



## RESEARCH ARTICLE

### Effect of Medical Grade Chitosan Powder with Xenogenic Mesenchymal Stem Cell for Full Thickness Wound Healing in Rat Model

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#### ABSTRACT

The present study was conducted in eighteen adult wistar rats (n=18) divided into two equal groups (n=9) to evaluate the healing potential of medical grade chitosan powder and xenogenic mesenchymal stem cell. One full thickness skin wound (20 x 20 mm<sup>2</sup>) was created on dorsal thoracic region of the rats. Topical application of paraffin gauze over the wound acted as control (Group A). In second group (Group B), the animals were treated with medical grade chitosan powder and xenogenic stem cell locally. Findings including clinical examinations, gross observations, exudation, granulation tissue, peripheral swelling and the contraction rate of the wounds were recorded on days 0, 3, 7, 14, 21, 28 in both groups. Early granulation tissue formation with reduced exudation and peripheral swelling was observed in group B and proved better than group A. In group B, complete wound healing was observed on day 19-20, but in group A, healing was completed by day 27-28, however, the percent of wound contraction was similar to gross findings.

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## INTRODUCTION

Disruption of the cellular or anatomical continuity of the normal organ structure is known as wound. Healing involves migration, infiltration, proliferation and differentiation of several cell types like keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets which are culminate as an inflammatory response, new tissue the formation of and wound closure (Barrientos *et al.*, 2008).

Chitosan is a 1, 4-linked polymer of glucosamine (2-amino-2-deoxy--D-glucose) and lesser amounts of N-acetylglucosamine. It is a derivative form of chitin (poly-N-acetylglucosamine) which is the second most abundant biopolymer after cellulose. It is biocompatible, biodegradable, hemostatic, anti-infective and, more importantly, it accelerates wound healing (Ishihara *et al.*, 2002; Conti *et al.*, 2000).

The term mesenchymal stem cells (MSCs) apply to adult fibroblast like cells that differentiate along multiple mesenchymal pathways when exposed to proper stimuli (Caplan, 2006). Currently, there are two basic delivery methods: systemic infusion of the cells into vascular circulation and direct application of therapeutic cells to

the wound sites. The source of donor tissue can be xenogenic, allogenic, or autogenic. With this background, the present study was designed with the objective to evaluate potential effect of medical grade chitosan powder with xenogenic mesenchymal stem cell on wound healing in wistar rats.

## MATERIALS AND METHODS

This study was permitted by Institute Animal Ethics Committee (IAEC), Indian Veterinary Research Institute, Izatnagar (Uttar Pradesh) India. Eighteen clinically healthy of either sex adult wistar rats (n=18) were used in this study. Each animal was caged individually and provided for free access to water and a standard diet. The animals were acclimatized to approaching, handling and animal house conditions for a period of 10-15 days prior to the study. They were randomly divided into two groups *viz*, Group A and Group B of nine animals each.

Collection of bone marrow sample was done from New Zealand White rabbit by 18 G bone marrow biopsy needle from the posterior aspect of iliac crest (Figure 1). Then Isolation and culture of mesenchymal stem cells (r-MSC) was done in low glucose stem cell media in

Department of Bio Technology stem cell laboratory Division of Surgery, IVRI. The cells (BM-rMSC) was passaged several times to increase the cell population up to 3<sup>rd</sup> passage (Figure 2). Once population established, they were characterized by mesenchymal stem cells specific markers (positive and negative CD markers) during proliferation by RT-PCR. Then these cells were ready to use locally at site of wound.

### Surgical procedure

The rats were anaesthetized using xylazine HCl (6 mg/kg) and ketamine HCl (60 mg/kg) body weight administered intramuscularly. The animals were placed in sternal position on the operative table. The animal and operation area was aseptically prepared starting from the dorso-caudal part of the shoulder on the dorsum to the caudal part of the last rib. On the prepared area, a 20x20 mm<sup>2</sup> full thickness skin defect was created aseptically. The animals in each group were treated as mentioned in table 1. The wounds were observed for 28 days or till complete healing of wound. The efficacy of the wound healing was measured on the basis of following parameters.

### Clinical observations

- a) *General behavioural changes:* Feeding pattern and general behavioural changes in all the rats was observed daily during the observation period.
- b) *Rectal temperature:* Rectal temperature was recorded daily up to 7 post-operative days in all rats.

### Gross observations

The wound site was examined grossly on days 0, 3, 7, 14, 21 and 28 or till completion of healing for the evaluation of following parameters:

- a) *Measurement of wound area:* Wound area was measured at day 0, 3, 7, 14, 21 and 28 or till completion of healing.
- b) *Wound contracture:* Wound contraction was measured on 0, 3, 7, 14, 21, and 28 postoperative day as a percentage reduction in wound area. Progressive decrease in the wound area was monitored periodically by tracing the wound margins on a tracing paper and the area was assessed by using a graph paper. The mean wound area and the mean percentage of wound contraction for each interval were calculated for each group (Bohling *et al.*, 2004).

$$\% \text{ contraction} = 100 - \frac{\text{Total wound area on day}_n}{\text{Original wound area on day}_0} \times 100$$

Where n is: 0,3,7,14,21 and 28 day.

- c) *Exudation:* The degree of exudation at the site of repair was graded on 1-4 scale as per standard method (Bigbie *et al.*, 1991). 1 = None (Apparently dry wound); 2 = Mild exudates (Wound is moist, no oozing on pressing the wound); 3 = Moderate exudates (Wound is moist, slight oozing on pressing the wound); 4 = Extreme exudates (Exudates is visible and pressure lead to extensive exudation).

- d) *Evaluation of granulation tissue:* Granulation tissue evaluation was graded on 1-4 scale as per standard method (Bigbie *et al.*, 1991). 1 = Granulation tissue depressed below the skin edge; 2 = Granulation tissue

proliferated to the level of skin edge; 3 = Granulation tissue elevated above skin edges; 4 = Granulation tissue elevated above skin edges, projecting over the border of epithelium.

- e) *Colour of granulation tissue:* Colour of the wound depicts the status of healing and was scored as: 1=Pale yellow; 2=Pale red and 3=Pink.

- f) *Time of appearance of granulation tissue:* It was recorded as the first day when the granulation tissue was observed.

- g) *Colour Digital Image Processing:* Colour photographs were taken on days 0, 3, 7, 14, 21 and 28 or till completion of healing with the help of digital camera. Analysis of shape, size, irregularity and colour of the lesion was determined.

### Histomorphological observations

The biopsy specimens from the site were collected on days 7, 14, and 28 for the histomorphological evaluation. The section was stained with Hematoxylin and Eosin (H & E) for evaluation of inflammation, epithelialization and neovascularization. The H&E sections were evaluated microscopically by using histological scoring system (Ghamsari *et al.*, 1996). Special staining for collagen fibers was done by using Mason's Trichome stain.

### Statistical analysis

The data was analyzed using the suitable statistical program for Social analysis (SPSS) for windows (Sneador and Cochran, 1989). One way ANOVA (analysis of Variance) and Duncan multiple range test (DMRT) were used to compare the means at different time intervals among different group. Student paired 't' test was used to compare the mean value at different time interval with their base value in each group. The subjective data generated from the scoring of various parameters were analyzed using Kruskal Wallis test.

## RESULTS

The rats in both groups remained dull on first postoperative day and assumed a hunched back posture, while resting in their cages. They started taking feed and water partially within 24 hour after surgery. In all the animals feed and water intake became normal by 3<sup>rd</sup> postoperative day. It has been observed that rats generally rest in dorsal recumbency. The rats of group B started resting on dorsal recumbency from day 13, whereas, in group A it was from day 21. It indicates the normalcy and healing was progressing well in the experimental group.

Significant (P<0.05) increase in rectal temperature for first 3 days post operative was recorded in both groups. The hyperthermia recorded in the early postoperative period started returning to normal as soon as the animals recovered from surgical stress. On day 3, temperature was significantly increased within group A.

Wound contraction has been used to monitor wound healing. Wound area decreased gradually as the healing progressed. Control wound healed completely by 27-28 days leaving a large scar indicating the existence of severe contraction. Group B took 19-20 days for complete healing and it was with minimum contraction leaving a little scar than control group. Mean  $\pm$  SE of the total

**Table 1:** Treatment protocol in groups

Group	Treatment
A	Wound area was covered with standard dressing material/ Dermafin (Control)
B	Wound area was covered with Chitosan Powder (Medical grade) and xenogenic mesenchymal stem cell injected locally at site of wound, protected with standard dressing material on day 0 (figure 3)

**Table 2:** The mean  $\pm$  SE of wound area (mm<sup>2</sup>) at different time interval in both groups

Groups	Time interval (Days)					
	0	3	7	14	21	
A	425.00 $\pm$ 6.30	359.33 $\pm$ 9.04**	273.67 $\pm$ 10.02 <sup>b**</sup>	113.16 $\pm$ 11.24 <sup>b**</sup>	37.05 $\pm$ 12.16**	Healed
B	476.11 $\pm$ 5.54	327.67 $\pm$ 16.37**	188.33 $\pm$ 22.09 <sup>a**</sup>	32.83 $\pm$ 5.19 <sup>a**</sup>	Healed	Healed

**Table 3:** The mean $\pm$  SE of the percentage contraction of the wound area (mm<sup>2</sup>) in both groups at different time intervals

Groups	Time Interval (Days)					
	3	7	14	21	28	
A	15.38 $\pm$ 2.02 <sup>a**</sup>	35.52 $\pm$ 2.41 <sup>a**</sup>	73.38 $\pm$ 2.62 <sup>a**</sup>	91.37 $\pm$ 2.73**	Healed	
B	32.62 $\pm$ 3.10 <sup>b**</sup>	63.35 $\pm$ 4.38 <sup>c**</sup>	93.65 $\pm$ 1.01 <sup>b**</sup>	Healed	Healed	

**Table 4:** The mean  $\pm$  SE of degree of exudation scores at different time interval in both groups

Groups	Time Interval (Days)					
	0	3	4	5	6	7
A	1.0.0 $\pm$ .00	3.22 $\pm$ .22 <sup>b**</sup>	2.55 $\pm$ .17**	2.22 $\pm$ .14*	1.55 $\pm$ .17 <sup>b</sup>	1.22 $\pm$ .14
B	1.00 $\pm$ .00	2.44 $\pm$ .17 <sup>a**</sup>	2.44 $\pm$ .17**	2.00 $\pm$ .00	1.00 $\pm$ .00 <sup>a</sup>	1.00 $\pm$ .00

**Table 5:** Mean  $\pm$  S.E values of score for granulation tissue level in excisional wounds in both treatment groups.

Groups	Time interval(days)			
	3	7	14	21
A	1.33 $\pm$ .16	2.22 $\pm$ .14**	2.42 $\pm$ .14 <sup>a**</sup>	3.66 $\pm$ .16 <sup>a**</sup>
B	1.88 $\pm$ .26	2.55 $\pm$ .17**	3.33 $\pm$ .16 <sup>b**</sup>	4.00 $\pm$ .00 <sup>b**</sup>

Means with different alphabets within a column are significantly different. (P<0.05); \* Significantly different from 3 day value (P<0.05); \*\* Highly significant difference from 3 day value (P<0.01).

**Table 6:** Mean  $\pm$  S.E values for start of granulation tissue in excisional wounds in various treatment groups

Granulation start	Days
A	3.21 $\pm$ 0.15 <sup>a</sup>
B	2.11 $\pm$ 0.24 <sup>b</sup>

Means with different alphabets within a column are significantly different. (P<0.05); The time (day) of appearance of granulation tissue seen grossly was significantly (P<0.05) less in groups B than A.

wound area (mm<sup>2</sup>) of the skin wounds at different time interval are presented in table - 2. On day 7 and 14, wound areas were significantly (P<0.05) decreased in group B as compared to group A. Original wound was created as 20 mm x 20 mm dimensions; however, almost all the wounds expanded to various extents and had an area greater than the 40 mm<sup>2</sup>. Significant (P<0.05) increase in percent of contraction was observed in both the groups during the observation period. On day 7 and 14, wound area was significantly (P<0.05) contracted in group B as compared to group A. Mean  $\pm$  SE of the total wound area contraction at different time interval are presented in table 3.

Moderate exudation at the site was observed up to day 3-5 in all the treated as well as control wounds. But in animals of group A showed a significantly higher (P<0.001) values of exudation on days 3 and 6 respectively as compared to group B (table 4). As the healing progressed the inflammation subsided gradually and therefore, no exudation was observed on day 7 and onwards except in group A.

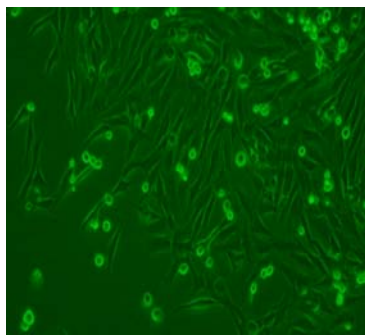
Granulation tissue was first observed in group B on day 2-3 and in group A on day 4. On day 12-14 granulation tissue was almost fully covered the wound in group B, whereas, wound area was covered by the granulation tissue in group A on day 21-22. Rats in group B showed significantly higher granulation tissue values (P<0.05) on day 14 and day 21 as compared to group A (Table 5). The time (day) of appearance of the granulation tissues seen grossly was significantly (P<0.05) less in group B than A (table 6).

Creation of excisional wounds resulted in variable extent of bleeding and formation of clot. The clot was dried and formed a cover over the wounds, which rendered the evaluation of colour of granulation tissue difficult in some of the animals. The scab, which covered the underlying granulation tissue, was detached in majority of the wounds before 17 postoperative days in group B. At this junction the classical 'shiny, beefy, red' to pink granulation tissue indicating healthy healing progress was evident. The scar became paler with passage of time, which indicated stage of maturation. The maturation was much earlier in group B than A.

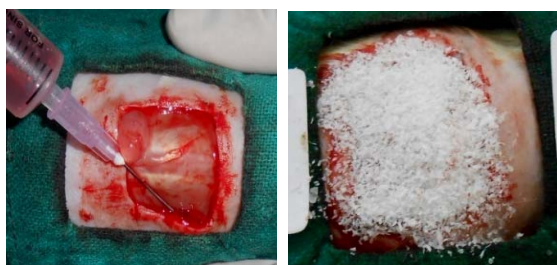
The coloured digital photographs of wounds in rats of group A revealed that the wounds were covered with soft and fragile pinkish mass having mildly desiccated top surface on day 3. On day 7, the surface became desiccated and somewhat necrosed. By day 14, thick granulation appears and size of wound decreases. By day 21, crust shed off and pinkish area appears. By day 27-28, the wound was healing leaving large scar. In rats of group B, on day 3, the wound was covered with brownish border having good granulation tissue. On day 7, the wound was more desiccated, completely brown in colour with thick granulation and size was nearly same. On day 14, wound contraction was very fast. On day before 21, the crust detached off leaving a raw granular pinkish tissue. On day 28, the wound healed up completely by severe contraction leaving no scar. Normal epithelization with very good hair growth on wound area was observed on day 28, resembling normal skin (figure 4).



**Fig. 1:** Collection of bone marrow from the iliac crest of a rabbit.



**Fig. 2:** Confluency of rMSC on day 12, 60-70% (40 x)



**Fig. 3:** Application of medical grade chitosan powder with xenogenic mesenchymal stem cell in Group B.

### Histopathological observations

**Group A:** On day 7, the necrosed surface was detached and the edges of the wound had partial epithelisation. The granulation tissue (fibroplasia and neovascularization) was severe and has less dense, thin and worst arranged collagen fibres and presence of mainly mononuclear cells (lymphocytes and macrophages) and less number of neutrophils. On day 14, severe proliferation of fibroblasts and neovascularization was observed. On day 28, a high degree of collagen deposition was observed with complete epithelisation but arrangement of collagen fibre in the wound area was however unorganized (Figure 5 and 6).

**Group B:** By day 7, fibroblast proliferation became more prominent, the necrosed surface was detached and the edges of the wound had partial epithelisation. The granulation tissue (fibroplasia and neovascularization) was mild and had very less dense, thin and worst arranged collagen fibres and presence of mainly mononuclear cells (lymphocytes and macrophages) and less number of neutrophils shows excessive inflammation. By day 14, epithelisation was partial, inflammation decreased, denser, thick and better arranged collagen fibres. By day 28,

epithelisation was almost completed covering the granulation tissue. Hair follicles were also observed with arranged collagen fibre.

### DISCUSSION

The animals of group B started resting on dorsal recumbency from day 13, whereas, in group A, it was from day 21. These results were in accordance with other workers (Kaarthick, *et al.*, 2011). It indicates the normalcy and healing was progressing well in the experimental group. 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 (Gangwar *et al.*, 2006).

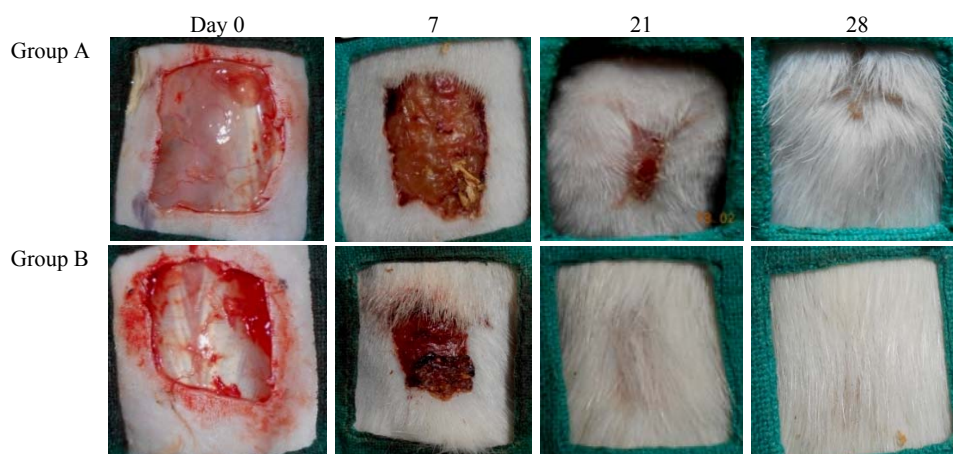
Significant ( $P < 0.05$ ) increase in temperature for first 3 days post surgery was recorded in both groups which may be due to foreign body reaction, surgical trauma and stress to the animals following skin wound. Pyrexia of variable degree in postoperative days has also been reported after the repair of full thickness skin defects with different materials in rabbits (Gangwar *et al.*, 2006; Purohit *et al.*, 2008) and rats (Kaarthick, *et al.*, 2011). The increase in temperature may also be attributed to the action of endogenous or leukocytic pyrogen produced by granulocytes, monocytes and macrophages (Atkins *et al.*, 1960).

Wound contraction has been used to monitor wound healing. Wound area decreased gradually as the healing progressed. The most important cell, the fibroblast attained the peak approximately on day 7 from injury and is responsible for initiating the angiogenesis, epithelisation and collagen formation. Control wound healed completely by 27-28 days leaving a large scar indicating the existence of severe contraction. Group B took 17-18 days for complete healing, but it was with minimum contraction leaving a little scar than control group. Wound contraction is the centripetal displacement of the wound edges that facilitates its closure after trauma. This process is carried out by myofibroblasts that contain  $\alpha$ -actin from smooth muscle and is mediated by contractile forces produced by granulation tissue from wound (Neagos *et al.*, 2006). Wound healing rate is defined as the gross epithelisation of the wound bed. Wound contraction was assessed by percent retention of the original wound area (Schalleberger *et al.*, 2008).

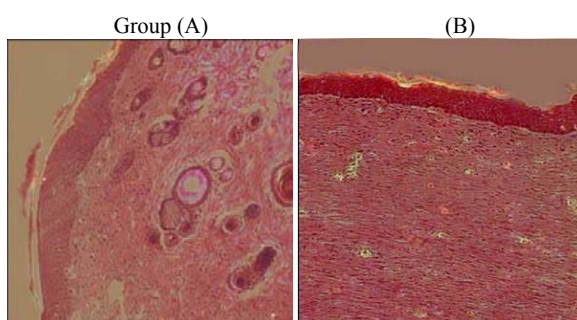
Moderate exudation at the site was observed up to day 3-5 in both groups. Exudation may be due to inflammatory reaction at the site in response to surgical trauma. As the healing progressed the inflammation subsided gradually and therefore, no exudation was observed on day 7 and onwards except in group A. A significant decrease in exudation after full-thickness wounds treated with small intestinal sub mucosa compared to untreated wounds in rat model was reported by (Kim *et al.*, 2005). According to (Wang *et al.*, 2007), a reverse correlation was detected between the survival area of the skin graft and the degree of exudation of the graft bed.

Granulation tissue was first observed in group B on day 2-3 and in group A on day 4 post surgery. On day 12 - 14, granulation tissue was almost fully covered the wound area in group B, whereas, wound area was covered by the granulation tissue in the control group (A) on day 21 - 22.

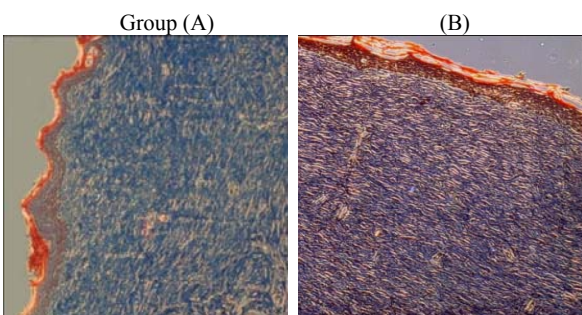




**Fig. 4:** Gross observations of the wounds in group A and B on day 0, 7, 21 & 28.



**Fig. 5:** Histopathology by using H & E stain on day 28.



**Fig. 6:** Histopathology by using Masson's Trichome stain on day 28.

Granulating tissue can generally be divided into two types, healthy and unhealthy granulating tissues. It is well known that healthy granulating tissue develops only in the absence of foreign bodies such as bacteria, debris, and so forth. Formation of healthy granulating tissue which is closely related to angiogenesis is a very important factor in wound healing (Clark and Denver, 1985). Similar finding was also obtained in this study in group B.

The colour of the wound changed from white to dark brown and finally dark on subsequent time intervals. There was not so much difference in wound area of group A and B from day 0 to 3. On day 7; a layer of scar was present on both groups. There was drastic reduction in wound area from day 7 to day 14 in group B in comparison to group A. On day 19 - 20 healing was complete in group B leaving a very little scar, whereas, in

control group (A) healing was complete in 27 - 28 days with abundant scar. Similar findings also have been reported after the repair of full thickness skin defects in rabbits (Purohit *et al.*, 2008) and in rats (Kaarthick *et al.*, 2011).

In the present study the histopathological samples collected on day 7, 14, and 28 were subjected to Hematoxylin and Eosin staining. Masson's Trichrome staining was also done to assess the collagen formation. On day 7, post implantation, moderate to severe inflammation was present in all the groups; however, it was minimum in groups B. The early control of inflammation, as in case of groups-B might facilitate the progress to the next phase of wound healing. On day 14, epithelisation and neovascularization was faster in group-B as compared to groups-A. On day 28, the collagen fibre arrangement was almost similar to normal skin in group-B. In group-B, hair follicle and skin glands could also be seen as in case of normal skin. Similar findings were also observed by Perme (2008).

Full thickness skin wound healing occurs by granulation tissue formation, contraction and epithelisation (Fossum *et al.*, 2007). Porous chitosan scaffolds present a promising approach for tissue engineering applications (Hong *et al.*, 2006). In experimental animal models, chitosan was shown to influence all stages of wound repair (Howling *et al.*, 2001). The haemostatic activity of chitosan can be seen in the inflammatory phase. It also interacts with and regulates the migration of neutrophils and macrophages acting on repairing processes such as fibroplasia and epithelialization (Ishihara *et al.*, 2002; Howling *et al.*, 2001). Chitin and chitosan derivatives are well-tolerated, effective adjuvants with considerable potential for clinical practice (Zahroff, 2006). The mesenchymal stem cells proved useful for repair and regeneration of a variety of mesenchymal tissues such as bone, cartilage, muscle and the cells produce useful growth factors and cytokines that may help repair additional tissues (Pittenger, 2008). Injection of bone marrow derived mesenchymal stem cells around wound and their application to the wound bed in an excision wound model enhanced healing significantly in normal and diabetic mice (Wu *et al.*, 2007). Clinical trials in human beings suggested that

direct application of bone marrow derived cells leads to dermal rebuilding and closure of non healing chronic wounds of more than 1 year duration (Badiavas and Falanga, 2003). In this study, medical grade chitosan powder with xenogenic mesenchymal stem cell to accelerated wound healing in comparison to control group. In conclusion, medical grade, sterilized chitosan powder with help of mesenchymal stem cell showed better healing potentiality in comparison to standard dressing material (dermafin) for repair of full thickness skin wounds in rat model.

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