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Development of *in situ* gels of nano calcium oxide for healing of burns

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ABSTRACT

Background: Wound closure is the prime objective in the treatment of deep and extensive burns, where the dermis layer is partially or completely destroyed and the inherent capability of spontaneous re-epithelialization is greatly reduced or absent leading to high mortality rate. Scar formation as a result of burn wounds results in aesthetic and functional impairment, causing emotional distress in patients. Induced regeneration of skin could be a solution to improve the quality of life of burned patients.

Materials and methods: Nano calcium oxide (NCO) was prepared by thermal decomposition method. *In situ* gels (ISG) were formulated using micronized xanthan gum and NCO in different concentrations by geometric mixing. The formulations were evaluated for their particle size, surface morphology, elemental composition, clarity, pH, gelling and in *vivo* burn wound healing properties.

Results: The average particle size and polydispersity index of NCO was found to be 345.3 nm and 0.23 respectively. Three formulations were prepared, each containing varied concentrations of NCO (12.5, 25 and 50 ppm). The SEM analysis of the formulation exhibited particle sizes ranging from 1 nm to 468 nm and EDX characterization showed intense peaks of calcium and oxygen. The percentage of wound size reduction was significantly increased in wounds treated with prepared *in situ* gels in contrast to commercial product (calcium alginate dressing) and control (untreated) group. With an increase in concentration of NCO, the rate of healing also increased. *In situ* gels containing 50 ppm of NCO exhibited a better rate of wound healing in comparison to other formulations, within a period of 15 days. Histopathological analysis indicated that *in situ* gels had better wound healing properties.

Conclusion: *In situ* gels of NCO were developed as a formulation for effective treatment of burns and to aid in skin regeneration.

1. Introduction

Burn injuries are among the most challenging ones to manage as significant fluid loss and extensive tissue damage, resulting from deep wounds, can impair multiple vital functions performed by skin. Local tissue damage caused by wound infection is a common complication, while systemic inflammatory and immunological responses might lead to a higher predisposition to fatal sepsis and multiple organ failure. In such cases, timely medical intervention is imperative to reduce the mortality rates associated with burn injuries [1].

Minor burn injuries can be managed by appropriate first aid and good dressings to obviate the need for specialist review or surgery. In most cases of massive injuries, the impact of scar tissues on the body is devastating and only plastic surgery procedures can reduce the deformity and/or improve the appearance of the tissues [2,3]. Despite the use of a wide range of wound dressings and topical medications, wound healing is still a challenge as wound management is mainly restricted to wound repair rather than restoration of the tissue (tissue regeneration).

Abbreviations: CaO, calcium oxide; ISG, *in situ* gel; NCO, nano calcium oxide; NC, negative control; ppm, parts per million; PC, positive control; SEM-EDX, scanning electron microscopy and energy dispersive X-ray microanalysis; SD, standard deviation; DLS, Dynamic Light Scattering

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Therefore, there is a need for effective wound therapies to provide patients with long-term relief and scarless wound healing [4,5].

Wound healing is a calcium-mediated process and calcium is one of the most important ions involved in the process of wound healing [6]. Calcium influx into cells regulates inflammatory cell infiltration, fibroblast proliferation and keratinocyte migration. It maintains normal homeostasis of skin, regulation of keratinocyte proliferation and differentiation. The release of ionized calcium into the wound bed promotes cell proliferation and subsequent enhanced wound healing. Thus by increasing the concentration of ionized calcium in the wound bed, the process of healing can be accelerated [7,8]. Studies have shown that Calcium oxide (CaO) has potential application for bone tissue engineering as it promotes cell viability and osteogenic differentiation. It also has significant antimicrobial and antifungal activities and serves as a root canal filling material in dentistry as it aids in mineralization, pulpal and periodontal tissue healing process. The important role of CaO on tissue biological response is primarily due to its conversion to Calcium hydroxide and subsequent chemical dissociation into calcium and hydroxyl ions [9–16] and the presence of CaO in nano form can improve its bioavailability at the site of application [17]. Traditionally, Lime Liniment (Linimentum Calcis) a mixture of lime water (dilute aqueous solution of calcium hydroxide) and olive oil/linseed oil has been used in the treatment of burn wounds for its antibacterial and wound healing properties [18–21] and studies carried out on mice have shown that they are beneficial in treatment of third-degree burn wounds compared to routinely used silver sulfadiazine therapy [22].

The present study focuses on the development and characterization of *in situ* gels (ISG) of nano calcium oxide (NCO) that could effectively deliver ionized calcium to the site of burns to aid in healing and assist skin regeneration, which was evaluated using a rat burn model.

2. Materials and methods

Micronized xanthan gum (200 mesh) was purchased from Shakti enterprises, India. Citric acid was obtained commercially from Thermo Fischer Scientific India Pvt Ltd., India. Fluid Thioglycollate medium and Soyabean Casein Digest medium were procured commercially from Himedia laboratories Ltd., India. All other reagents and chemicals used were of analytical reagent grade.

2.1. Preparation and characterization of nano calcium oxide (NCO)

NCO was prepared by thermal decomposition of calcium nitrate in a muffle furnace maintained at 450 °C. The particles were then characterized by ZetaPALS.

2.1.1. Determination of particle size and polydispersity

Particle size and polydispersity index of NCO was determined using NanoBrook ZetaPALS (Brookhaven Instruments Corp., USA) based on the principle of Dynamic Light Scattering (DLS). The data was analyzed with ZetaPALS Particle Sizing Software Ver.5.23. Dilute suspension of NCO in methanol was sonicated for 45 min before measurement.

2.2. Formulation of in situ gels (ISG)

10 mg of NCO was mixed with 100 g of micronized xanthan gum by geometric mixing method to obtain a stock mixture containing 100 ppm of NCO. The stock was then suitably diluted with xanthan gum to obtain mixtures containing 12.5, 25 and 50 ppm of NCO. Three formulations ISG 1, ISG 2 and ISG 3 were prepared, each containing 12.5 ppm, 25 ppm and 50 ppm of nano calcium oxide respectively, along with micronized xanthan gum (Table 1).

Table 1
Formulation of *in situ* gels of nano calcium oxide.

Ingredients	Formulations		
	ISG 1	ISG 2	ISG 3
Nano Calcium oxide stock added (g)	2.5	5.0	10
Micronized xanthan gum (g)	17.5	15.0	10
Total quantity(g)	20	20	20

2.3. Evaluation of in situ gels

2.3.1. Scanning Electron Microscopy and Energy Dispersive X-ray microanalysis (SEM-EDX)

Particle size and surface morphology of the formulations was determined by SEM and the elemental composition analyzed by EDX (Carl Zeiss AG - ULTRA 55, Germany). This is a combined analytical technique used to visually capture and represent a detailed image of the substance while quantifying the elemental composition of the sample [23]. The samples were affixed to aluminium stubs with double sided adhesive tape and sputter coated with a thin layer of gold in an argon atmosphere prior to imaging. Micrographs at different magnifications were recorded to study the morphological and surface characteristics of the particles.

2.3.2. Clarity

The clarity of the formulations after gelling was determined by visual observation against a black and white background.

Clarity was coded as + Turbid; + + Clear; + + + Very clear/glassy

2.3.3. Gelling properties

The gelling properties of formulations were assessed by visual inspection, by addition of the powder into a petri plate containing few millilitres of water, maintained at 32 − 34 °C [24].

The gelling capacity was graded as

+ No gelation

+ + Immediate gelation and remains for few minutes

+ + + Immediate gelation and remains for extended period of time

2.3.4. pH

pH of all the formulations was determined using digital pH meter (Model ATC Chemlabs, India). The measurements were carried out in triplicate and average values were calculated.

2.4. Sterilization of Formulations

Since the formulations are intended to be used on open wounds, sterility is essential. The formulations were sterilized by gamma irradiation at a dose not less than 25 kGy (Microtrol Sterilization Services Pvt Ltd., India).

2.4.1. Sterility Test

Sterility test was performed to ascertain the sterile nature of formulations. The sterilized formulations were incubated for not less than 14 days at 30 − 35 °C in Fluid Thioglycollate medium to detect the presence of bacteria and at 20–25 °C in Soyabean Casein Digest Medium to detect the presence of fungi. Microbial growth was determined readily by visual examination [25].

2.5. In vivo Burn Wound Healing Studies

Female Wistar Albino rats weighing approximately 150 − 225 g were used to evaluate the *in vivo* burn wound healing efficacy of *in situ* gels. The protocol of the study was performed in accordance with the European community guidelines as accepted principles for the use of experimental animals and all the experiments were performed after

Table 2
Grouping of animals for burn wound model.

Group	Treatment given	Number of animals
1	Control (untreated)	6
2	Standard (DynaGinate)	6
3	ISG 1	6
4	ISG 2	6
5	ISG 3	6
Total number of animals used		**30**

Fig. 1. Fabricated thermostat assisted device for burn creation.

obtaining the approval of Institutional Animal Ethical Committee (IAEC) of Faculty of Pharmacy (IAEC Ref No: XIX/MSRFPH/M-06/07.11.2017). The rats were fed with standard pellet diet and water *ad libitum*. During the experiment, the rats were maintained under a controlled environmental condition with 12 h of light and dark cycle. 30 rats were randomly divided into five groups (n = 6 per group) (Table 2).The rats were anaesthetized by i.p injection of a mixture of ketamine hydrochloride (60 mg/kg) and lignocaine (16.5 mg/kg). The dorsal hair was carefully shaved with an electric razor and the skin was sterilized with 70 % ethanol. The wound site was marked and divided into two sections. Second degree burn wounds (partial thickness burns) were inflicted with a thermostat assisted device, indigenously fabricated on the lines of models described earlier [26,27], with certain modifications. The device had a round copper base of diameter 2.2 cm, capable of achieving a temperature range of 50 − 200 °C when heated electrically (Fig. 1). A digital thermocouple type temperature sensor was placed 2 mm above the heated copper base to monitor its temperature. The device was heated for 3 min to obtain a scalding temperature of 120 °C and held in contact with the skin of the rats for 5 s to create partial thickness burns measuring 2 cm in diameter [28–31]. Immediately after each burn infliction, the wound was cooled with gauze embedded in isotonic saline at 22 °C [32]. All wounded rats were separately maintained in individual cages and the formulations applied every 48 h. The wound healing activity of *in situ* gels and commercial product (DynaGinate) treated animals were compared with control animals. On the 3rd, 6th, 9th, 12th, and 15th day post wounding, the wound size was measured using Vernier caliper. Percentage size reduction of each wound was calculated using the following equation.

$$\%\,\text{Wound size reduction} = \frac{\text{Area of wound on initial day} - \text{Area of actual wound contracted}}{\text{Area of wound on initial day}} \text{x } 100$$

2.5.1. Histopathological analysis

After 15 days of treatment, animals were anaesthetized by i.p injection of a mixture of ketamine hydrochloride (60 mg/kg) and lignocaine (16.5 mg/kg). The healed skin specimens were carefully trimmed and stored in 10 % v/v neutral buffered formalin. Sections of 3 − 4 µm were prepared by embedding with paraffin and stained with hematoxylin-eosin for microscopic examination. The sections were evaluated in terms of collagen formation, fibroblast proliferation, keratinisation and epithelisation [33]. In addition, angiogenesis, congestion, edema, epithelialization, fibroblast proliferation, intensity and extent of inflammation (cell infiltration), mononuclear and/or polymorphonuclear cells, necrosis, ulceration, neovascularization and the pattern of collagen depositions in the dermis were qualitatively evaluated.

2.5.2. Statistical analysis

All data were expressed as mean ± S.D. The obtained results were interpreted statistically using ANOVA Instat graph pad software and compared using Dunnet test to determine the probability value. $p < 0.05$ was considered significant. All statistical analysis was performed using GraphPad Prism version 5.1.

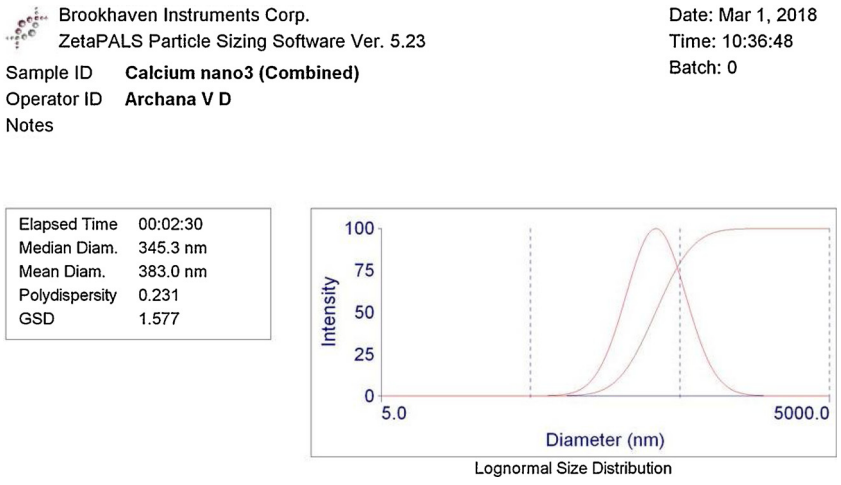
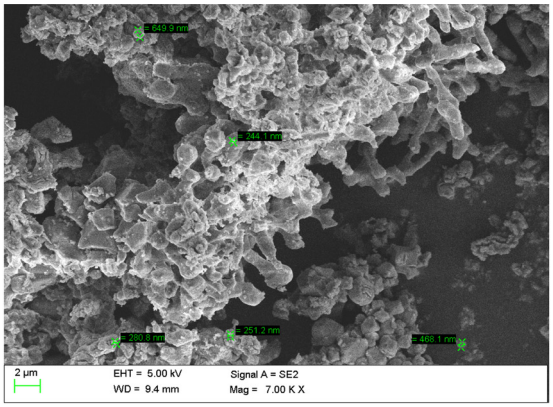
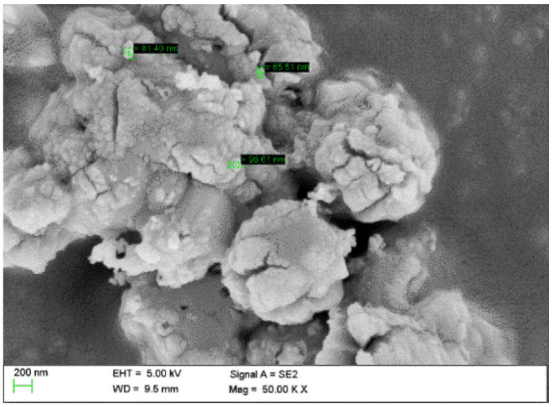


Fig. 2. Particle size distribution and polydispersity of NCO.





Element	Weight %	Atomic %
O	43.44	65.80
Ca	56.56	34.20

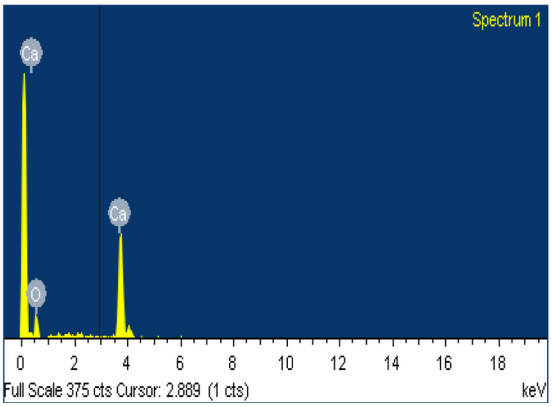


Fig. 3. SEM micrographs and EDX analysis report of *in situ* gels of NCO.

3. RESULTS

3.1. Characterization of Nano Calcium oxide

3.1.1. Determination of particle size distribution and polydispersity

The average particle size was found to be 345.3 nm and the average polydispersity index value 0.23. (Fig. 2).

3.2. Formulation and Evaluation of in situ gels of NCO

Three formulations were prepared, each containing varied concentrations of nano calcium oxide and micronized xanthan gum.

3.2.1. Scanning electron microscopy and energy dispersive X-ray microanalysis (SEM-EDX)

The SEM images of the samples at 7KX and 50KX magnifications as well as the EDX spectra and analysis report is shown in Fig. 3. The particle size of NCO particles ranged from 1 nm to 468 nm and EDX analysis showed intense peaks of calcium and oxygen.

3.2.2. Clarity, gelling capacity and pH

All the formulations were clear and exhibited immediate gelation that remained for an extended period of time (Table 3). The pH ranged from 7.17-7.34.

Table 3
Clarity, gelling capacity and pH.

Formulation Code/Parameter	ISG 1	ISG 2	ISG 3
Clarity	+ +	+ +	+ +
Gelling capacity	+ + +	+ + +	+ + +
pH	7.34	7.28	7.17

3.3. Sterility Test

Sterility testing was carried out by direct inoculation method. After 14 days of incubation at 30 − 35 °C Fluid Thioglycollate medium and Soyabean Casein Digest medium, the formulations showed no microbial growth whereas the positive controls (containing *Staphylococcus aureus* and *Candida albicans*) showed turbidity. (Fig. 4a and b)

3.4. In vivo Wound Healing Studies

3.4.1. In vivo burn wound healing

At the end of the treatment period, all treated groups exhibited an efficient keratinocyte migration and acceleration in re-epithelialization process. There was no indication of necrosis, inflammation or hemorrhage on the animals and all the rats survived throughout the study period. The observed healing activity showed best results with ISG 3 with 99.10 ± 0.22 %. All tested formulations as well as the standard were significant in comparison to the control (Table 4; Figs. 5 and 6). The wounds appeared to be healed and completely sealed 9 days post wounding (Fig.7).

3.4.2. Histopathological studies

The results revealed that control group showed damaged dermis with epidermal detachment, scabs formation of necrotic tissue remnants, hyperemic vessels. Hair follicles, sebaceous glands and phagocytic cell infiltration were not observed (Fig. 8). The tissue section showed densely inflamed connective tissue with chronic inflammatory cells between the collagen fibers, indicating incomplete wound healing. The epidermis close to the wound showed lining stratified squamous epithelial cells with thickened edges and keratin layer covering the surface. There was formation of greater extracellular matrix and collagen fibres in the dermis with infiltration of inflammatory cells.

Group treated with DynaGinate dressing showed epidermis with

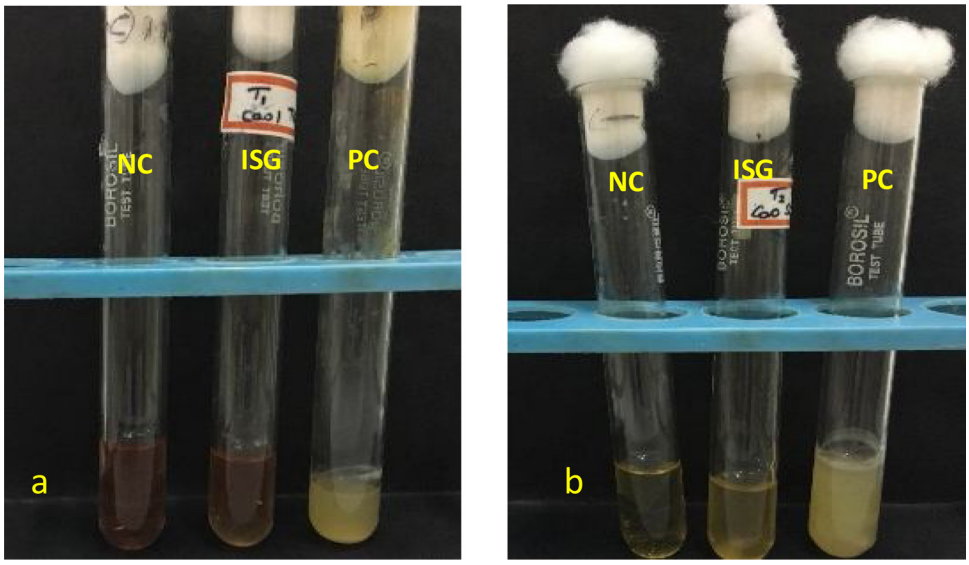


Fig. 4. Sterility testing of formulations a) Testing of bacteria b) Testing of fungi where NC: Negative control; ISG: *in situ* gel; PC: Positive control.

wound area covered with normal stratified squamous epithelial cells containing the five layers namely stratum basale, stratum granulosum, stratum spinosum, stratum lucidum and stratum corneum. Dermis showed numerous collagen fibres with extracellular matrix and formation of new blood vessels with red blood cells.

Formulation treated groups showed thickened epidermis with five layers of stratified squamous epithelium (namely stratum basale, stratum granulosum, stratum spinosum, stratum lucidum and stratum corneum) over the wound area indicating wound healing. Dermis layer showed normal architecture of collagen fibres with sweat, sebaceous glands and hair follicles similar to standard group. Epithelialization and fibroblast infiltration was found in all groups.

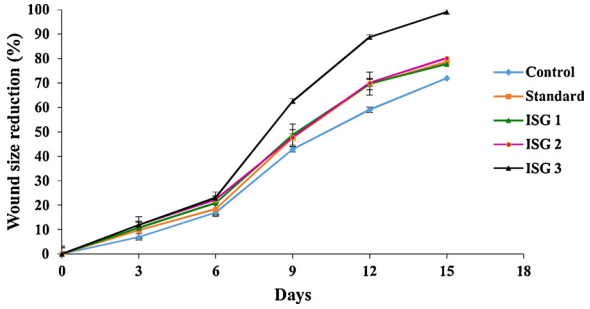


Fig. 5. Size reduction of the burn wound treated with *in situ* gel formulations and standard product in rat dorsum. Each value represents the Mean ± SD (n = 6).

4. Discussion

In the present study, burn dressings were prepared by simple admixture of nano calcium oxide and micronized xanthan gum. The powder mixture forms an *in situ* gel instantaneously on contact with burn wound exudate. Nano Calcium oxide prepared by thermal decomposition was evaluated for its particle size and subsequently used in the formulation of *in situ* gels. The results of particle size determination of NCO indicated that the particles were monodispersed with a narrow range of homogenous distribution. On exposure to moisture, *in situ* gels containing 12.5, 25 and 50 ppm of NCO formed clear gels immediately. The results from SEM analysis indicated the presence of pores on the surface on *in situ* gels. The porosity can be attributed to the presence of xanthan gum, which will produce an optimal level of moisture on the wound, thus creating a favorable environment for wound healing [34]. Burn wounds treated with *in situ* gels containing 50 ppm of nano calcium oxide (Formulation ISG 3) showed significant wound healing in comparison to marketed (DynaGinate) and all other formulations. The wound closure and epidermal regeneration was intensified with the increase in concentration of NCO and lowering of pH of formulations. Similar studies carried out on mice inflicted with third degree burns showed that the presence of calcium hydroxide improved the rate of healing, induced faster vascularization, granulation tissue growth, maturity and epithelialization [35,36]. pH also plays a key role in wound healing and studies have shown that acute and chronic wound environment progresses from an alkaline to a neutral state and then to acidic state during healing [37–44]. The results are in line with studies demonstrating that decrease in pH of topical wound medications promotes contraction, epithelialization and scar formation of the wounds [45]. All the *in situ* gel treated groups showed clearly developed epithelialization, angiogenesis and hair follicles compared to other groups. The tissue was composed of dense collagen fibres, fibroblasts with

Table 4
Data for statistical analysis of wound healing activity.

Group	Day 3	Day 6	Day 9	Day 12	Day 15
Control	6.97 ± 2.72	17.05 ± 1.30	42.85 ± 1.13	59.06 ± 1.093	71.95 ± 1.05
Standard	9.82 ± 2.53^{ns}	18.37 ± 3.12^{ns}	47.74 ± 3.04^{ns}	69.54 ± 3.06**	78.70 ± 2.36**
ISG 1	10.78 ± 2.70^{ns}	20.87 ± 4.44^{ns}	48.61 ± 4.54 *	69.71 ± 4.69**	77.82 ± 4.70**
ISG 2	11.92 ± 3.15*	22.29 ± 1.53*	47.86 ± 1.37^{ns}	70.19 ± 1.43**	80.30 ± 1.43**
ISG 3	11.95 ± 2.38*	23.11 ± 1.57*	62.63 ± 0.58**	88.87 ± 0.22**	**99.10 ± 0.22****

The values are expressed as Mean ± SD (n = 6), where ns – (non-significant) $p > 0.05$.
* $p < 0.05$.
** $p < 0.01$, when compared to experimental control.

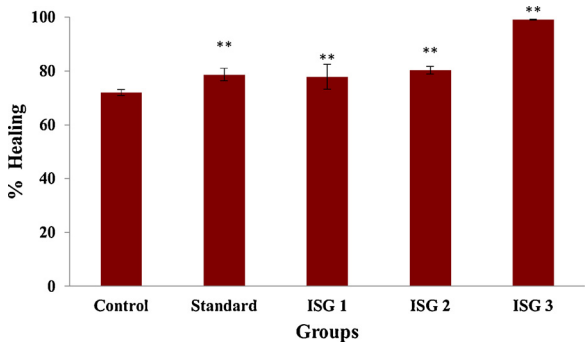


Fig. 6. Percentage rate of healing of burn wound treated with *in situ* gel formulations and standard product in rat dorsum. Values are expressed as mean ± SD (n = 6). One way ANOVA followed by Dunnet test where ns - (non-significant) $p > 0.05$, * - $p < 0.05$, ** - $p < 0.01$, in comparison with control.

round to oval nuclei and thin walled blood vessels. It also showed dense fibrous tissue with thick collagen bundles, fibroblasts and scattered inflammatory cells and the appearance was almost identical to that of normal tissues.

The observations of histopathological analysis further strengthen the results obtained from wound contraction data. However, the lack of a comparison between the *in situ* gel formulation and a standard reference product may be regarded as a limitation to our investigation.

5. Conclusions

In situ gels have an advantage of ease of application, as there is no pressure exerted on the burn wound, avoiding discomfort to burn patients. It also has an added advantage of obviating the need for removal of dressings. Formulations are fine powders, which when sprinkled on the burn/wound exudate forms an *in situ* gel at the site of application. The presence of micronized xanthan gum aids in faster gel formation and maintains the formulation in fine state. In conclusion, a wound care system that is dermatologically acceptable for effective treatment of burns has been developed successfully.

Further investigations can be conducted in human volunteers and higher concentrations of nano calcium oxide can be explored for assessing wound healing potential. The effect of nano calcium oxide in combination with anti-infective agents can also be investigated.

Consent for publication

We certify this manuscript has not been published elsewhere and is not submitted to another journal. All authors have approved the manuscript and agreed with submission to Journal of Wound Medicine.

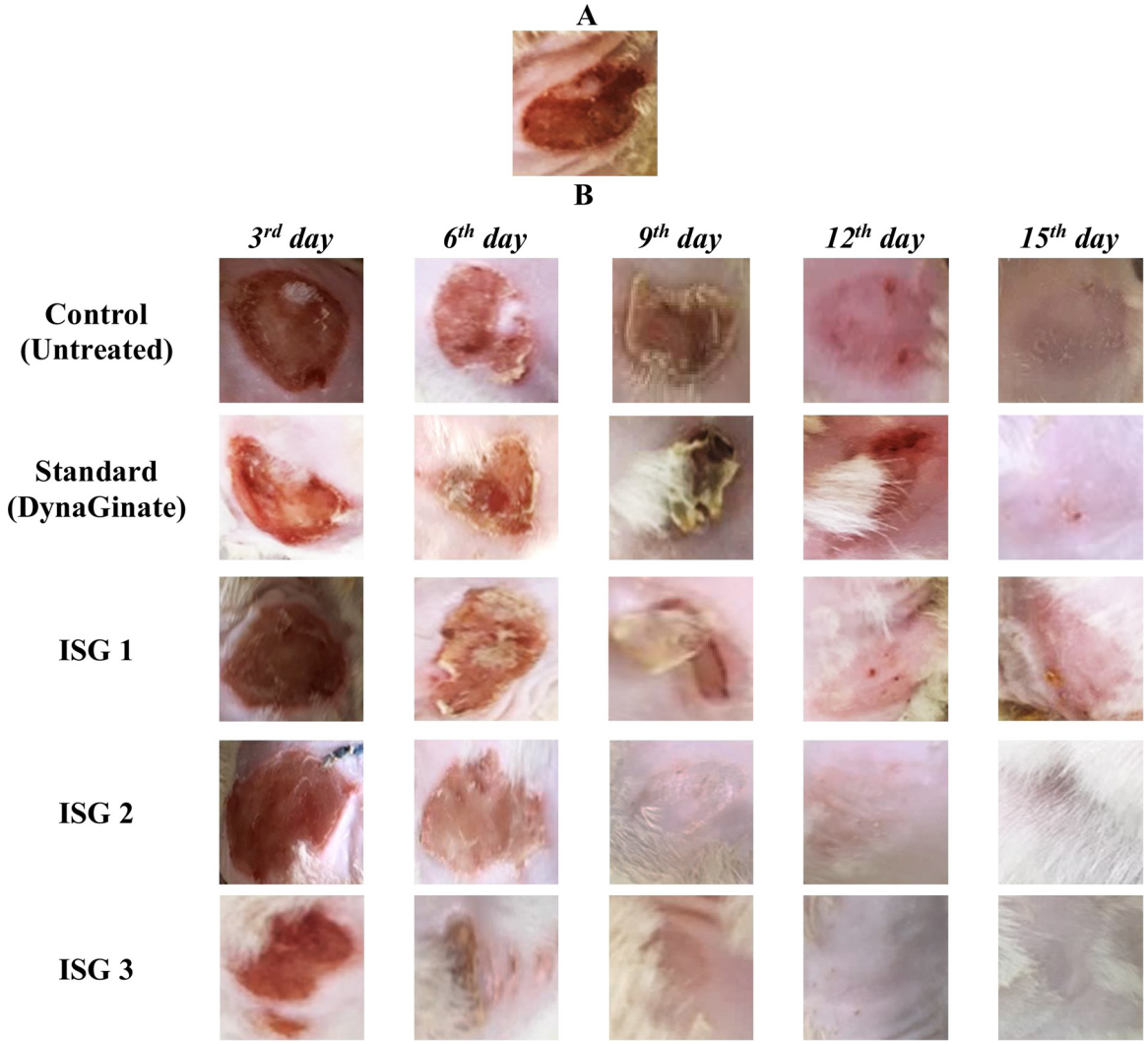


Fig. 7. Representative images of A) Burn wound created with fabricated heating device. **B)** Treatment of burn wound with control, standard and *in situ* gel formulations upto 15 days post wounding. The scale bar for all images is 10mm.

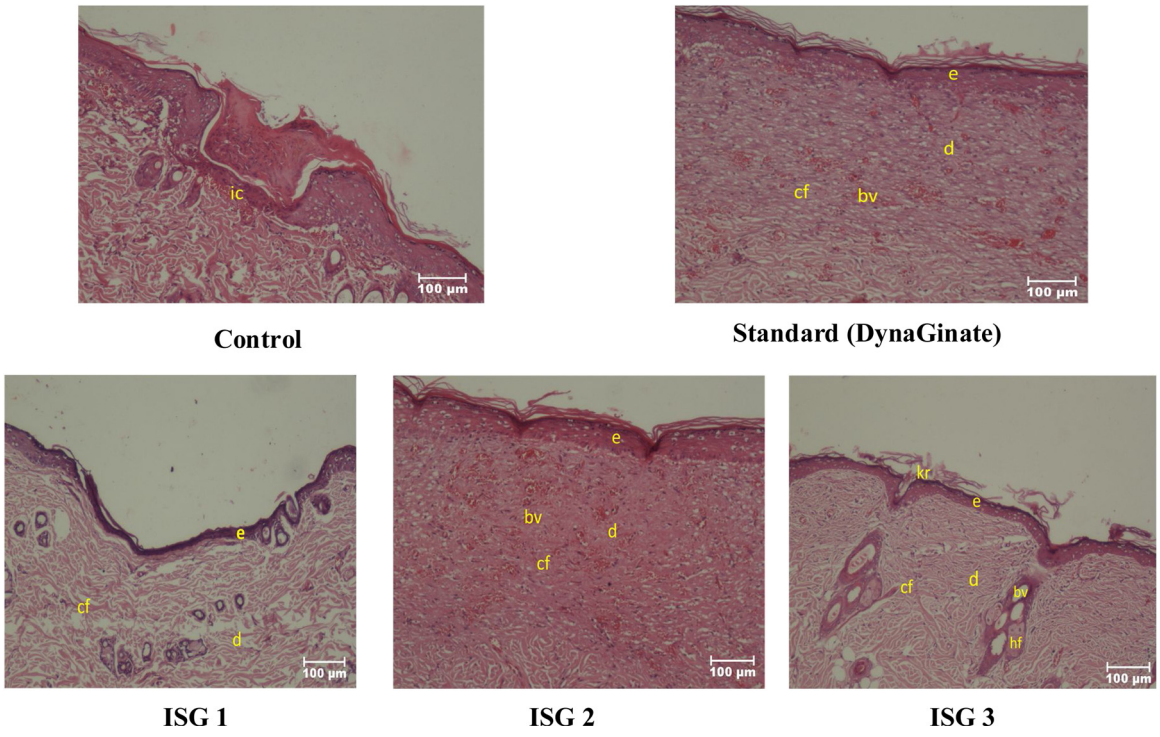


Fig. 8. Photomicrographs showing the section of skin tissues with H& E staining at day 15 for control, standard and *in situ* gel treated groups. The figure shows; e: epidermis; d: dermis; Kr: keratinocytes; cf: collagen fibres; hf: hair follicles; bv: blood vessels; ic: inflammatory cells.

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Declaration of Competing Interest

The author(s) declare that they have no competing interests

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