and 28. The HIS was extracted from the blood on day 28 and was used as a positive control in ELISA and sera samples of day zero as a negative control. The intensity of the color reaction was recorded at 492 ηm using an automatic ELISA reader. The cell-mediated immune response was assessed by LST. For this, spleen was collected aseptically from the implanted animals of the two groups on day 45 post-operatively, and splenocyte culture was performed [24].

For histopathological evaluation, the biopsy specimens were collected on days 3, 7, 14, and 28 postimplantation. The biopsy specimens were fixed in 10% formalin saline. The implants were then processed for paraffin embedding technique to get 5 µ thick paraffin section. The sections were stained with hematoxylin and eosin to evaluate the healing process.

Results

Native FSB matrix showed cellularity and a compact fiber arrangement both in light microscopic (Figure-2a) and SEM observations (Figure-2b). Whereas decellularization with 0.5% SDC for 24 h revealed a complete loss of cellularity and mild derangement of the dense fiber architecture providing adequate porosity for cellular and vascular proliferation without damaging the basic architecture of FSB matrix (Figure-3a and b). The protocol adopted for isolation and culture of G-BMdc was found optimum and non-toxic for the cells as we could maintain the cells up to third passage without affecting the survivability and proliferative potential of the cells. Bone marrow mononuclear cell fraction contains heterogeneous cell population and G-BMdc was isolated on the basis of plastic adherence property. The non-adherent cells were discarded on 3rd day after culture and adherent cells grew as fibroblast-like cells by day 4 (Figure-4a), which further formed symmetric colonies and attained 80-90% confluence by day 16 (Figure-4b). After the first passage, there was a rapid proliferation of the cells by the 3rd week, we were able to collect the third passage cells. Third passage cells were used for seeding over the matrix. Morphological assessment of the matrix by SEM on day 7 post seeding revealed an enough amount of attachment and proliferation of G-BMdc over acellular swim bladder (Figure-5).

Animals of both the groups started taking feed and water partially within 24 h of surgery. However, they were slightly dull for first 2 days of surgery. In all the animals, feed and water intake became normal

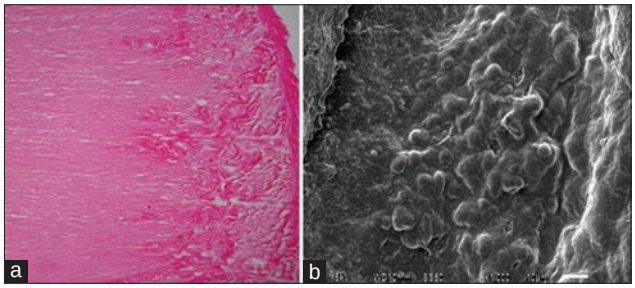

Figure-2: (a) Microscopically native fish swim bladder (FSB) showing cellularity and compact fiber arrangement (hematoxylin and eosin stain, ×40), (b) native FSB showing cellularity and compact fiber arrangement (scanning electron microscopy observation, ×500).

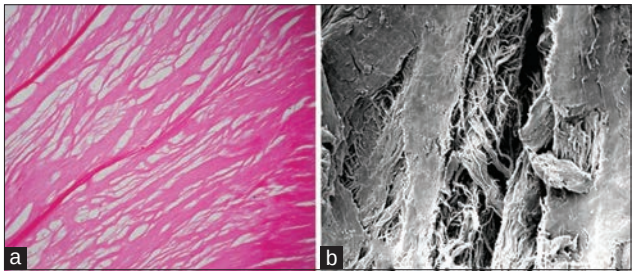

Figure-3: (a) Decellularized fish swim bladder showing loss of cellularity and dense fiber architecture providing adequate porosity (hematoxylin and eosin stain, ×40), (b) scanning electron microscopy observation showing loss of cellularity and dense fiber architecture providing adequate porosity (×3000).

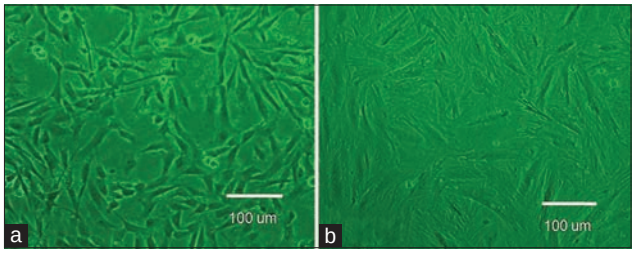

Figure-4: (a) Spindle-shaped fibroblastoid cells started to attach on day 4, (b) goat bone marrow-derived cells showing 80-90% confluence by day 16.

by day 3 post-operative. Animals of both the groups assumed a hunched back posture up to day 11 while resting in their cages, instead of their natural resting posture of dorsal recumbency. The temperature of adjacent normal skin was found to be significantly higher ($p<0.001$) in both the groups on day 3 when compared to the temperature of the matrix. But graft temperature showed an increasing tendency by day 7 post-operative (Figure-6). No significant difference in temperature was observed between the two groups. Macroscopic observations show that in both the groups the upper layer of matrix was dried on day 14 and sutures were removed. This dried layer detached off, and lower layer merged with newly formed ECM covering the remaining surface of the wound (Figure-7). Epithelization and hair growth was already started at the margins of the wound, reducing the total wound area. In the healing period, we could grossly appreciate a better rate of wound closure in Group II than Group I. By day 28, the wound healed almost completely in both the groups with no scar i.e., healing was with minimum contraction, but mainly by regeneration and replacement.

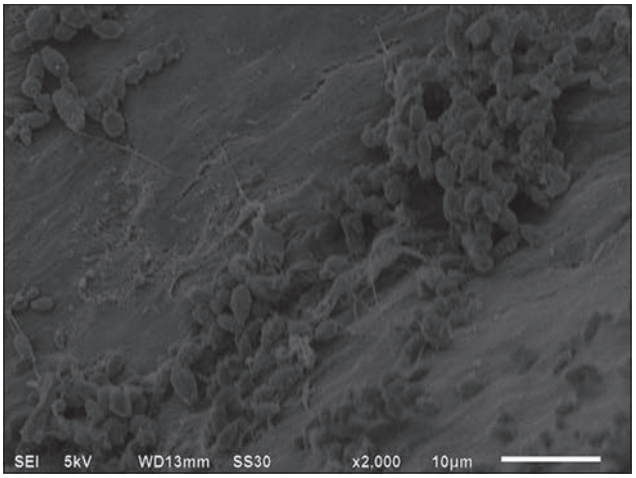

Figure-5: Attachment and proliferation of goat bone marrow-derived cells over acellular swim bladder by day 7 (scanning electron microscopy observation, ×2000).

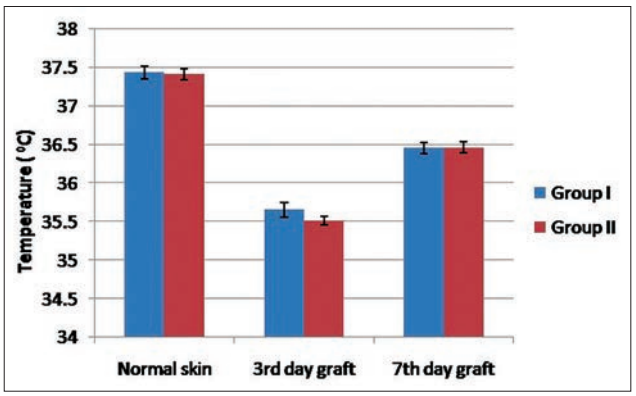

Figure-6: Mean ± standard error temperature (°C) of the graft and adjoining skin in different groups at different time intervals.

There is a relative rise in antibody titer from day 0 to 28 post implantation in both the groups, but it declined by day 35. A significant ($p<0.001$) increase in antibody response (on days 21 and 28 post implantation) was observed in Group I, as compared with Group II (Figure-8a). But when we compared the humoral immune response of both, the groups with respective negative controls (zero-day serum sample-before implantation) and positive control (HIS), immune response in both the groups were significantly ($p<0.001$) lower than that of hyper immune sera (Figure-8b). The results of LST of both the two groups, in comparison with stimulation index values of Con A and PHA are presented in Figure-9. Group I showed a significant ($p<0.001$) amount of stimulation when compared to Group II.

The results of histological observations at different time interval are presented in Figure-10. In both groups, the biomaterials were surrounded by an intense inflammatory reaction on days 3 and 7, but in latter stages of healing the inflammatory reaction subsides. By day 14, in both groups the implanted matrix disintegrated into the host tissue completely. However, some neo collagen formation was more evident in Group II. By day 21, dense and haphazardly arranged

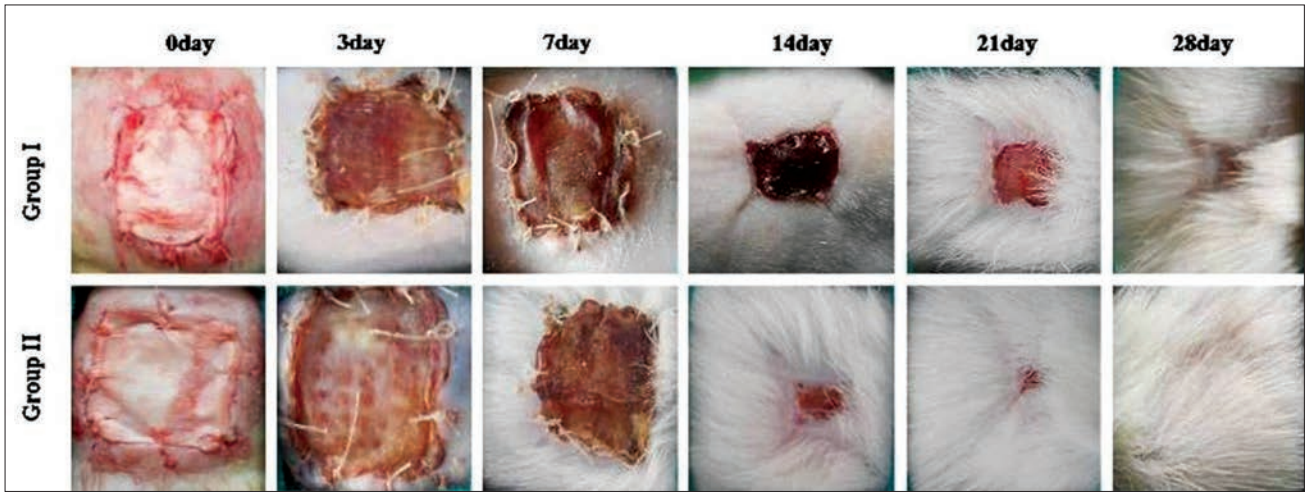

Figure-7: Macroscopic observations of healing at different time intervals.

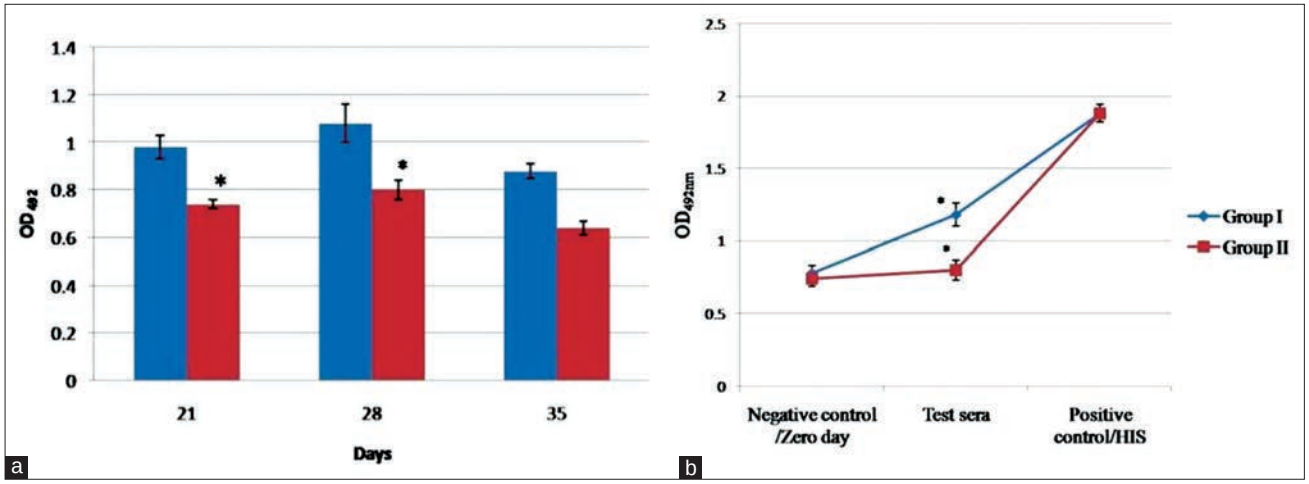

Figure-8: (a) Mean ± standard error absorbance at 492 nm wavelength enzyme-linked immunosorbent assay (ELISA) reading to compare immune response between groups at different time interval, *differ significantly (p<0.001) from immune response of Group I, (b) mean ± standard error absorbance at 492 nm wavelength ELISA reading to compare immune response between treatment groups (decellularized) and native fish swim bladder (hyper immune serum [HIS]), *differ significantly (p<0.001) from HIS.

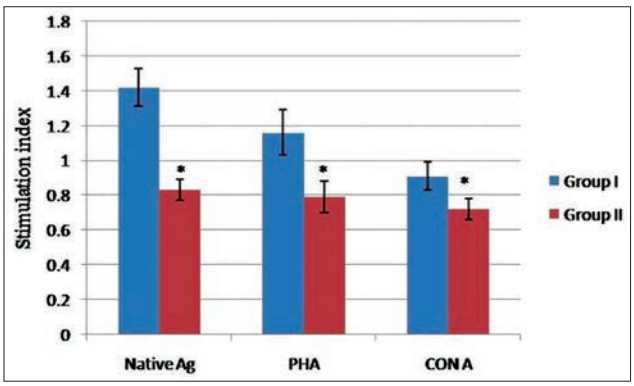

Figure-9: Mean ± standard error stimulation index values to compare cell-mediated immune response in both the two groups, *differ significantly (p<0.001) from Group I.

collagen fibers were observed in Group I. In Group II, the neoformed collagen gets matured, and parallely arranged collagen fibers with neovascularization were seen. There was complete epithelization. By day 28, in Group I, the arrangement of collagen fibers was regular in most of the wound area but not fully. In Group II collagen fiber arrangement and epithelization resembled to normal skin with the appearance of some hair follicle and optimum neovascularization.

Discussion

Ionic detergents are effective for solubilizing both cytoplasmic and nuclear cellular membranes [25]. SDC is one of the most commonly used ionic detergents for decellularization [26]. The technique for making biomaterials acellular was found successful as complete acellularity and adequate porosity of the swim bladder was obtained and was found non-toxic for the BMdc to grow and proliferate.

It has been observed that rats generally rest in dorsal recumbency. The animals of Group I and II started the resting on dorsal recumbency from day 11 onward. It indicates the normalcy and progressive healing. Dullness, depression, and partial anorexia observed in the immediate post-operative period (1-2 days) may be attributed to surgical trauma (pain) and inflammation at the site of reconstruction [27]. The warmth of the graft implanted at the surgically created wounds was significantly (p<0.001) lower than normal skin temperature up-to day 7 post-operatively, as during this period the grafts remains avascular. Similar findings have been reported after the repair of full-thickness skin defects with acellular matrix in rabbits [21,28] and rats [29]. The graft temperature increases on day 7 onward. The vasodilatation of the local wound vessels beneath the grafted tissue leads to increased blood flow resulted in an increase in the temperature of the graft [28].

Complete healing took place in about 28 days but was with minimum contraction in Group II animals. Adult mammalian skin wounds close by a combination of wound contraction, scar formation, and induced regeneration. Wound contraction establishes a plane tensile stress field in a skin wound which appears to be required for scar formation. Blocking of wound contraction by biologically active scaffolds leads to induced regeneration. Scaffolds appear to block contraction by interfering with the number and organization of myofibroblasts [30].

The color of the implanted matrix changed from white to dark brown in the course of healing and finally dried up upper layer got detach on removal of sutures. Lack of vascularization and continuous loss of moisture contents of the upper layer graft led to this. Similar findings have been reported after the repair of full-thickness skin defects in rabbits [28]. But at the same time it fulfilled our intention of protecting the lower layer from desiccation. By the time of removal of the upper layer, the lower target layer has got disintegrated and merged with the newly formed ECM/neoformed collagen of wound site but by blocking the contraction, thus served our purpose.

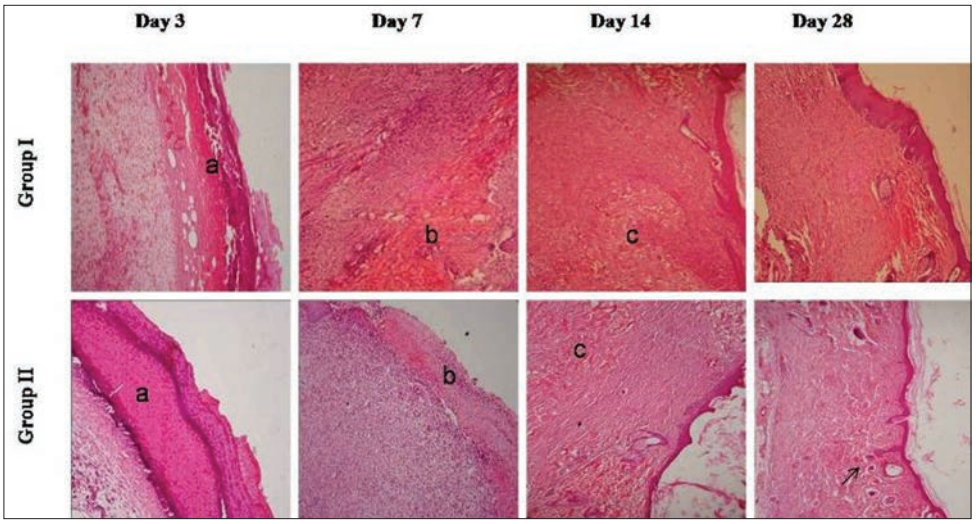

Figure-10: Histological appearance of cross section of wound area (hematoxylin and eosin stain, ×40), (a) Implanted matrix, (b) disintegration of matrix in host tissue, (c) neo collagen deposition, arrow - neovascularization.

The ECM represents nature's scaffold for tissue development and tissue repair. Collagen is the most abundant protein within the normal ECM [2]. Abundant collagen rich FSB matrix acting as a mimic of the normal ECM with the G-BMSc proliferating over it. In G-BMdc seeded matrix, the epithelization, neovascularization, and collagen fiber arrangement and its density resembled that of normal skin by day 28. However, in Group I, healing was completed by day 28, it does not attained that optimum quality of healing as exhibited by Group II in its histomorphological analysis. In gross morphological analysis, it almost appeared similar to Group II in its performance with only a slight lagging behind in the rate of wound closure.

In the present study, the animals of Group I (acellular matrix implanted group) show significantly higher immune response as compared to Groups II both in terms of humoral and cell-mediated immunity. The least immune response was in serum samples of day zero, where no grafts were used. Significantly ($p<0.001$) higher immune response in Group I animals may be due to the implanted acellular matrix, but it was not significant, when compared to the positive control (hyper immune sample). This result clearly explains the antigenic roll of cellular components and the effects of decellularization in putting down the immune response. The immune reaction elicited by these biomaterial even after making it acellular is due proteins of native ECM. Even if the cell matrix construct (G-BMdc seeded matrix) of Group II also have the same biomaterial as matrix, surprisingly they elicited a negligible immune response as compared to Group I animals. This explains the immuno modulatory property of BMdc. Adult bone-marrow-derived mesenchymal stem cells are immunosuppressive and prolong the rejection of mismatched skin grafts in animals [31]. Three broad mechanisms contribute to this effect. First, mesenchymal stem cells itself are hypo immunogenic, often lacking major histocompatibility complex II and costimulatory molecule expression. Second, these stem cells prevent T cell responses indirectly through modulation of dendritic cells and directly by disrupting natural killer cells, as well as CD^{8+} and CD^{4+} T cell function. Third, mesenchymal stem cells induce a suppressive local microenvironment through the production of prostaglandins and interleukin-10 as well as by the expression of indoleamine 2, 3,-dioxygenase, which depletes the local milieu of tryptophan [32].

Conclusion

Our study shows that treatment with 0.5% SDC for 24 h resulted in complete acellularity and adequate porosity of FSB. Repeated washing with PBS resulted in the removal of residual SDC, and the prepared acellular matrix was found non-toxic for the BMdc to grow and proliferate. Acellular FSB itself have wound healing potency, restricting the rate of contraction to minimum. However, seeding with BMdc increases the wound healing potency as it modulated the immune response to a significantly negligible level, thereby enhancing the graft take and quality of healing, avoiding immune-mediated inconveniences in the host. The BMdc-seeded acellular FSB matrix was found to be a novel biomaterial for wound management.

Authors' Contributions

RV, NK, AKS, SS and S conceptualized the study. NK and SKM obtained the funding RV, DDM and MN performed, analyzed the experiments, and wrote the initial draft of the manuscript. NPK did the expert analysis of histopathology. NK, AKS, SS, SKM and S reviewed and edited all subsequent drafts of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors acknowledge the financial assistance received from the Department of Biotechnology,

Ministry of Science and Technology, New Delhi, India and Junior Research Fellowship received from ICAR, New Delhi, India to carry out this research work.

Competing Interests

The authors declare that they have no competing interests.

References

1. Clark, R.A.F., Ghosh, K. and Tonnesen, M.G. (2007) Tissue engineering for cutaneous wounds. *J. Invest. Dermatol*., 127: 1018-1029.
2. Badylak, S.F. (2002) The extracellular matrix as a scaffold for tissue reconstruction. *Semin. Cell. Dev. Biol.*, 13(5): 377-383.
3. Bellows, C.F., Albo, D., Berger, D.H. and Awad, S.S. (2007) Abdominal wall repair using human acellular dermis. *Am. J. Surg.*, 194(2): 192-198.
4. Harth, K.C. and Rosen, M.J. (2009) Major complications associated with xenograft biologic mesh implantation in abdominal wall reconstruction. *Surg. Innov*., 16: 324-329.
5. Pascual, G., Sotomayor, S., Rodriguez, M., Kohler, B.P. and Bellon, J.M. (2012) Repair of abdominal wall defects with biodegradable laminar prostheses: Polymeric or biological. *Plos One*, 7(12): 1-11.
6. Gulati, A.K. and Cole, G.P. (1994) Immunogenicity and regenerative potential of acellular nerve allograft to repair peripheral nerve in rats and rabbits. *Acta Neurochir*., 126(2-4): 158-164.
7. Kumar, N., Gangwar, A.K., Sharma, A.K., Negi, M., Shrivastava, S., Mathew, D.D., Remya, V., Sonal, S, Maiti, S.K., Devi, K.S., Kumar, V., Ramteke, P.W., Kaarthick, D.T. and Kurade, N.P. (2013) Extraction techniques for the decellularization of rat dermal constructs. *Trends Biomater. Artif. Organs.*, 27: 102-103.
8. Gilbert, T.W., Sellaro, T.L. and Badylak, S.F. (2006) Decellularization of tissues and organs. *Biomaterials*, 27: 3675-3683.
9. Lee, C.H., Singla, A. and Lee, Y. (2001) Biomedical application of collagen. *Int. J. Pharm*., 221: 21-22.
10. Van der Laan, J.S., Lopez, G.P., Van, P.B., Nieuwenhuis, P., Ratner, B.D., Bleichrodt, R.P. and Schakenraad, J.M. (1991) TFE-plasma polymerized dermal sheep collagen for the repair of abdominal wall defects. *Int. J. Artif. Organs*, 14(10): 661-666.
11. Dong, S.W., Ying, D.J., Duan, X.J., Xie, Z. and Yu, Z.J., Zhu, C.H., Yang, B and Sun, J.S. (2009) Bone regeneration using an acellular extracellular matrix and bone marrow mesenchymal stem cells expressing C bfa1. *Biosci. Biotechnol. Biochem.*, 73(10): 2226-2233.
12. Cheng, H.L., Loai, Y., Beaumont, M. and Farhat, W.A (2010) The acellular matrix (ACM) for bladder tissue engineering: A quantitative magnetic resonance imaging study. *Magn. Reson. Med.*, 64(2): 341-348.
13. Boer, U., Lohrenz, A., Klingenberg, M., Pich, A., Haverich, A. and Wilhelmi, M. (2011) The effect of detergent based decellularization procedures on cellular proteins and immunogenicity in equine carotid artery grafts. *Biomaterials*, 32(36): 9730-9737.
14. Kheir, E., Stapleton, T., Shaw, D., Jin, Z., Fisher, J. and Ingham, E. (2011) Development and characterization of an acellular porcine cartilage bone matrix for use in tissue engineering. *J. Biomed. Mater. Res. A.*, 99(2): 283-294.
15. Stapleton, T.W., Ingram, J., Fisher, J. and Ingham, E. (2011) Investigation of the regenerative capacity of an acellular porcine medial meniscus for tissue engineering applications. *Tissue Eng. Part. A.*, 17(1-2): 231-242.
16. Zhao, Y., Zhang, Z., Wang, J., Yin, P., Zhou, J., Zhen, M., Cui, W., Xu, G., Yang, D. and Liu, Z. (2012) Abdominal hernia repair with a decellularized dermal scaffold seeded with autologous bone marrow derived mesenchymal stem cells. *Artif. Organs.*, 36(3): 247-255.
17. Kumar, V., Kumar, N., Sharma, A.K., Maiti, S.K., Gangwar, A.K., Singh, H., Shrivastava, S., Sonal, S and Rai, R.B. (2012) Acellular buffalo small intestinal submucosa and fish swim bladder for repair of full-thickness skin wounds in rabbit. In proceeding of: 9th World Biomaterials Congress, Chengdu, China. Abstract No.:52. p104.
18. Gangwar, A.K., Kumar, N., Sharma, A.K. Devi, S., Negi, M., Shrivastava, S., Mathew, D.D., Remya, V., Sonal, S., Arundeep, P.S., Maiti, S.K., Kumar, V., Kaarthick, D.T., Kurade, N.P. and Singh, R. (2013) Bioengineered acellular dermal matrix for the repair of full thickness skin wounds in rats. *Trends Biomater. Artif. Org.*, 27: 67-80.
19. Rose, C., Mandal, A.B. and Joseph, K.T. (1998) Characterization of collagen from the swim bladder of catfish (*Tachysurus maculates*). *Asian Fish Sci.*, 11: 1-10.
20. Le Blanc, K., Tammik, L., Sundberg, B., Haynesworth, S.E. and Ringdén, O. (2003) Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand. J. Immunol.*, 57(1): 11-20.
21. Kumar, V. (2010) Acellular buffalo small intestinal submucosa and fish swim bladder for the repair of full thickness skin wounds in rabbits. M. V. Sc. Thesis Submitted to Indian Veterinary Research Institute, Deemed University, Izatnagar, Bareilly, Uttar Pradesh, India.
22. Boyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.*, 21: 77-89.
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193(1): 265-275.
24. Kruisbeek, A.M. and Shevach, E.M. (2004) Proliferative assays for T cell function. In: Coligan, E., Margulies, D.H., Shevach, E.M. and Strober, W., editors. Current Protocols in Immunology. Unit 3.12. John Wiley, New York. p1-3.
25. Seddon, A.M., Curnow, P. and Booth, P.J. (2004) Membrane proteins, lipids and detergents: Not just a soap opera. *Biochim. Biophys. Acta*, 1666(1-2): 105-117.
26. Woods, T. and Gratzer, P.F. (2005) Effectiveness of three extraction techniques in the development of a decellularized bone-anterior cruciate ligament-bone graft. *Biomaterials*, 26(35): 7339-7349.
27. Gangwar, A.K., Sharma, A.K., Kumar, N. Maiti, S.K. Gupta, O.P. Goswami T.K. and Singh, R. (2006) Acellular dermal graft for repair of abdominal wall defects in rabbits. *J. S. Afr. Vet. Assoc.*, 77(2): 79-85.
28. Purohit, S. (2008) Biocompatibility testing of acellular dermal grafts in a rabbit model: An *in-vitro a*nd *in-vivo* study. Ph.D. Thesis Submitted to Deemed University, I.V.R.I., Izatnagar, Bareilly (UP).
29. Kaarthick, D.T. (2011) Repair of cutaneous wounds using acellular diaphragm and pericardium of buffalo origin seeded with *in-vitro* cultured mouse embryonic fibroblasts cells in rat model. M.V.Sc. Thesis Submitted to Deemed University, I.V.R.I., Izatnagar, Bareilly (UP).
30. Yannas, I.V. (2005) Similarities and differences between induced organ regeneration in adults and early fetal regeneration. *J. R. Soc. Interface*, 2(5): 403-417.
31. Blanc, K.L., Fibbe, W., Frassoni, F., Locatelli, F. and Ringdén, O. (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet*, 363(9419): 1439-1444.
32. Ryan, M. Barry, P.F., Murphy, M.J. and Mahon, P.B. (2005) Mesenchymal stem cells avoid allogeneic rejection. *J. Inflamm*., 2: 8.

****