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Biopolymeric nifedipine powder for acceleration of wound healing

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ABSTRACT

Nine biodegradable polymeric powders of chitosan; with or without gelatin; containing nifedipine (NF), were prepared via spray drying for acceleration of wound healing. The angle of repose of powders ranged from 30° to 40° for F_3 and chitosan/gelatin (C_2), respectively. Upon spray drying, the mean particle size (PS) of chitosan was greatly reduced from 294 to 3.4 μm, while all formulae showed a PS near 3 μm. Specific surface area of the powders ranged from 0.06×10^5 to 2.03×10^5 cm^2 g. Powders exposure to glutraldehyde (GA) vapors gave smaller particles with higher densities with F_4 showing a mean PS of 0.4 μm. NF dissolution pattern in PBS, was highest from F_2 with 33% released after 30 min while F_3 showed only 4.5% NF released after the same period. Exposure to GA showed a reduction in NF release especially with F_5 showing 30% release after 3 h. Tensile strength of 12 days-post incision wound in rats showed a maximum value of 4.7 kg cm^{-2} for F_3 compared with 3.3 kg cm^{-2} for the control. Excision wounds treated with F_3 also showed a fully developed epithelium with normal keratinization, and an average wound contraction of 99% compared with 79.5% for the control.

1. Introduction

The process of skin wound healing is represents a dynamic and well-ordered biological process (Santoro and Gaudino, 2005). This process occurs in different phases: inflammation, proliferation and maturation. It is characterized by distinct but overlapping events such as blood vessels disruption and extravasation, coagulation, infiltration of inflammatory cells, deposition of granulation tissue and collagen, epithelialization, wound area contraction and tissue remodeling (Baie and Sheikh, 2000; Jurjus et al., 2007). The initially randomly distributed collagen fibers become cross-linked and aggregated into fibrillar bundles, which gradually provide the healing tissue with increasing stiffness and tensile strength (Bailey et al., 1975).

Nifedipine (NF) is a dihydropyridine calcium channel blocker, which blocks the trans-membrane influx of calcium ions into muscle cells causing dilatation of blood vessels and decreased total peripheral resistance leading to decreased systemic blood pressure. Interestingly, systemic use and topical application of NF has been reported in few publications to promote wound healing. In a previous study it was demonstrated that NF enhanced skin wound healing as evidenced by increase in tensile strength of 10 days-old granulation tissue in incision wounds (Bhaskar et al., 2005). Likewise, the oral administration of NF enhanced the stability of colonic anastomoses during the first postoperative week due to increased deposition of collagen fibers in the wound area (Ugurlu et al., 2003). In a case report, Torsiello and Kopacki (2000) applied NF powder in a Pluronic–lecithin organogel base to treat a number of patients with difficult-to-heal wounds that are refractory to standard forms of treatment. They observed a decrease in healing time with no adverse effects on applying 80 mg NF powder twice daily. The only effect that has been noted is the dryness of the skin surrounding the wound. Nevertheless, none of these studies evaluated the impact of different formulations with different release rates on the ability of NF to accelerate wound healing or the effect of the selected doses of NF that accelerated wound healing on systemic blood pressure.

Chitosan is a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine (Picker-Freyer and Brink, 2006). Studies on the different biomedical applications of chitosan have been intensified since 1990 due to its low cytotoxicity, antimicrobial activity and excellent biodegradable properties in the human body (Kumar et al., 2004; Elgindy et al., 2010, 2011a). The biopolymer, chitosan, has good hemostatic properties with natural biocompatibility and biodegradability that render it suitable for wound management. Consequently, chitosan gained approval in the United States and Europe for use in bandages. Experimentally, chitosan hemostatic products have been tested by the United States Marine Corps (USMC) to quickly stop bleeding thus increasing the survival of

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pigs subjected to lethal arterial wounds by reducing blood loss (Pusateri et al., 2003). Similarly, Xie et al. (2008) reported that chitosan hemostatic dressing can be used in laparoscopic partial nephrectomy procedures as a primary or supplemental material for controlling parenchymal hemorrhage and sealing the renal collecting system in the animal model used.

Dai et al. (2009) reported that chitosan acetate bandage effectively controlled the growth of bacteria in burns and prevented the development of systemic sepsis. Recently, Zeng-xiao et al. (2010) prepared chitosan/silk fibroin composite nano-fibrous membranes for wound healing applications. An interesting new matrix was proposed by Liu et al. (2004), that crosslinks chitosan, gelatin and hyaluronic acid, generating a mechanically resistant porous matrix able to support fibroblast growth.

Surface area and porosity are important functional properties of the powders that can affect drug release (Elgindy et al., 2011b). Ehtezazi et al. (1999) found that decreasing porosity of the poly (lactic acid) microspheres decreased the release rate of the active ingredients and a long-term release was observed. Klose et al. (2006) reported that high porosities of poly (lactic-co-glycolic acid) based microparticles do not only lead to increased drug mobility, but can also alter the underlying mass transport mechanisms.

The process of wound healing has two components, the formation of new tissues and the protection from microbial invasion during the healing process. Nifedipine is expected to promote the former while chitosan is known to offer the later. The aim of the present work is to verify the idea that the combination between NF and chitosan as a spray dried powder would prove a superior treatment for rapid wound healing if given concomitantly in one formula. Therefore the biomedical evaluation of the proposed formulae was tried in order to determine their ability to accelerate wound healing.

2. Materials and methods

2.1. Materials

Nifedipine (NF) was kindly supplied by El Amriya Pharmaceutical Company (Alexandria, Egypt). Chitosan (CS, 150 kDa), gelatin, hamotoxylin and eosin, Masson trichrome stain were obtained from Sigma–Aldrich (St. Louis, USA). All other chemicals were of analytical grades and used without further purification.

2.2. Preparation of the powdered samples

NF (400 mg) was dissolved in 100 mL of isopropanol/water mixture (1:1) in a beaker wrapped with aluminum foil. The drug solution was added to 400 mL of chitosan (1%, w/v) in 1% (v/v) acetic acid either alone or containing 1% (w/v) of gelatin. In formula F_3, the drug was directly suspended in the prepared chitosan solution. The solutions were stirred with a magnetic stirrer during feeding to a Minispray Dryer B 290 (Buchi, Switzerland). Inlet temperature was adjusted at 135 °C and the outlet at 87–90 °C with an air flow rate of 439 L/h. The prepared powders, containing 10% (w/w) NF, were stored in a desiccator till further testing.

A certain weight of formulae F_1, F_2 and F_3 was exposed in a desiccator to glutraldehyde fumes for 12 h at room temperature for cross-linkage of the particle surface. The powders were then exposed to a slight air current for 24 h for removal of the aldehyde traces. The composition of each preparation is given in Table 1.

2.3. Characterization of the powders

2.3.1. Morphology observation

The surface topography of the prepared powders was examined using a JEM-100S scanning electron microscope (JOEL, Japan). Samples were mounted on metal stabs using double-sided adhesive tape, coated with approximately 10–20 nm gold film for 20 s under vacuum using a sputter coater and then examined. Scans were performed at an acceleration voltage of 10 kV.

2.3.2. Powder surface area and particle size

The powder particle size (P.S.) and specific surface area (S.S.A.) were measured using NOVA 1000 series (Quantachrome, USA) Laser particle size analyzer. Samples were dispersed in 100 mL distilled water and agitated at 100 rpm prior to measurement.

2.3.3. Powder flowability and bulk density

The flow properties of the powders were measured using a Flowability Tester, BEP2 (Copley, UK). An average of three readings was calculated. The bulk volume was obtained by pouring an amount of one gram of the powder in a 10 mL graduated cylinder, the volume of powder was recorded and the bulk density was calculated. Tapped density was measured using a Tapped Density Tester, JV1000 (Copley, UK). Each experiment was repeated in triplicate and the average was calculated.

2.3.4. Moisture uptake

An accurately weighed amount of three selected formulae (F_1, F_2, and F_3) was placed in a watch glass in a desiccator filled with water at room temperature. After 24 h the powders were re-weighed to calculate the percent moisture uptake. An average of three readings was calculated for each formula.

2.4. In vitro NF dissolution

The NF content of the prepared powders was assayed spectrophotometrically after powder digestion (25 mg) in 250 mL PBS. The average of three determinations was taken.

Table 1
The composition and physical parameters of the prepared powders (values are mean ± S.D., $n = 3$).

Formula	Composition	Density (g cm^{-3})		e	θ (°)	P.S. (μm)	S.S.A. ($\times 10^5$, cm^2 g^{-1})
		Bulk	Tapped				
C_0	Native chitosan	0.22 ± 0.008	0.28 ± 0.010	22	19 ± 1.0	294.0 ± 6.50	0.06 ± 0.001
C_1	CS	0.19 ± 0.010	0.23 ± 0.008	18	35 ± 1.5	3.42 ± 0.02	–
C_2	CS/G	0.19 ± 0.007	0.24 ± 0.008	21	40 ± 0.5	3.37 ± 0.01	0.70 ± 0.002
F_1	CS/NF	0.19 ± 0.008	0.24 ± 0.009	21	36 ± 0.5	3.54 ± 0.03	1.77 ± 0.002
F_2	CS/G/NF	0.04 ± 0.001	0.06 ± 0.001	33	32 ± 1.0	4.46 ± 0.01	1.87 ± 0.001
F_3	CS/NF susp.	0.20 ± 0.005	0.22 ± 0.006	22	30 ± 0.5	3.55 ± 0.01	1.67 ± 0.003
F_4	CS/NF/GA	0.32 ± 0.010	0.38 ± 0.012	16	39 ± 0.5	0.40 ± 0.01	1.99 ± 0.002
F_5	CS/G/NF/GA	0.12 ± 0.006	0.15 ± 0.008	20	38 ± 0.2	2.96 ± 0.01	2.03 ± 0.004
F_6	CS/NF susp./GA	0.33 ± 0.010	0.38 ± 0.010	17	39 ± 0.3	0.49 ± 0.01	1.89 ± 0.001

e: percent porosity; θ: angle of repose; P.S.: particle size; S.S.A.: specific surface area; CS: spray dried chitosan; G: gelatin; NF: nifedipine; NF susp.: nifedipine suspension; GA: glutraldehyde.

The release of NF from the prepared powder was investigated using the USPXXIV dissolution rate apparatus, Type II (Pharmatest, Germany) with a paddle speed of 100 ± 2 rpm. A known weight of the powder (equivalent to 7 mg drug) was added to 400 mL PBS (pH 7.4) at 35 ± 0.5 °C placed in the dissolution rate umber glass vessels wrapped in foil. At appropriate time intervals, 5 mL samples were withdrawn and immediately replaced with an equal volume of pre-warmed PBS at the same temperature. All samples were filtered through a 0.45 μm membrane filter. The amount of drug released was assayed spectrophotometrically (T80, UV/VIS Spectrometer, PG Instruments Ltd., UK) at 340 nm for NF content after proper dilution with PBS. The percentage cumulative amount of drug released at each time interval was plotted against time. The experiment was carried out in triplicate and the average NF released was calculated. The polymers used showed no interference with the spectrophotometric assay of NF at 340 nm.

2.5. In vivo testing

2.5.1. Animals

Female Wister rats (140 ± 10 g, $n = 72$, Faculty of Pharmacy animal facility, Alexandria, Egypt) were housed individually and randomly assigned to 12 groups (6 rats each) in the present study. The first 6 groups were used for testing the tensile strength of incision wounds, and the next 6 groups were employed for the assessment of % wound area contraction and wound area histology. All experiments were performed in strict accordance with Institutional Animal Care and Use guidelines. Animals were kept under controlled environmental conditions (12:12 h light/dark cycle and room temperature 22 ± 2 °C) and had free access to standard laboratory food and water throughout the study.

2.5.2. Measurement of rat systolic blood pressure

Systolic blood pressure (SBP) and heart rate (HR) were measured before, 24 h and 3 days after inflicting the wounds as well as at the 12th day at the end of the experiment in conscious restrained animals (7.00–10.00 AM), using non-invasive blood pressure (NIBP) system in conjunction with a PowerLab system and Labchart pro 7 software (AD Instrument, Australia). A specialized tail cuff and pulse transducer (Pan Lab, Spain) were employed for blood pressure measurement based on the periodic occlusion of tail blood flow. Animals were trained on the restrainer and tail cuff for measuring the blood pressure for 3 days before commencement of experiment. Four determinations were made each time for each rat, and the means were used for further analysis.

2.5.3. Surgical procedures

The dorsal and lateral areas of the skin of the rats were shaved with an electric animal clipper (Golden A5, Oster, USA). The skin was prepared by scrubbing with dilute solution of cetrimide–chlorhexidine (1:30) followed by 70% ethanol. Wounds infliction and all other surgical procedures performed at the end of the experiments were performed under thiopental sodium (50 mg/kg, intra-peritoneal) anesthesia (Raahave, 1974). Except for the drugs under study, no local or systemic therapy was provided to animals bearing the wounds.

2.5.3.1. Incision wound. Two 3 cm long para-vertebral incisions were made using surgical blade (No. 15) through the entire thickness of skin at a distance of about 2 cm from the midline on each side of the depilated back of the rat. After mopping the wound dry, the powder formula (equivalent to 50 mg NF/kg of rat body weight) was divided and applied inside the wounds. Intermittent sutures were placed 1 cm apart, using a surgical nylon thread and then removed on the 7th day. On the 12th post-wounding day standardized strip specimens (10 mm × 40 mm) were excised and trimmed with a guillotine from the middle of each wound at right angles to the long axis of the wound. The specimens were transferred to a PBS until mechanically tested for tensile strength (TS). Prior to the testing, the width and thickness of the wound strips were measured by a digital caliber (Mitutoyo, Tokyo, Japan). The tensile strength was measured using a texture analyzer (Instron, UK) and calculated by dividing the maximum load at rupture (N) by the cross sectional area of the specimen. The increase in TS is used as a measure of wound healing.

2.5.3.2. Excision wounds. Two circular pieces (each 80 mm^2 in area) with full skin thickness were excised from the dorsal intercapsular regions. The powder formula (equivalent to 2.5 mg NF) was sprinkled on each wound on day zero and maintenance doses equivalent to 1.5 mg NF at days 3, 6 and 9. Wounds were covered with gauze and a porous 3M Micropore™ adhesive tape after each powder application. Wound contraction was monitored by measuring the wound area, planimetrically, every 3 days till the 12th day and calculated as % reduction in wound area. Then the wound half closure time (WC_{50}) was calculated by Litchfield and Wilexon method (Prasad and Dorle, 2006).

On the 12th post-wounding day specimens for histological examination were collected from each group by the full-thickness skin excision of the wound area and fixed in 10% neutral formaldehyde. Wound sections were stained with hamotoxylin and eosin (H&E) as well as Masson trichrome stain and examined microscopically for epithelialization, keratinization and neovascularization (Yeo et al., 2000). The results were visually quantified by numbering the results from 1 to 5, with 5 standing for maximum similarity and 1 standing for least similarity from the normal tissue around the wounded area in untreated wounds (Taranalli and Juppest, 1996). The staining intensity of the collagen fibers with Masson trichrome was graded under × 100 magnification as follows: 1 = completely negative staining intensity; 2 = lower staining intensity; 3 = moderate staining intensity; 4 = slightly higher staining intensity; 5 = considerably higher staining intensity (Kim et al., 2009).

2.6. Statistical analysis

All the values were expressed as mean ± standard deviation (S.D.). The data was statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test with equal sample size. The difference was considered significant when P-values < 0.05 using Graph pad prism, version 3.02.

3. Results and discussion

3.1. Physical characteristics

3.1.1. Scanning electron microscopy

The scanning electron microscopy (SEM) showed a major change in particle shape of chitosan upon spray drying (Fig. 1a and b) where the flaky appearance of chitosan (C_0) had changed to the standard spherical appearance of spray dried products (C_1). Spray dried particles seemed hollow with the characteristic "notch" in the center. The incorporation of gelatin with chitosan (C_2) did not show a significant change in particle shape with the exception of showing more corrugated surface with deeper "notches" (Fig. 1c). The prepared NF formulae showed similar particle shape (Fig. 1d and e) but with a relatively smoother surface with the disappearance of the surface notches indicating more solid and less hollow particles. Formula F_3 (containing NF suspension) showed both spherical (drug-loaded particles) and notched (CS) particles (Fig. 1f). Exposure of the powdered formulae to glutraldehyde (F_4–F_6) gave rise

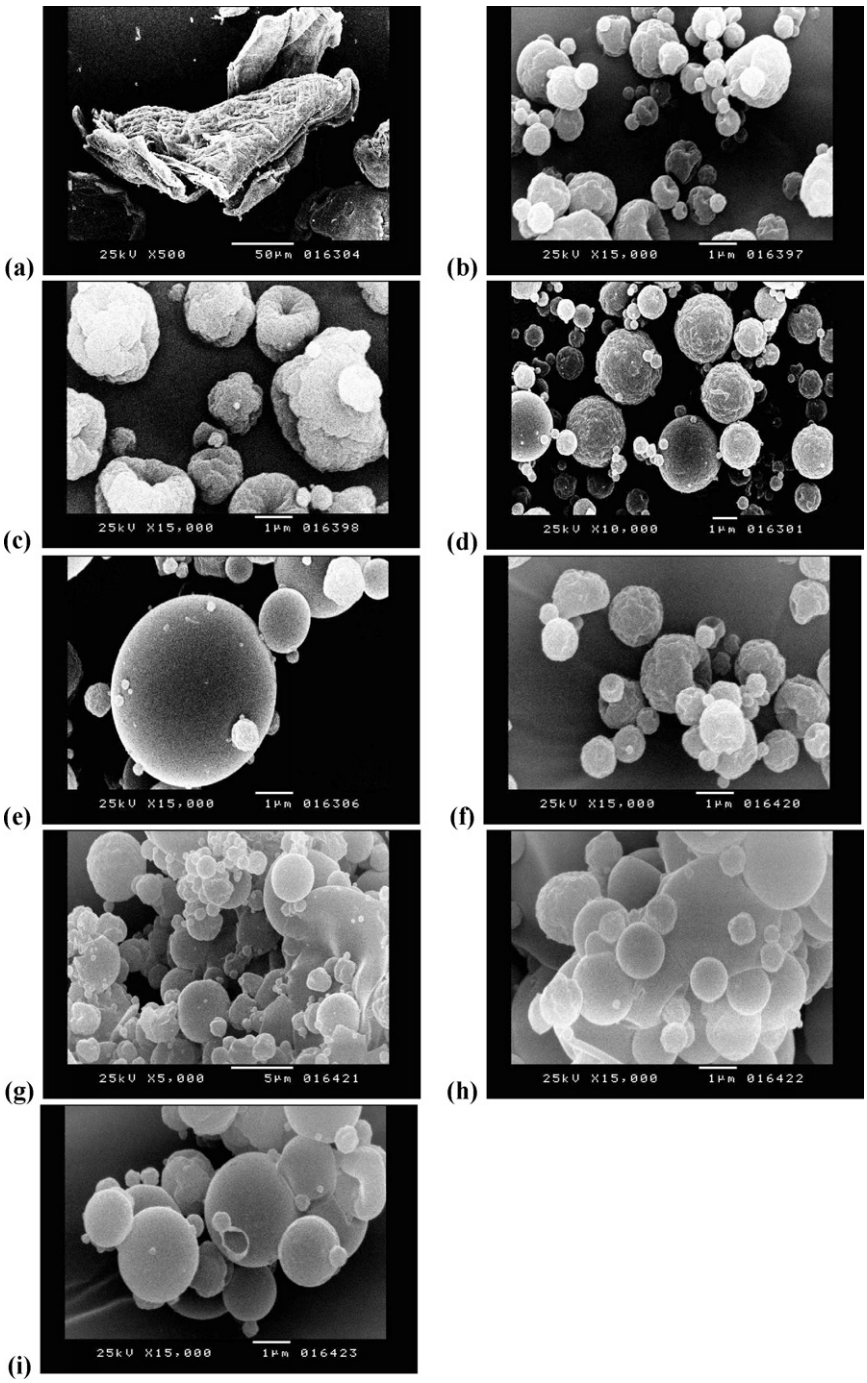

Fig. 1. Scanning electron micrographs for different biopolymeric powdered formulae (Table 1): (a) C_0, (b) C_1, (c) C_2, (d) F_1, (e) F_2, (f) F_3, (g) F_4, (h) F_5 and (i) F_6.

to very smooth-surfaced particle aggregates (Fig. 1g–i) which may be due to cross-linkage of the surface polymer chains.

3.1.2. Powder flowability and bulk density

Studying the flow properties of the prepared powders revealed that native chitosan powder had the best flow with an angle of repose (θ) of 19°, while the flow of the prepared powders was relatively good ($\theta < 45°$) with θ ranging from 30° for F_3 to 40° for C_2. This change in flowability upon spray drying of chitosan may be attributed to the relatively small particle size of the prepared powders that ranged from 3.37 μm for C_2 to 4.46 μm for F_2 compared with 294 μm for the native chitosan (C_0) (Table 1).

The spray dried chitosan (C_1) powder showed a minor reduction in the bulk density compared with the native chitosan (C_0) which may be attributed to the formation of relatively porous chitosan spheres as evidenced by the SEM pictures (Fig. 1). Incorporation of gelatin (C_2) showed no significant change in the density from C_1. Formula F_2 (containing NF/chitosan/gelatin) was very fluffy with very low bulk and tapped densities of 0.04 and 0.06 g cm^{-3}, respectively. This fluffiness was evidenced with the high percent porosity (33%) compared with that of 18% in case of spray dried chitosan, C_1 (Table 1).

Upon calculating the specific surface area, a maximum value was seen with F_2 with a value of 1.9×10^5 cm^2 g^{-1}. The exposure to glutraldehyde (GA) vapors caused "shrinkage" of the particles due

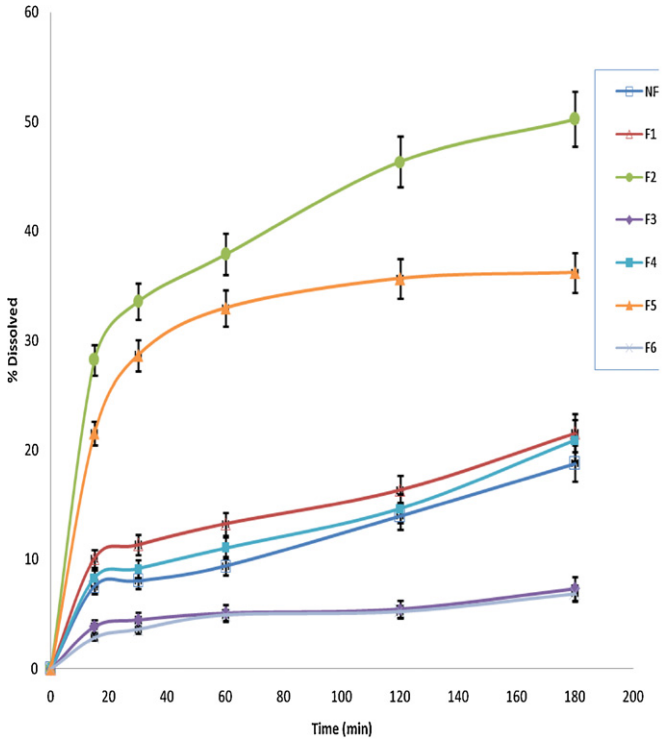

Fig. 2. In vitro nifedipine dissolution for biodegradable powdered formulae (Table 1) in PBS (pH 7.4) at 35 °C.

to cross-linkage of the surface polymer which was reflected in the increased density, specific surface area and decrease of the mean particle size of the three formulae reaching a mean particle size of 0.4×10^5 μm for F_4 (Table 1).

3.2. NF dissolution

The calculated NF content was 10 ± 0.3% (w/w) for all formulae except for F_2 and F_5 it was 5 ± 0.1% (w/w) which represent about 100% of the claimed drug content. Drug dissolution in PBS (pH 7.4) at 35 °C was very low showing about 8% NF dissolved after 30 min. This dissolution pattern was slightly increased in case of F_1 showing about 10% NF released after the same period. Upon incorporation of gelatin (F_2), the drug dissolution was greatly increased showing 33% of the drug dissolved in the first half an hour (Fig. 2).

The relatively rapid NF release from formulae F_1 and F_2 may be due to drug deposition in a very fine form within the prepared powder. The rapid release of F_2, containing gelatin, can be also explained by its calculated large specific surface area (1.9×10^5 cm^2 g^{-1}). After 3 h F_2 showed about 50% drug release compared with only 19% of the native drug, such a release could be expected to increase the systemic side effects of the drug. In order to delay drug release from the powders, NF was suspended in the polymer solution during spray drying (F_3), which gave rise to polymer-coated drug particles that showed only 4.5% NF dissolved after 30 min.

Exposure to glutraldehyde vapors was another method to reduce drug release from all formulae (F_4–F_6) by increasing the barrier properties of the polymeric coat after cross linkage (Fig. 2). These formulae were not selected for in vivo testing to avoid the arguments concerning toxicity of residual aldehyde in the powder. Calculating the in vitro release parameters (Table 2) showed that DE_{60} ranged from 9.94 to 75.04 mg min for F_6 and F_2, respectively. The rate of NF dissolution at 5 min (RDR_5) showed a maximum value of 4.63 for F_2 and a minimum of 0.48 for F_6. The percent NF dissolved after 30 min was 8.03 for native drug with the maximum value of 33.54% for F_2. The calculated values of the time required for 25% NF release ($T_{25\%}$) exceeded 12 h for F_3 and F_6 with the least value of 12 min observed with F_2.

Table 2
In vitro dissolution parameters of NF from biodegradable powdered formulae in PBS (pH 7.4) at 35 °C (values are mean ± S.D., $n = 3$).

Formula	DE_{60}[a]	RDR_5[b]	PD_{30}[c]	$T_{25\%}$[d]
NF	19.30 ± 0.95	1.00	8.03 ± 0.07	246 ± 2.4
F_1	26.45 ± 0.87	1.50 ± 0.02	11.34 ± 0.08	208 ± 1.8
F_2	75.04 ± 1.20	4.63 ± 0.05	33.54 ± 0.14	12 ± 0.4
F_3	12.21 ± 0.17	0.60 ± 0.01	4.46 ± 0.02	779 ± 3.4
F_4	24.71 ± 0.46	1.14 ± 0.04	9.15 ± 0.02	222 ± 1.5
F_5	37.31 ± 0.22	3.97 ± 0.01	28.63 ± 0.12	20 ± 0.7
F_6	9.94 ± 0.12	0.48 ± 0.01	3.57 ± 0.01	770 ± 1.2

[a] DE_{60}: % dissolution efficiency of NF after 60 min.
[b] RDR_5: ratio of NF dissolved from each formula to that of the NF/chitosan mixture after 5 min.
[c] PD_{30}: % NF dissolved after 30 min.
[d] $T_{25\%}$: time required for 25% NF release.

3.3. Moisture uptake

The moisture uptake after 24 h of exposure to water vapors at room temperature was 128%, 132% and 120% for F_1, F_2 and F_3, respectively. Such a high moisture uptake capacity would be favorable in excision wounds to remove wound exudates. Nevertheless, it may represent a drawback when applied inside incision wounds as it may lead to wound swelling hindering re-epithelialization and wound closure.

3.4. Effect of different formulae on systolic blood pressure and heart rate

Since the minimum NF concentration required for wound healing is not known, our preliminary studies have shown that a total dose of 7 mg NF per rat (3.5 mg applied to each wound) would be rather safe with minimum side effects. Such a dose, when fully absorbed from the broken skin, is equivalent to 50 mg NF/kg rat body weight.

In the current study, rats were randomized among different groups based on their baseline SBP and HR prior to surgery such that the average SBP and HR of these rats were approximately similar in different groups (118.28 ± 10.24 and 362.53 ± 31.43, $n = 72$), respectively.

At 24 h following infliction of incision wound, rats treated with NF or NF containing formulae (F_1, F_2 and F_3) showed SBP and HR significantly lower than their baseline values. However, the decline in SBP and HR attained by F_2 was significantly less than that of NF alone or F_1, which can be attributed to the higher dissolution rate of F_2. Alternatively, at 24 h following excision wounds, only rats treated with NF and F_1 showed significant decreases in SBP with no significant effect on HR in any treated group compared to baseline values (Fig. 3). These discrepancies in the hemodynamic responses to different formulae in case of incision and excision wound can be attributed to the mode of application of the drug.

3.5. In vivo wound healing

Wound healing is an interaction of a complex cascade of biochemical and cellular events that generates resurfacing, reconstitution and restoration of the tensile strength of injured skin (Oberyszyn, 2007).

For evaluation of the wound healing capability of the preparations, three parameters were measured; tensile strength

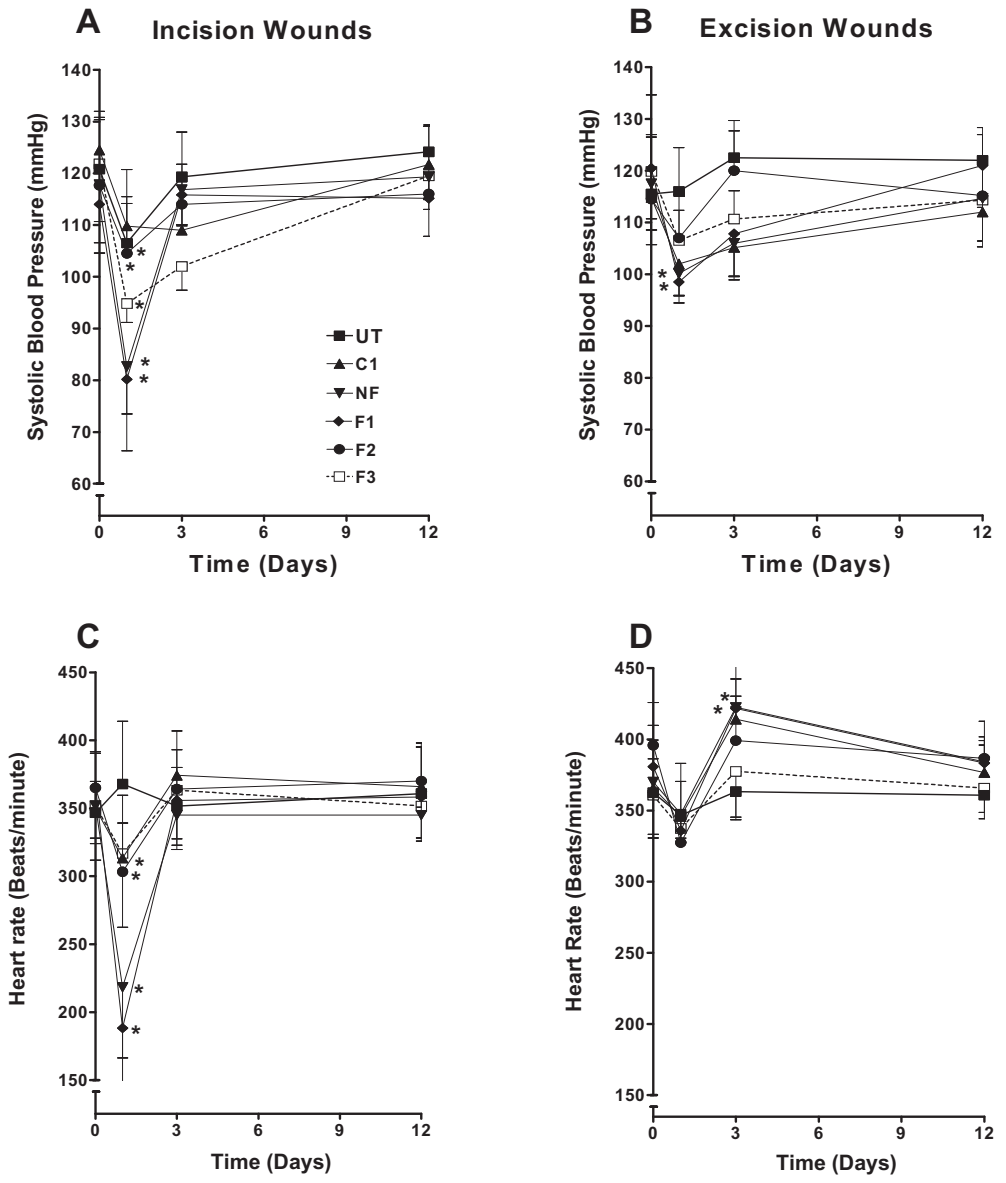

Fig. 3. Systolic blood pressure for rats subjected to incision wounds (panel A) and excision wounds (panel B) and their corresponding heart rate responses (panel C and D). Values are mean ± S.D. of six observations. Values are significant (*) at $P < 0.05$ as compared to the baseline values before surgery.

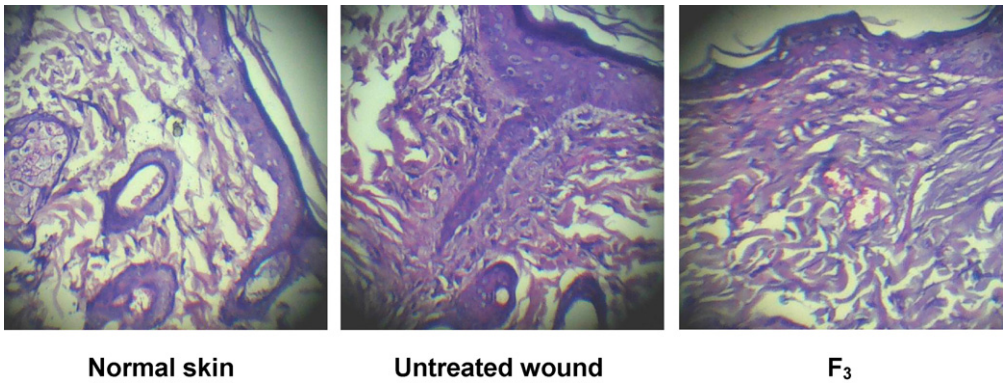

Fig. 4. Effect of F_3 on incision wound in rat skin.

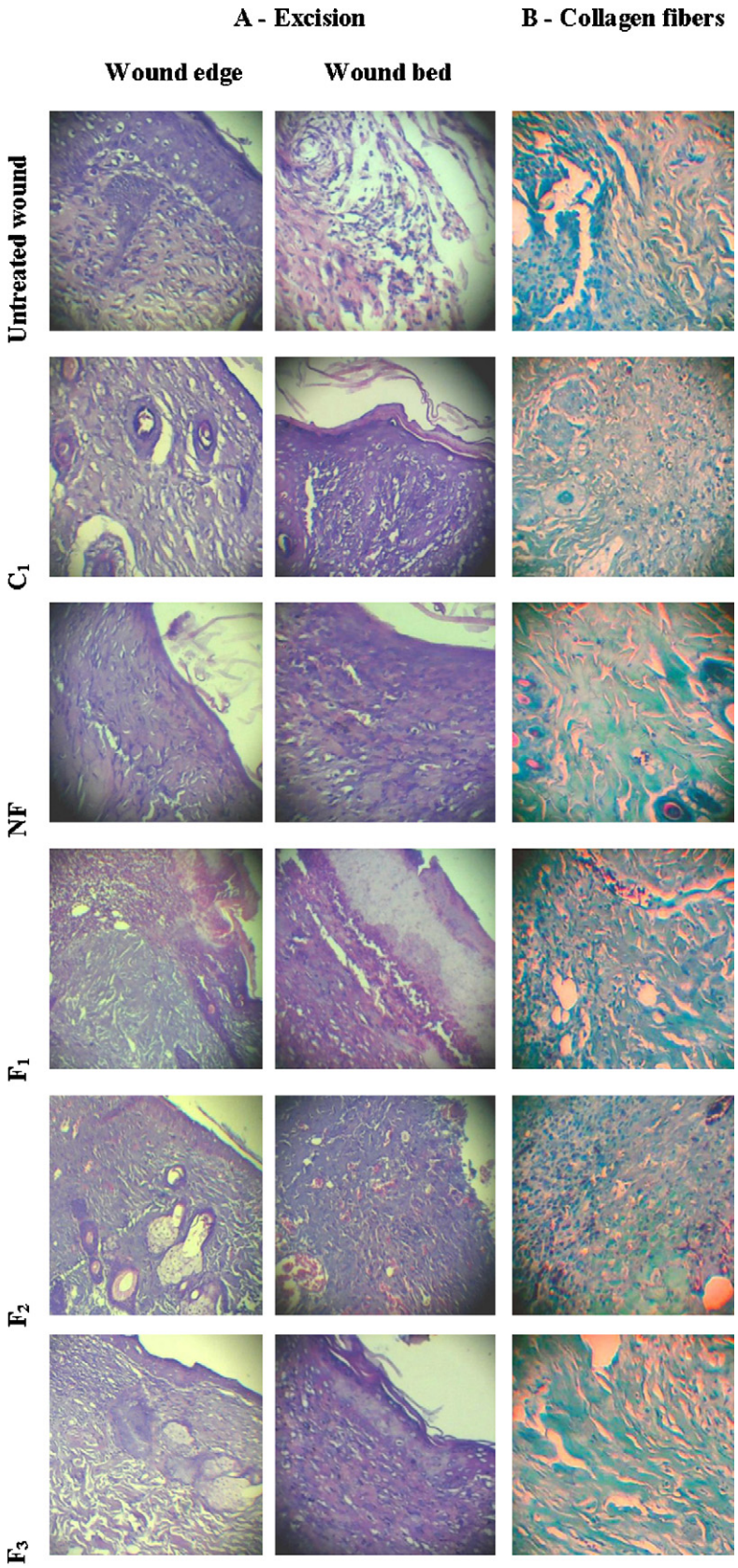

Fig. 5. Effect of different NF formulae on excision wound in rat skin and collagen fiber formation.

Table 3
Effect of biopolymeric NF powders (Table 1) on excision wound area contraction and half closure time together with the TS of incision wound during 12 days (values are mean ± S.D., $n = 6$ observations in each group).

Day	% wound area contraction					
	Control	C_1	NF	F_1	F_2	F_3
Day 3	30.3 ± 7.1	36.2 ± 2.1	37.8 ± 1.9	37.0 ± 4.4	47.0 ± 7.1	42.5 ± 2.5
Day 6	40.0 ± 9.6	49.4 ± 6.2	51.2 ± 8.7	64.8 ± 6.6	49.5 ± 8.9	55.2 ± 8.7
Day 9	69.8 ± 7.2	82.0 ± 0.8	85.9 ± 8.2	94.4 ± 3.8	93.4 ± 2.1	76.9 ± 5.8
Day 12	79.5 ± 1.3	92.1 ± 0.9	97.5 ± 0.9	99.0 ± 0.9	97.4 ± 0.5	99.1 ± 1.7
WC_{50}	6.93	5.75	5.45	4.94	5.07	5.39
TS ± S.D.	3.3 ± 0.2	1.8 ± 0.1#	5.9 ± 0.2#	3.0 ± 0.1	3.1 ± 0.1	4.7 ± 0.2#

WC_{50}: half closure time in days; TS: tensile strength (kg cm^{-2}) at 12th day of treatment. Values are significant (#) at $P < 0.05$ as compared to the untreated group (control).

Table 4
Histological examination of excision wounds treated with different formulae (Table 1) at the end of 12 days (values are mean ± S.D., $n = 6$ observations).

	Control	C_1	NF	F_1	F_2	F_3
Keratinization	0.60 ± 0.52	4.20 ± 0.52#	2.80 ± 0.75#	0.80 ± 0.82	1.40 ± 1.05	4.20 ± 0.52#
Epithelialization	1.20 ± 0.89	3.60 ± 0.52#	2.60 ± 0.52#	1.40 ± 0.75	2.00 ± 0.89	4.40 ± 0.55#
Neovascularization	2.60 ± 0.82	1.60 ± 0.55	4.60 ± 0.52#	4.00 ± 0.89#	4.00 ± 0.89#	3.60 ± 0.52
Collagen fibers	2.40 ± 0.55	1.60 ± 0.55	4.80 ± 0.41#	3.00 ± 0.98	2.60 ± 0.55	4.40 ± 0.55#

Values 5 refer to maximum similarity and 0 refers to least similarity of wound from normal tissue. Values are significant (#) at $P < 0.05$ as compared to the untreated group (control).

measurements on incision wounds, percent wound contraction on excision wounds and histopathological studies for both wounds.

3.5.1. Incision wound

These wounds are created in animals to mimic the conditions encountered in the surgical patients. The general appearance of the wounds revealed some swelling of the wound site at the first three days in case of wounds treated with C_1, F_1 and F_2 suggesting relatively poor healing when visually compared with the untreated and NF-treated wounds. This swollen appearance can be attributed to the high moisture uptake of these formulae.

Tensile strength measurements (TS) reflect the quality and speed of tissue regeneration and collagen fibers deposition and remodeling. Powder C_1 did not impart improvement for wound healing with a calculated tensile strength of 1.8 kg cm^{-2}. On the other hand, native NF powder as well as F_3 significantly increased the wound TS from 3.3 for the untreated wounds to 5.9 and 4.7 kg cm^{-2} for NF and F_3, respectively, indicating a significant improvement. Whereas the TS values for incision wounds treated with formulae F_1 and F_2 were close to the untreated group indicating a slight improvement in wound healing (Table 3).

Healing of closed incisional wounds is best determined by the histopathological studies together with the tensile strength measurements (Ziv-Polat et al., 2010). Fig. 4 shows the histological studies on different formulae. The figure provides a good evidence of suitability of F_3 for promoting healing of wounds. The photomicrographs for the section of incision wound treated with F_3 showed proliferation of epithelial tissue covering the wound area together with remodeling of well-developed collagen fibers that almost resembled normal tissue. Sections obtained from incision wounds treated with F_3 revealed almost complete healing with nearly full resolution of the granulation tissue, normal tissue architecture, and normal collagen fibers alignment.

Based on data presented in the current work, it is obvious that the best results for healing of incision wounds were obtained with F_3 with a higher tensile strength after 12 days, minimum wound swelling during healing and minimum adverse effect on rat hemodynamics.

3.5.2. Excision wounds

Wound contraction is a factor which indicates the rate of reduction of unhealed area during the course of treatment. A greater reduction is a measure of the efficacy of medication. Table 3 records the percent reduction of excision wounds area of the different animal groups over a period of 12 days. Wound contraction was relatively rapid at the first 3 days with F_2 and F_3 showing about 47% and 42% contraction, respectively, compared with 37% contraction for F_1. Alternatively, at the 12th day all wounds treated with NF or NF containing formulae showed more than 97% wound contraction compared to 79.5% for the untreated wounds.

At the 12th day following surgery, the histological examination of the dorsal excision wounds in rats treated with F_3 showed that these sections had a fully developed epithelium with normal keratinization at the wound margin and center (Fig. 5). However, even though remodeling of the collagen fibers at the wound margins was remarkable, the granulation tissue was not fully resolved at the wound center (Fig. 5, panel A – F_3). Nevertheless, the highly significant staining intensity of the collagen fibers and their near to normal size, shape and orientation (Fig. 5, panel B – F_3) indicates a significant healing potential of this formula.

On the other hand, sections taken from untreated wound (Fig. 5, panel A – UT) showed absence of the epithelium, infiltration of large number of leukocytes in the granulation tissue and areas with a loose ground substance that lacked the presence of well-defined collagen fibers (Fig. 5, panel B – UT). The epithelialization and keratinization of excision wounds receiving other studied treatments (C_1, NF, F_1 and F_2) showed significantly a lower tendency to completion of epithelialization and keratinization compared to F_3 (Table 4). Collagen fibers formed in the wound bed of wounds treated with C_1, F_1 and F_2, are much smaller and have a random appearance compared to normal tissue. The collagen fibers from wounds treated with C_1 had the least staining intensity and were ill-defined, which can explain the significantly low tensile strength of incision wounds treated with C_1 as well.

The tendency for remodeling of the collagen fibers at the wound margin from wounds treated with F_1 and F_2 was less marked than that in case of wounds treated with F_3.

In contrast, collagen fibers from wounds treated with native NF showed the highest staining tendency that was even greater than normal unwounded skin, with much thicker fibers. This over deposition of collagen fibers, characteristic of NF, as demonstrated in other studies performed on various tissue types (Kataoka et al., 2001; Frolov, 2003) is not favorable concerning the cosmetic

appearance of the wound as it increases the tendency for hypertrophic scar formation. Accordingly, the slowing of the deposition rate of collagen of NF by incorporating it with chitosan as in F_3 seems to be the most optimum for better scarless wound healing outcome.

4. Conclusion

The present study established the first evidence that the combination of spray dried chitosan with nifedipine can be superior to either of them alone in the acceleration of wound healing. The prepared formula F_3, where NF is in suspension, was proven to have a high wound healing activity promoting post-wound tensile strength more than other tested formulae. The data obtained demonstrated the importance of the formulation technique and physicochemical properties of the formula on its capacity to accelerate wound healing and avoid unwanted adverse effects. The results of the current work indicates that F_3 can be suggested as a powdered formula to accelerate the healing of excisional together with incisional wounds significantly better than free NF with minimal side effect on hemodynamics.

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