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Microporous Biodegradable Polymeric Sponge for Surgical Haemostasis and Wound Healing

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ABSTRACT

Purpose: Conventional Hemostats used to control bleeding during surgeries are not biodegradable and cause hemorrhage while removal. To address this challenge, we have developed absorbable surgical hemostat which will be biodegradable, haemostatic and will also help in wound healing.

Methods: Biodegradable sponges were prepared by freeze drying 5% porcine gelatin solution, using formaldehyde as crosslinking agent. Internal structure of developed sponges was characterized *in-vitro* by Scanning Electron Microscopy (SEM) and was further tested for its water absorption capacity as well as biodegradation. Preclinical evaluation was performed for skin irritation, haemostasis, wound healing and biodegradation *in-vivo*.

Results: Optimized freeze drying process resulted in microporous, absorbable gelatin sponges which were biodegradable *in-vitro* in pepsin solution. The SEM images revealed that crosslinked Gelatin sponges were uniform and microporous. Excision and Incision wound model of rats showed enhanced haemostasis as well as wound healing without causing hemorrhage and exhibited complete biodegradation within 3-4 weeks on implantation without showing any irritation or toxicity.

Conclusion: Porcine gelatin could be formulated as microporous, absorbable gelatin sponge which will act as biodegradable surgical hemostat and it can also be helpful in wound healing.

Keywords: Hemostat, Microporous, Biodegradable, Lyophilization

INTRODUCTION

Among the various problems arising in the field of medicine and surgery, one of the most critical problems is to minimize the presence of blood around a surgical incision. The flow of blood is usually stopped or slowed by the application of a coagulating agent, such as thrombin or use of some nonbiodegradable sponges[1]. However both these methods have some limitations in closing of the wound and may cause hemorrhage. It is desired, therefore to eliminate these hazards by providing a biodegradable sponge which will be equally effective and convenient to use[2].

To promote wound healing, collagen plays a major role in haemostasis. Externally it is used in the foam and sponge form[3]. Collagen binds to the specific receptor site on platelet membrane which swells and release substances which help in haemostasis[4]. Collagen binds to fibronectin, causing platelet adhesion. It attracts monocytes which transform into macrophages. Macrophages release substances that result in fibroplasias and angiogenesis[5]. Collagen provides support for the growth of new capillaries. The presence of new capillaries is essential for the deposition of new fibers. Collagen directly supports the growth, attachment, differentiation and

migration of keratinocytes. By binding with fibronectin, collagen provides a provisional matrix for keratinocytes migration. It also helps in wound remodeling[6-7].

We selected Gelatin in place of collagen since it is a denatured type of collagen which is obtained by hydrolysis of collagen molecules. Gelatin has structural similarities as well as similar properties as of collagen so it also can be used as haemostatic and for wound remodelling. It is completely resorbable *in-vivo* and its physicochemical properties can be modulated due to existence of many functional groups[8]. Collagen is known to have wide biomedical applications but expresses antigenicity in physiological condition; gelatin has no such antigenicity. Gelatin has been used in wide variety of wound dressing[9-10]. Recently gelatin has shown to exhibit activation of macrophages and high haemostatic effect[11]. Gelatin is practically more convenient than collagen because a concentrated collagen solution is extremely difficult to prepare from the native collagen, and furthermore gelatin is far more economical than the collagen[12].

Gelatin is used in biodegradable materials. It can be converted into other forms such as sponges by crosslinking of gelatin. Physical crosslinking of gelatin film is generally carried out by thermal heating and ultraviolet irradiations. For chemical crosslinking of gelatin several crosslinking agents such as formaldehyde, glutaraldehyde, water soluble carbodiimides, diepoxy compounds, diisocynates are used. These agents form amide bridges within the amino acids present in the gelatin and cause crosslinking of the gelatin. The toxicity of crosslinking agent should be taken into consideration while development of gelatin sponges[13-14].

The aim of current study was to prepare crosslinked gelatin sponges and to investigate their physical, chemical, mechanical and morphological characteristics. The ultimate goal of this study is to evaluate the haemostatic and wound healing effect of these sponges compared with conventional wound dressing in order to prove their applicability to a new type of biodegradable wound dressing.

MATERIALS AND METHODS

Type A gelatin prepared by acidic treatment to porcine skin was procured from Nitta Gelatin (Canada) and had the following characteristics: gel strength on bloom scale 272, pH 4.7, viscosiy of 5% solution was 44mPas at 60^{o}C. It was used without further purification. Formaldehyde solution (37-41%), conc. sulphuric acid, sodium bicarbonate, hydrochloric acid and chromotropic acid were ordered from S.D.Fine chemicals, Mumbai.

Chromotropic acid reagent consisted of 50mg of chromotropic acid per 100ml of mixture of 9ml concentrated sulphuric acid and 4ml of water. TNBS (≥98% pure) was purchased from Sigma Aldrich. Double distilled water was used for all experimental work. All the chemicals utilized were ACS reagent grade.

All *in-vivo* studies mentioned here were carried out after approving the protocol from Animal ethical committee of C.U.Shah College of Pharmacy, Mumbai.

Preparation of crosslinked gelatin sponges

Aqueous solution containing 5% w/w porcine gelatin was prepared at 80^{o}C using double distilled water. Resulted solution was foamed with vigorous stirring and crosslinked with formaldehyde solution. It was further frozen at –40^{o}C overnight and frozen foam was freeze dried at -80^{o}C for 24 hours. Table 1 lists the conditions of sponge preparation and the density of final crosslinked sponge.

Table 1: Composition of Gelatin sponge

Sponge code	Gelatin (%)	Formaldehyde Wt% (w/v)[a]	Density (mg/cm3)[b]
GS1-x1	10	0.015	18.2
GS1-x2	10	0.03	18.5
GS1-x3	10	0.06	18.8
GS1-x4	10	0.12	20
GS2-x1	7	0.015	14
GS2-x2	7	0.03	14.2
GS2-x3	7	0.06	14.8
GS2-x4	7	0.12	15.1
GS3-x1	5	0.015	9.5
GS3-x2	5	0.03	10
GS3-x3	5	0.06	10.8
GS3-x4	5	0.12	12

[a] *Against volume of gelatin solution*
[b] *Density = weight (1cm x 1cm x 0.3cm) / volume of Sponge*

Morphology of gelatin sponges

Using Scanning Electron Microscopy (SEM), crosslinked gelatin sponges were analyzed to observe the internal structure of the sponge. The average diameter of pores and wall thicknesses (average distance between neighboring pores) were also measured by geometrical observation on screen using image analyzer. Effect of gelatin and formaldehyde concentrations on pore size and wall thickness was studied by plotting surface response curves and was interpreted in terms of percent contribution of each factor and from equations obtained from Stat-Ease Design-Expert v.7.1.6 software.

Water uptake ability

To measure the equilibrium water uptake ability of the sponge, preweighed dry sample was immersed in distilled water. It was kneaded gently between the fingers until thoroughly wet and until all the air was removed taking care not to break the tissue. After the bulk water was removed, the weight of wet sample was measured. The procedure was repeated with 5 different pieces of the same sponge. Then the water uptake ability was determined according to the following equation:

$$\textit{Water uptake ability} = [(Ww - W_{d)} / W_d] \quad \text{---------- 1}$$

Where W_w and W_d represents the weight of wet and dry sample respectively.

Measurement of degree of crosslinking

Crosslinking degree was measured by modified TNBS method[15]. The reaction of TNBS with primary amino groups of gelatin was used to chemically detect the absorbance of uncrosslinked groups in the crosslinked sponges by UV spectroscopy (SPECORD 205). Then the crosslinking degree could be obtained from the differences between the absorbance values before and after crosslinking[16-17]. Following equation was used for determination:

$$\textit{Crosslinking degree (\%)} = 1 - \left[\frac{\textit{Absorbance of crosslinked sponge}}{\textit{Absorbance of uncrosslinked sponge}} \right] x\ 100 \quad \text{--------2}$$

Residual formaldehyde content

Piece of sponge weighing about 50mg was added to 100ml of water and macerated for at least 2 hours, shaking occasionally. About 0.5ml of the supernatant liquid was transferred to a glass stoppered test tube and 10 ml of chromotropic acid solution was added, the tube was stoppered and heated in a water bath for 30 minutes.

The absorbance of the resulting solution was taken at 570nm. It should not be more than that of a solution obtained by repeating the operation using 0.5 ml of solution containing 0.001% w/v of formaldehyde, CH_2O in place of supernatant liquid.

***In vitro* biodegradation**

Pieces of sponges (1cm x 1cm x 0.3cm) were immersed in 5ml of phosphate buffered saline (PBS pH 7.4) containing 6 µg ml^{-1} of pepsin. After incubation at 37^0C, the sponges were repeatedly washed with distilled water and freeze dried. This was carried out after every 6, 12, 24, 36, 60 and 72 hour. The changes in weight of sponges were monitored after each time interval. The time taken for total biodegradation of sponge was determined[18].

The average biodegradation time of three determinations was noted as the reading.

Pre-clinical evaluation:

a) Skin irritation testing

Primary skin irritation studies on the optimized formulations were performed using **New Zealand White** albino rabbits in accordance with the guidelines of the Consumer Product Safety Commission. Formalin and Vaseline gauze dressing were used as positive control and negative control respectively. The scores were recorded as per the Draize patch test[19].

b) Haemostasis and wound healing

The dorsal hairs of Wistar rats were shaved and the animals were anaesthetized with Ketamine HCl (40-60mg/kg) intraperitonialy. After disinfection of the skin, full thickness excision wound of 1cm diameter was prepared by excising the dorsal skin of all Wistar rats. Sterile prepared gelatin sponges of $1cm^2$ each were fixed with the help of adhesive tape. Excision wound of rats of control group was kept open without application of sponge.

Haemostatic activity was determined by measuring and comparing bleeding time of both the groups. Wound healing was also determined by observing wound status for 12 days. Morphometric analysis of the healing wound was done by measuring the size of the wound for both the groups.

c) In-vivo biodegradation

The dorsal hair of Wistar rats was shaven and the animals were anaesthetized with Ketamine HCl (40-60mg/kg) intraperitonialy. After disinfection of the skin, incisions were made on the back of Wistar rats to expose subcutaneous layer and sterile gelatin sponge of $1cm^2$ was implanted into the incision of a Wistar rat. These rats were sacrificed on the 1^{st}, 2^{nd}, 4^{th}, 7^{th}, 14^{th} and 21^{st} postoperative day and observed for the biodegradability of the sponge after opening the wound. The time taken for total biodegradation of sponge was determined[18].

RESULTS AND DISCUSSION

Factorial design approach was used to optimize formulation and process variables in development of microporous sponges.

The effect of gelatin concentration and formaldehyde concentration on pore size and wall thickness are graphically shown in figure 1a and 1b respectively.

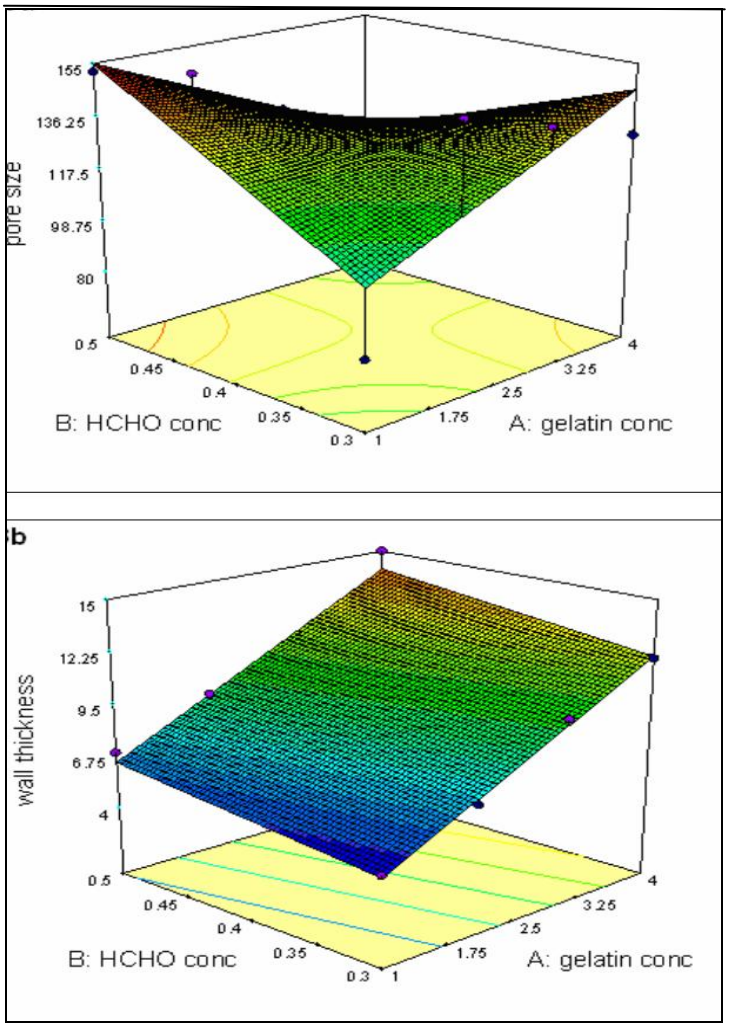

Figure 1: Effect of polymer and crosslinking agent on (a) pore size and (b) wall thickness of gelatin sponge

Contribution of gelatin concentration, formaldehyde concentration and both the parameters on pore size were 0.022%, 3.10% and 49.56% respectively; which was calculated from Eq.3 using surface response curve. This proved that both the parameters are responsible for affecting pore size of sponge.

Pore size (µm) $= -29.25 + 56.90 \times \text{gelatin conc.} + 397.50 \times \text{formaldehyde conc.} - 143.00 \times \text{gelatin conc.} \times \text{formaldehyde conc}$ ----------3

Pore size of the sponge depends on both gelatin and formaldehyde concentration, this can be explained with the fact that amide bridging within the gelatin molecules determines its pore size which may increase due to increase in crosslinking agent in the formulation. The highly porous structure of gelatin sponges are also considered to contribute to enhancement of wound healing by immobilizing overflowing hemorrhagic exudates within their

network structures at the instant of wound, thus preventing the formation of mobile body fluid packets and rapid dehydration of the wound bed.

Contribution of gelatin concentration, formaldehyde concentration and both the parameters on wall thickness were 85.90%, 8.77% and 0.036% respectively; which was calculated from Eq.4. This proved that gelatin concentration is the major parameter affecting wall thickness of sponge.

Wall thickness (µm) = 0.25 + 2.25 x gelatin conc. + 7.50 x formaldehyde conc. + 0.5 x gelatin conc. x formaldehyde conc ----------4

Wall thickness of gelatin sponge depends on the concentration of gelatin molecules since they undergo crosslinking forming amide bridges. Pore size and wall thickness determines water uptake ability of sponge. More the wall thickness more will be the capacity to retain the water inside.

Figure 2a represents microscopic image of gelatin sponge which showed crosslinking of gelatin sponge and figure 2b surface morphology of gelatin sponges having pore size within range of 60-120µm and wall thickness in the range of 3-5µm.

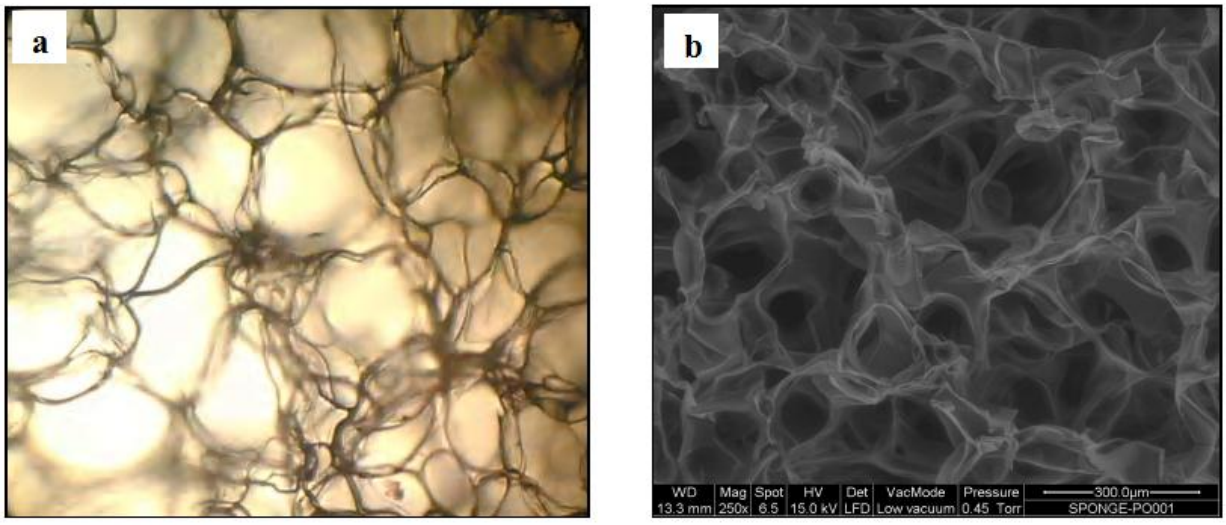

Figure 2a: Microscopy and 2b: SEM of gelatin sponges

Water uptake ability of gelatin sponges

Prepared gelatin sponges absorbed water within 30 sec and were saturated within 5 min. Water uptake ability of gelatin sponge was up to 40 times (Table 2). In other words, 1g of sponge bound 30-40 g of water.

Table 2: Characteristics of Gelatin sponges

Sample Code	Average Pore Size (µm)[a]	Wall Thickness (µm)	Water uptake[b] (g H_2O/ g sponge)
GS1-x2	57.8 ± 0.8	25.2 ± 0.5	28 ± 2.3
GS2-x2	79.2 ± 0.6	19.8 ± 0.2	32 ± 2.6
GS3-x2	156 ± 5.8	5 ± 0.5	40 ± 2.5

[a] *Average pore size was calculated by measuring the size of 60 pores by the image analyzer tool.*
[b] *Water uptake ability was calculated from the weight difference between the wet state and dry state of sponge.*

Crosslinking degree measurement and determination of residual formaldehyde

Crosslinking degree measures the extent of crosslinking which determines degradation of gelatin sponge. It was found to be increased with increase in concentration of formaldehyde used. More the crosslinking degree more will be the resistance towards degradation.

Residual formaldehyde content was evaluated for each batch of formulation described in table 1 to determine the safe and effective concentration of formaldehyde to be used as crosslinking agent for development of gelatin sponge having good fluid uptake ability and complete biodegradation.

Formaldehyde concentration of 0.03% w/v at the 5% gelatin concentration was found to give gelatin sponge of having 40 times water uptake ability.

***In-vitro* biodegradation**

Biocompatibility of the material and their degraded products is a prerequisite for resorbable haemostat. To check biocompatibility of gelatin sponge, *in-vitro* biodegradation studies were carried out.

Collagenase is naturally present in healing wounds which breaks down collagen molecules. Pepsin is used as substitute for evaluation degradation mechanism in denatured collagen which is gelatin. The degradation behavior *in-vitro* as a function of degree of crosslinking was investigated using series of samples with increasing cross-linking density. Formulations with varying crosslinking degree were checked for biodegradation time and plotted as described in figure 3. It showed that increased crosslinking degree reduces the biodegradation rate of gelatin sponge.

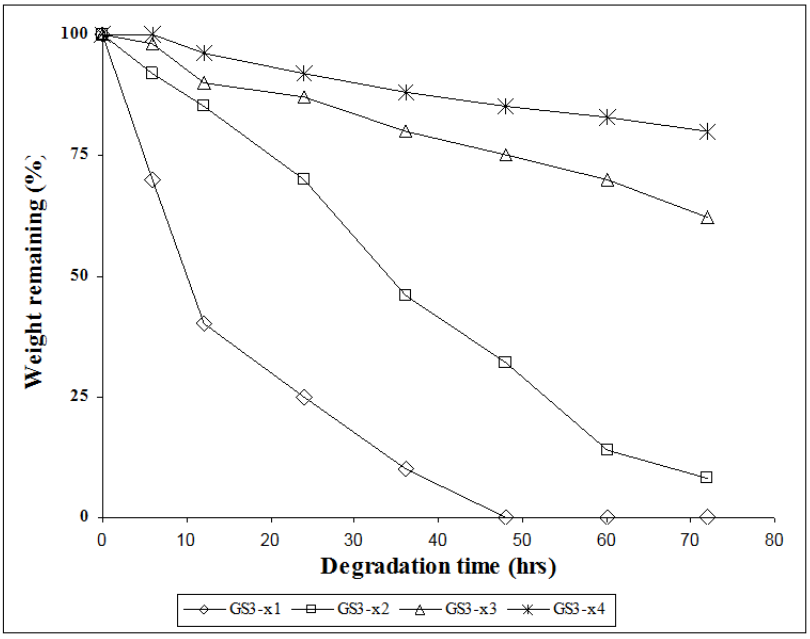

Figure 3: *In-vitro* degradation pattern of gelatin sponges with varying crosslinking degree

Prepared plain gelatin sponge formulation which was found to be degraded totally in 72 hours were selected further for pre-clinical evaluation[13, 18].

Pre-clinical evaluation of surgical hemostat

a) Skin irritation testing

The scores of erythema and edema were totaled for intact and abraded skin for all rabbits at 24 and 72 hours. The primary irritation index was calculated based on the sum of the scored reactions divided by 24 (2 scoring

intervals multiplied by 2 test parameters multiplied by 6 rabbits). The developed formulation showed no erythema or edema on the intact and abraded rabbit skin. The primary irritation index of the formulation was calculated to be 0.00. Thus the formulations found to be nonirritant to the rabbit skin. Standard scoring system for potential irritants and actual observations of the gelatin sponges are shown in table 3a and 3b respectively.

Table 3a: Standard scoring system for topical testing of potential primary irritants

Skin Reactions, Erythema & Scar Formation	Value
Erythema formation	
No erythema	0
Very slight Erythema	1
Well defined Erythema	2
Moderate to severe Erythema	3
Severe Erythema (beet redness) to slight scar formation	4
Edema formation	
No edema	0
Very slight edema	1
Well defined edema	2
Moderate edema	3
Severe edema (raised more than 1mm and extending beyond area of exposure)	4

Table 3b: Score for skin irritation test of Absorbable Gelatin Sponge formulation in Rabbits.

Formulation	Score	
	Erythema	Edema
Positive control (Formalin)	4	4
Prepared Gelatin Sponge	0	0

b) Hemostasis and wound healing

Application of gelatin sponge to bleeding site of excision wound of the rat showed dramatic reduction in bleeding time (Figure 4) as compared to control.

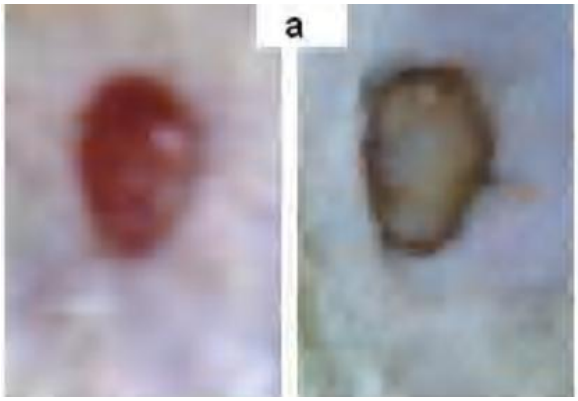

Figure 4: Haemostasis achieved 1minute after application of gelatin sponge.

The gross finding of measurement of wound size showed that total wound healing was observed within 15 days after application of gelatin sponge.

The morphometric results of wound recovery on 5th and 12th postoperative day are shown in figure 5. After 12 days, wound recovered with intact epidermis.

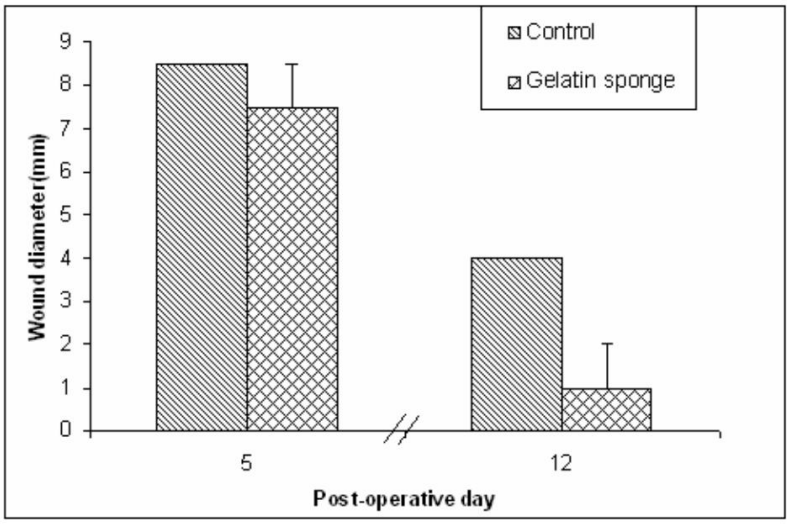

Figure 5: The morphometric results of wound recovery on 5th and 12th postoperative day

c) In-vivo biodegradation

In vivo biodegradation of sponge was determined by implanting the gelatin sponge subcutaneously (Figure 6) and observing for the degradation on 1st, 2nd, 4th, 7th, 14th and 21st postoperative day.

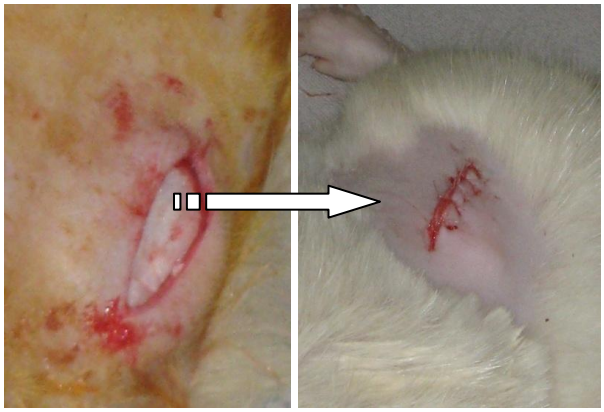

Figure 6: Subcutaneous implantation of gelatin sponges

Transparent gelatin mass was observed in 7th postoperative day whereas degraded mass of gelatin was present after 14th postoperative date. Complete biodegradation was observed after 21st postoperative day without showing any other adverse effects on the operated rat.

Developed microporous sponge degraded inside the body within 3 week period without showing any visible toxic effects.

CONCLUSION

This work shows that microporous biodegradable polymeric sponge can be prepared by crosslinking of gelatin using formaldehyde as crosslinking agent which can be used as surgical hemostat and helps in wound healing. Physicochemical, morphological and biological properties were characterized by TNBS assay, SEM, *in vitro* biodegradation. The developed sponge has pore size of 50-150 µm. Water uptake ability test showed that the sponges could bind 30-40 times the weight of the original sponge. Pre-clinical evaluation on gelatin sponge

confirmed that they can facilitate hemostasis as well as help in wound healing and is completely biodegradable within 3 weeks of implantation without showing any adverse effects.

In-vitro and *in-vivo* evaluation of developed formulation of gelatin sponge demonstrated the potential of macroscopic gelatin sponge as biodegradable advanced wound management tool for surgical haemostasis and wound healing.

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