Optimization, characterization, and efficacy evaluation of 2% chitosan scaffold for tissue engineering and wound healing

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ABSTRACT

Objective: To develop a chitosan-based scaffold and carry out a complete comprehensive study encompassing optimization of exact chitosan strength, product characterization, toxicity evaluation, in vitro validation in cell culture experiments, and finally in vivo efficacy in animal excision wound model.

Materials and Methods: Developed chitosan scaffolds (CCSs) were optimized for tissue engineering and wound healing efficacy by means of microstructure, toxicity, and biocompatibility evaluation. Results: Scanning electron microscope (SEM) studies revealed that porosity of CS decreased with increase in chitosan concentration. Chemical stability and integrity of scaffolds were confirmed by Fourier transform infrared studies. Highest swelling percentage (500%) was observed in 2%, while lowest (200%) was observed in 1% CS. Reabsorption and nontoxic property of optimized scaffold were established by enzymatic degradation and MTT assay. Enzymatic degradation suggested 20–45% of weight loss (WL) within 14 days of incubation. Cytotoxicity analysis showed that scaffolds were noncytotoxic against normal human dermal fibroblast cell lines. Significant cellular adherence over the scaffold surface with normal cellular morphology was confirmed using SEM analysis. In vivo efficacy evaluation was carried out by means of reduction in wound size on Sprague-Dawley rats. Sprague-Dawley rats treated with optimized scaffold showed ~ 100% wound healing in comparison to ~80% healing in betadine-treated animals within 14 days. Histological examination depicted advance re-epithelization with better organization of collagen bundle in wound area treated with 2% CS in comparison to conventional treatment or no treatment.

Conclusion: This study, thus, reveals that 2% CCSs were found to have a great potential in wound healing.

KEY WORDS: Chitosan, histopathology, scaffold, tissue engineering, wound healing

Wound healing is a multifaceted process, which involves multiple factors and biological events comprising of multiple complex cellular and biochemical processes.¹,² The healing process involves a successive progression of inflammation, proliferation of different types of cells, and tissue remodeling.³,⁴ Besides this, tissue engineering has also emerged as a promising approach to treat the lost or malfunctioning tissues.⁵-⁷ Tissue engineered skin surface uses engineering, as well as biological cascade,⁸ to promote organ and tissue regeneration to recover their function.⁹,¹⁰

The standard tissue engineering approach is the development of a biodegradable scaffold.¹¹ An ideal scaffold should be nontoxic, biocompatible, having optimal mechanical strength, and should provide a favorable microenvironment for cell growth and proliferation.¹²-¹⁸ With this approach, porous three dimensional scaffolds may be used and can play an important role in new tissue formation. In the last two decades, several natural and synthetic biomaterials have been developed for tissue engineering and wound healing applications.¹⁹ Research is still going on to develop suitable biomaterial that promotes physiological restoration of skin and diminishes or inhibits scar formation.¹⁶,¹⁷

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Nature has provided a significant set of biomaterials such as chitosan, alginites, and hyaluronic acid with great potential to be used in tissue engineering and wound healing applications.\cite{13,19} It is already reported that chitosan and its oligomers can be used as an ideal material for promotion of wound healing since they possess specific biological properties like hemostatic, granulation, epithelization, and biodegradability,\cite{20,21} which can be processed into membranes, sponges, meshes, and scaffolds.\cite{22,23} Chitosan as natural cationic polysaccharide is composed of glucosamine (GlcN) and N-acetylglucosamine (GlcNAc) residues\cite{24,25} and serves an important role in wound healing and regenerative medicine.\cite{26,27} It is a biodegradable polysaccharide, which is degraded slowly by lysozymes, chitinase, and chitosanase into harmless oligomers that significantly activate the wound healing process.\cite{28,29} Another advantage is that chitosan resembles with various glycosaminoglycans, which are distributed throughout the human body and are essential for cell morphology and differentiation.\cite{30,36} Chitosan also provides a suitable microenvironment for wound healing and cellular proliferation by behaving like an extracellular matrix\cite{11,32} and aids in tissue growth,\cite{33,34} initiates proliferation of fibroblast cells, and stimulates collagen synthesis.\cite{35} It also possesses antimicrobial properties against a variety of bacteria, fungi, and algae, preventing wound infection.\cite{27,36} In view of the significant advantages offered by chitosan, we thought it worthwhile to select it as a base biomaterial for the development of the scaffold for wound healing and tissue engineering. Although a lot of work has been reported on chitosan with respect to its wound healing properties, a complete comprehensive study encompassing optimization of exact chitosan strength in any such scaffold, product characterization, toxicity evaluation, in vitro validation in cell culture experiments, and finally in vivo efficacy in animal wound model is probably warranted.

In the present study, various types of chitosan scaffolds (CSs), with varying concentration of chitosan, were fabricated to obtain desired porosity. Optimization of chitosan concentration suitable for preparing the CS for wound healing and tissue engineering was established by means of standardization of pore size, chemical stability, enzymatic degradation, and biocompatibility. Physicochemical characterization of the developed CSs was carried out in terms of scanning electron microscopy (SEM), atomic force microscopy (AFM), Fourier transform infrared (FTIR), swelling index, and enzymatic degradation. Biological efficacy of CSs was evaluated by means of support in cellular viability, proliferation, and adhesion on scaffold surface. Biocompatibility was validated by MTT assay and cell culture studies. In addition, in vivo wound healing efficacy studies were carried out on Sprague-Dawley rats by creating excision wound model. The outcome of the study may improve the understanding toward the use of CSs for wound healing and tissue engineering.

**Materials and Methods**

**Ethics statement**

All animal procedures and care protocols were followed as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals regulations and the experimental protocol was approved by the Institutional Animal Ethics Committee.

**Material**

Chitosan (low viscous) was procured from Sigma-Aldrich, USA, povidone-iodine ointment (Betadine®) was purchased from Win-Medicare Pvt., Ltd., New Delhi. Glacial acetic acid and calcium chloride were procured from Merck India Ltd., Mumbai, all other chemicals were of analytical grade and used as obtained.

**Experimental animals**

Female Sprague-Dawley rats weighing (200–250 g) were obtained from the Experimental Animal Facility of Institute of Nuclear Medicine and Allied Sciences, DRDO, Delhi. All animals were housed in the standard environmental conditions of temperature 21–23°C, humidity 45–50%, a 12 h light and 12 h dark cycle in polypropylene cages, and were provided with a standard feed from Golden Feed Ltd., Delhi, India, and water ad libitum.

**Preparation of chitosan scaffolds**

Different concentrations (1–5%) of chitosan powder were dissolved separately in dilute acetic acid (1% w/v), and the reaction mixtures were kept at 25°C under continuous stirring at 250 rpm on a magnetic stirrer. After 2 h of continuous stirring, 1% w/v of calcium chloride (as electrolyte) was added to all the reaction mixtures and the stirring was continued overnight. The chitosan gels thus obtained were poured into 35 mm diameter circular polypropylene Petri dishes to obtain the desired shape of CS and kept at −20°C for the next 24 h. The samples were then lyophilized at −50°C under vacuum. Different types of scaffolds thus obtained were stored at 4°C till further evaluation.

**Physicochemical characterization**

**Physical characterization of chitosan scaffold**

Physical evaluation of different types of CSs was carried out using dried samples (after lyophilization) by the naked eye.

**Scanning electron microscopy**

Surface morphological study of CSs was carried out using SEM (Leo, VP-435, UK). Small size polymeric samples of ~5.0 mm² were dried overnight at 50°C on a double-sided adhesive tape separate. The dried samples were sputter coated with gold particles under reduced pressure conditions and observed under SEM at constant 15 kV accelerating voltage. Average pore size was also calculated in each cross-sectional image of the scaffold.

**Atomic force microscopy**

AFM micrograph of selected polymeric CS (synthesized using 2% of chitosan gel concentration) was carried out with AQ7
scanning probe microscopy ([SPM]; SPM – AFM, NTAF13 MDT, Switzerland) in contact mode, wherein a 2.0 mm² sample of the scaffold was observed under AFM. Nitride silicon cantilevers (OMCL-TR400PSA-1) with a spring constant of 0.02 N/m and a nominal tip radius of approximately 15 nm was used. The experiments were carried out under ultra-clean conditions at room temperature, and AFM imaging was performed in air scanning frequency of 1 Hz and a vertical deflection of 0.5 V. AFM images with 512 × 512 pixels were obtained at separate locations to ensure a high degree of reproducibility of experimental data.

**Fourier transform infrared spectroscopy**

Attenuated total reflectance-FTIR spectroscopy of vacuum-dried samples of chitosan and various types of CSs were recorded on a Perkin-Elmer spectrum one spectrometer in the range of 4000/cm to 650/cm in transmittance mode.

**Swelling studies**

The swelling characteristics of CSs were determined by immersing the lyophilized scaffolds (with calculated weights) in distilled water for 3 days at 37°C separately. The swollen scaffolds were removed at specific time intervals and weighed after removal of excess surface water using Whatman filter paper for 5 s. The SP was calculated using Equation 1.[9]

\[
SP = \left( W_w - W_d \right) / W_d \times 100 \quad \text{Equation (1)}
\]

Where \( W_w \) is the swollen weight of CS, and \( W_d \) is the dry weight of scaffold sample.

**Enzymatic degradation studies**

Stability of CSs was studied using enzymatic degradation. CSs were placed in pH 7.11 enzymatic solution containing 2 mg/ml of lysozyme and in pH 7.4 in phosphate buffered saline (PBS) as a control sample at 37°C separately. Medium was replaced with fresh one on every alternate day. At specific time intervals 7th and 14th day, scaffolds were taken out from the solutions and rinsed thrice with double distilled water to remove salts. Scaffolds were immersed in 100% ethanol for 2 h and dried for 1 day at room temperature before measuring the weight. The percentage WL (% WL) of scaffolds was calculated according to Equation 2.

\[
% \text{WL} = \left( \left( W_i - W_f \right) / W_i \right) \times 100\% \quad \text{Equation (2)}
\]

Where \( W_i \) is the initial dry weight of scaffold and \( W_f \) is the weight of dry scaffold after incubation in PBS or enzymatic solution.

**Biocompatibility studies**

**MTT assay**

Cytotoxicity of different types of scaffolds was investigated by MTT assay. The scaffolds were kept in 48 well plates and incubated with Dulbecco’s modified eagle medium (DMEM) for 24 h to improve cell adhesion efficiency of scaffolds. After 24 h, 1 ml of fibroblast cell suspension (in DMEM media) at a density of 5 × 10⁶ cells/ml and 20 μl of MTT solution (5 mg/ml) were added to each well. The plate was placed at 37°C with 5% CO₂ atmosphere incubator for 4 h to allow the formation of formazan crystals in live cells. The pale yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by mitochondria of living cells to insoluble purple formazan crystals.[9] All the samples were observed in enzyme linked immunosorbent assay plate reader on day 1, 3, and 5 for cellular viability.

**Cell adhesion analysis**

Normal human dermal fibroblast human dermal fibroblast cell lines were taken as model cell lines for cell adhesion studies of CSs. Fibroblast cells were seeded in T-flasks containing DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C with 5% CO₂, and 95% humidified atmosphere for 48 h. After 4–8 passaging, cells were seeded on scaffold samples. Before cell seeding, the scaffolds were sterilized with 70% (v/v) ethanol for 2 h. Prior to cell seeding, scaffolds were rinsed thrice with milli-Q water and then immersed in PBS buffer for 2 days. The scaffolds were then seeded, with cell suspension of fibroblast at a density of 5 × 10⁶ cells/ml and kept for incubation at 37°C in CO₂ atmosphere for 3 days. After 3 days of culture, scaffolds were lyophilized and observed using SEM for cell growth and adhesion.

**Experimental wound model**

Eighteen female Sprague-Dawley rats were randomly selected and divided into three groups (n = 6). The dorsal side of all experimental animals was shaved and sterilized with 70% ethanol. Excision wound of 2.0 mm × 2.0 mm was created by a punch biopsy from a predetermined shaved area on the dorsal surface of each animal. Animals were housed separately in presterile cages to avoid intra-animal interaction.

**Experimental design**

Animals of Group I were taken as a negative control and left untreated to the open environment to monitor the rate of wound contraction. Group II animals were taken as the reference standard and treated with the wound healing ointment (povidone iodine) while in the third group, all the six animals were treated topically with CS synthesized using 2% of chitosan gel till complete healing. Each formulation was replaced with the fresh formulation on an alternate day to avoid infection. The decrease in the wound area was monitored at specific time intervals. The wound contractions were measured by using the standard method of tracing a wounded margin on a tracing paper[30] and calculating the percentage reduction in the wound area. Area of the wound at day zero was considered as 100% to calculate the percentage reduction in the wound area. The percentage wound contraction was calculated using Equation 3.
Hematological and histopathological analysis

After 14 days of the experiment, all the eighteen animals were sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture in ethylenediaminetetraacetic acid vacinators. Collected blood samples were evaluated for hematological parameters such as hemoglobin, red blood cell, white blood cell, packed cell volume, and differential counts. The granulation tissue was excised on day 14 after wound creation and immersed in 10% buffered formalin for histopathological analysis. For toxicity analysis, kidney and liver tissue were also collected. Tissues were processed by the standard histopathological procedure to observe microscopic changes in skin, kidney, and liver. Briefly, skin, kidney, and liver tissue section were embedded in paraffin and 5 μm sections were cut separately. The sections were deparaffinized using xylene and ethanol and then washed with PBS and permeabilization solution (0.1 M citrate, 0.1% Triton X-100). The deparaffinized sections were stained with hematoxylin and eosin. Masson’s trichrome staining for collagen was also performed on paraffin sections of granulation tissue. The histopathological examination of tissues of animals applied with scaffold was observed under fluorescence microscope (Olympus BX 60) and compared with that of control and reference animals.

Statistical analysis

The statistical significance was determined by using one-way analysis of variance by GraphPad prism 5. Statistical significance value was set at $P < 0.05$.

Results and Discussion

Chitosan scaffold preparation

Different porous CSs having 1–5% of chitosan were found to be white to pale white in color with $33 \pm 1.5$ mm diameter, $5.3 \pm 0.5$ mm thickness, and $115 \pm 3$ mg weight.

Physiochemical characterization

Physical characterization

Physical properties of different types of CSs are shown in Table 1. CSs obtained using 1% chitosan were white in color, had poor mechanical strength, and highly porous surface. Scaffolds obtained using 2% chitosan were white had optimal mechanical strength, spongy texture with smooth and porous surface. Scaffolds obtained using 3.0%, 4.0%, and 5.0% chitosan gel were found to be pale white, harder surface with full of cracks over the surface just after lyophilization. The intensity of cracks on the surface of scaffolds increased with increase in the concentration of chitosan used during synthesis. On the other hand, scaffolds having 1% chitosan were found to possess highly poor mechanical strength and could not be used in wound healing since it was not possible to collect them intact. In view of that CSs synthesized using 2% of chitosan gel were found to have optimal physical characteristics, and hence selected for further studies.

Scanning electron microscopy

Surface morphology of dried CSs samples was evaluated using SEM micrographs as given in Figure 1. The polymeric hydrogel scaffolds synthesized using 1% of chitosan were found to be highly porous with various irregular segregated structures as shown in Figure 1a. Scaffolds synthesized using 2% of chitosan were porous with pore size varying from 80 to 200 μm and pore structure arranged in hexagonal symmetry as shown in Figure 1b. Three percent CSs were found to exhibit lesser porosity with irregular texture with polymeric agglomeration [Figure 1c]. CSs with 4% and 5% chitosan were found to possess limited porosity and irregular structures with broken sections over the surface as shown in Figure 1d and e, respectively. Similar finding was concluded in the physical observation by naked eye that 2% CSs were best by means of physical properties and selected for further studies.

 Atomic force microscopy

Scaffolds synthesized using 2% chitosan were further evaluated using AFM [Figure 1f] for in-depth evaluation of its intramicrostructure and porosity. AFM micrograph of a sample size 200 nm × 5 μm cross section was evaluated. Intramicrostructure of scaffolds showed pores within pore size ranging from 100 nm to 2.5 μm. This type of microenvironment would probably enhance the efficiency of blood clotting, cell adhesion, and wound healing.

Fourier transform infrared spectroscopy imaging measurement

Chemical stability of the CSs was confirmed using FTIR as shown in Figure 2. All the major peaks in CS samples matched with the standard chitosan powder samples, which confirmed the chemical stability and integrity of chitosan in the scaffolds. Figure 2e showed the IR spectra of chitosan powder with major characteristic peaks at 3253/cm and 2873/cm for O-H stretching; 1407/cm, 896/cm, and 656/cm for C-H bending; and 1633/cm, 1547/cm, and C = C stretching at 1069/cm. FTIR spectra of scaffold having 1–4% of chitosan corresponds to the major peaks as 3253/cm and 2873/cm for O-H stretching; 1407/cm, 1547/cm, and C = C stretching at 1069/cm.
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896/cm, and 656/cm for C-H bending; and 1633/cm, 1547/cm, and C = C stretching at 1069/cm as shown in Figure 2a-d, respectively. Uniformity in the major peaks confirmed the chemical stability of chitosan in CS samples independent of its concentration used in synthesis.

Swelling studies

The propensity of scaffolds to retain water is a key aspect of their efficacy to become biocompatible base material for skin tissue engineering.[39] Fluid retention in the microenvironment of the CSs not only affects their morphology and configuration but also cell growth.[40] Swelling characteristics of various types of scaffolds, with different concentration of chitosan, were observed as shown in Figure 3. Chitosan has abundant hydrophilic groups such as hydroxyl, amino, and carboxyl groups, which are able to retain water in its microstructure. It was found that the degree of swelling of CSs increased with increase in their microstructured spaces. As shown in Figure 3, swelling ratio of CS was found to be independent of chitosan concentration in the initial 1 h. However, after 2 h, the minimum swelling ratio of 200% was observed in 1% scaffold as compared to 400% in case of 3% and 4% chitosan samples. Two percent CS showed the highest swelling ratio of 500%, probably due to well-arranged pore structure of the chitosan as observed in SEM analysis.

Enzymatic degradation

Degradation or reabsorption of scaffold by the body is a very important parameter in tissue engineering and wound healing. A scaffold should degrade in a biocompatible manner and provide suitable microenvironment for new tissue formation.[41] Lysozyme is the main enzyme in wound exudation fluid that can hydrolyze the glycosidic bonds present in the chemical structure of chitosan. Therefore, in vitro degradation of CS was studied at physiological pH, with lysozyme concentration corresponding to the concentration in human serum.[42,43] The results are shown in Figure 4. The CSs placed in PBS solution (control) exhibited only 12 ± 2% WL in 14 days. On the other hand, in the case of enzymatic solution, a substantial WL of CSs was observed within the 1st week of experiment and the degree of degradation increased with time. In the presence of enzymatic solution, 2% CSs showed a % WL of 44 ± 3% after 14 days. On the other hand, a scaffold with 3% chitosan concentration had the percentage WL of 24 ± 2.5%. More enzymatic degradation in CS having

Figure 1: Scanning electron microscope and atomic force microscopy micrograph of the chitosan scaffolds. Scanning electron microscope micrograph (a-e) is of scaffold synthesized using 1%, 2%, 3%, 4%, and 5% of chitosan, respectively. Atomic force microscopy micrograph of pertains to 2% chitosan scaffold

Figure 2: Fourier transform infrared spectra (a-d) is of scaffold synthesized using 1%, 2%, 3%, and 4% and spectra (e) is of chitosan powder

Figure 3: The swelling ratio of chitosan scaffold in water distilled water at 37°C for 3 days. One, 2, 3, 4, and 5 corresponds to the respective percentage of the chitosan used in synthesis
2% chitosan is probably due to larger pore size compared to 5% CS. Moreover, the observed decrease in WL percentage of CS, with increase in chitosan concentration, was probably due to decrease in surface area available for enzymatic action.

**Biocompatibility study**

**MTT assay**

As shown in Figure 5, cell viability assay was carried out up to 5 days. After 24 h of cell seeding, cell growth was found better in the case of control sample compared to the test sample. On day 3, cell growth in both the samples reduced but a large population of cells still survived. On day 5, cell viability in the case of CS was found to be 2-fold greater than the control sample. The lesser cell viability on the 1st day in the test sample was probably due to longer time requirement of cellular attachment to CS surface. In the first 24 h, cell proliferation was higher in control as compared to the test samples. On the fifth day, cell proliferation in control sample reduced probably due to confluence attained within the first 24 h and subsequent detachment of dead cells. However, in the case of CS samples, cells proliferated at a slower rate initially as they required time to get attached to the surface of the matrix with a gradual increase in proliferation. The results indicated that chitosan matrix material did not induce any toxic effects on the fibroblast cells has the potential to support cell proliferation and can be a good tissue engineering and wound healing material.

**Cell adhesion analysis**

As can be seen from Figure 6, cellular adhesion is clearly visible over the CS surface. After 3 days of fibroblast cell culture in the CS, significant cell proliferation was observed. The fibroblasts were seen to spread over the surface of the scaffold and showed flattened morphology, confirming significant adherence to the surface. Some outward growth from the surface was also observed in SEM micrographs. It was probably due to interspace cellular growth inside the porous CS hydrogel. The results reaffirmed our findings suggesting that CS has a significant potential to be used as a biomaterial for tissue engineering and wound healing purposes.
Wound contraction

The rate of wound contraction for excision wound model was significantly higher in CS treated wounds as compared to control and standard treatment (using povidone iodine) on all the days of the treatment [Figure 7]. On day 2, 34% healing was observed in the treatment group in comparison to 18 and 28% in control and standard group, respectively. On the 14th day, 100% healing was observed in the case of the treatment group compared to 78% and 51% in standard and control groups, respectively. So whereas, CS application showed a complete wound contraction within 14 days, it was 8 days faster than the normal control group.

Hematological and histopathological analysis of wounded area

The tissue sample was collected for histopathological analysis on day 14 from the wound healed site by punch biopsy method as mentioned in experimental wound model [Figure 8]. The CS-treated group revealed significantly advanced re-epithelization and layering with continued basement membrane in addition to the better organization of collagen bundle [Figure 9]. The CS-treated animals showed reduced congestion, edema, and polymorphonuclear leukocytes infiltration. The histological studies showed an overall early recovery and regeneration. The animals of the standard treatment group were incompletely epithelized and showed unevenness of epidermis. There were greater inflammation and decreased collagen. The control group animals that did not receive any treatment showed non epithelized wound surface. No significant histopathological changes were observed in the kidneys and liver tissue of the rats during toxicity studies when compared to the control groups. Furthermore, all hematological parameters were found within the normal range among control, standard treatment, and test treatment groups, indicating the safety and nontoxicity of CS [Table 2].

Conclusion

The present work describes the fabrication of a series of porous CSs by lyophilization of chitosan hydrogel. Structural morphology, pore size, swelling index, and enzymatic degradation of CSs were evaluated. The optimized 2% CS was found to have a great potential in wound healing. The results were validated by in vitro cell culture findings followed by in vivo wound healing studies in Sprague-Dawley rats. Two percent CS was able to show complete wound contraction within 14 days, which was 8 days faster than the normal control group given no treatment. The histological evaluation of wound on the 14th day in CS-treated group showed more advanced re-epithelization and better organization of collagen bundles as compared to standard treatment and no treatment groups. It can therefore be inferred from the present findings that the developed 2% CS may serve as an important biomaterial in the management of wound healing.

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**Table 2: Profile of hematological parameters in rats**

|                | Control | Standard | Treatment |
|----------------|---------|----------|-----------|
| Hb (g/dl)      | 11.9±0.6| 9.8±0.8  | 10.4±0.7  |
| WBC (%)        | 55.33±1.52 | 58.66±1  | 55±1      |
| RBC (millions/cm²) | 5.98±0.22 | 5.97±0.2  | 5.83±0.2  |
| Hematocrit (%) | 54±1.9  | 57±3.3   | 55.8±2.5  |
| Platelet count (lakh/cm²) | 8.58±3.6  | 8.08±3.7  | 8.9±3.8   |

RBC: Red blood cell, WBC: White blood cell

*Figure 7: In vivo wound healing analysis by excision wound on Sprague-Dawley rats up to 14 days*
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Conflicts of interest

There are no conflicts of interest.

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