Preliminary Communication

Therapeutic Delivery

Electrospun wound dressing as a promising tool for the therapeutic delivery of ascorbic acid and caffeine

Laya Avizheh[1,2], Tahmineh Peirouvi[3], Kambiz Diba[4] & Anahita Fathi-Azarbayjani*[,2,5]

[1]Student Research Committee, Urmia University of Medical sciences, Urmia, Iran
[2]Department of Pharmaceutics, School of Pharmacy, Urmia University of Medical Sciences, Urmia, Iran
[3]Department of Anatomical Sciences, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[4]Department of Parasitology & Mycology, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[5]Solid Tumor Research Center, Urmia University of Medical Sciences, Urmia, Iran
*Author for correspondence: anahita@alumni.nus.edu.sg

Aim: The aim of this work is to formulate a wound dressing for the delivery of ascorbic acid and caffeine. **Method:** A wound dressing was developed from electrospun nanofiber containing ascorbic acid and caffeine. *In vitro* drug release was performed at 25°C and 32°C. Wound healing activity of the nanofiber mats was tested *in vivo* using rat model with skin excision. Antifungal activity of the dressing was tested on *Candida albicans* using the disc diffusion method. **Results & conclusion:** Zone of inhibition was 6.7 mm for caffeine dressing; however, inhibition zone increased to 16.7 mm for samples containing both caffeine and ascorbic acid. Animals treated with ascorbic acid showed collagen deposition and very few fibroblast cells. Blood vessels and fibroblasts were increased in caffeine-loaded dressings compared with the ascorbic acid group. The findings of the present work suggest the benefits of topical ascorbic acid and caffeine for its high wound healing effects.

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Keywords: ascorbic acid • caffeine • electrospinning • nanofiber • topical drug delivery • wound healing

Electrospinning is a simple and rapid method for the production of nanofiber drug-delivery systems from polymeric solutions. This method utilizes strong electrostatic forces to the polymeric solution or melt placed in a syringe pump. The electrified pendant drop will deform to form a Taylor cone. When the applied voltage surpasses the surface tension of the droplet, the solution is ejected from the tip of the droplet toward the collecting metal screen and while the solvent evaporates, the solid nonwoven nano/micro fibers are produced on the surface of the collector. Although this method is fast developing to the coaxial, modified coaxial, tri-axial, side-by-side and multiple-fluid processes, the simple blending electrospinning is still the mainstream for creating nanofibers and is easy to be scaled up into industry. The important publications should be cited. The developed nano to micro-sized porous nanofibers have high surface area to volume ratio and large capacity for drug loading. This fabrication technique is a well developed and cost-efficient method and consists of simple apparatus including syringe pump and high voltage power supply. Drug-loaded biodegradable and biocompatible polymers can be employed for wound healing, scar reduction as well as antimicrobial effect [1–7].

Skin wound healing is a complicated process which may result in the formation of scar. Various approaches have been employed to significantly enhance wound treatment and decrease scar formation [8]. The dermal wound healing process comprises of various stages, some of which are dependent on ascorbic acid. These include tissue matrix formation, wound contraction and epithelialization. Formation of new extracellular matrix requires deposition of collagen and attachment proteins. Epithelialization and myofibroblasts needed for wound contraction also depend on ascorbic acid for collagen synthesis. Pathogens breakdown by neutrophils and macrophages require ascorbic acid. These indicate the role of ascorbic acid in an effective wound-healing process [9]. It is known that topical antioxidants such as ascorbic acid and tocopherol can protect the skin against radiation-induced injuries. Ascorbic

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Table 1. Composition and rheology of polymeric solutions used in nanofiber production.

Formulation	PVA (w/v) (%)	Ascorbic acid (w/v) (%)	Caffeine (w/v) (%)	Viscosity (cp)	Torque (%)	Temperature (°C)
A	10	-	-	510.5 ± 11.3	85.3	26.8
B	10	2	-	511.1 ± 15.7	85.6	28.2
C	10	-	5	531 ± 13.8	89	28
D	10	2	5	558.3 ± 21.4	93.6	28.1

PVA: Poly vinyl alcohol.

acid acts as a reducing agent and scavenges free radicals and diminishes radiation-induced skin defects. The effect of ascorbic acid on the healing of excision wound in mice exposed to radiation has been studied. Pretreatment with intraperitoneal ascorbic acid enhanced fibroblast proliferation, increased vascularization and accelerated wound-healing process [9]. Topical application of 10% w/w ascorbic acid solution on human burn wound significantly enhances wound healing [9,10]. It may act as a skin penetration enhancer when co-administered with photoprotctive agents [11].

Caffeine provide favorable anticellulitis and anti-aging effects in various topical and transdermal applications. It also stimulates hair growth and promotes wound healing. Coffea Arabica seed oil can help to increase collagen and elastin production. Caffeine has also been reported to protect against sun and reduce skin roughness and wrinkle formation. Topical application of coffee oil extract on cutaneous wound incision show fast wound-healing rate in rat model [12–14].

To the best of our knowledge, the effect of ascorbic acid and caffeine-loaded nanofibers on wound healing has not been reported previously. The current study aims to develop a versatile and easy to use nanofiber wound dressing and evaluate its healing potential on excisional wound in rat model. *In vitro* characterization, drug release, stability and antifungal effects were also investigated.

Materials & method

Materials

Ascorbic acid was a gift from Osveh pharmaceutical, Iran. Caffeine sodium benzoate was obtained from Merck. Chitosan with average molecular weight of 140,000–220,000 Da and deacylation of >75% and poly vinyl alcohol (MW 89,000–98,000) were obtained from Sigma-Aldrich, Singapore.

Electrospinning

Polymeric solutions with and without the drugs where prepared as listed in Table 1. Briefly, poly vinyl alcohol (PVA) solutions (10% w/v) were prepared in distilled water while stirring on a heater stirrer at 70°C till a clear solution was obtained. After cool down to room temperature (RT), ascorbic acid and/or caffeine sodium benzoate (a freely water soluble caffeine salt) were dissolved prior loading to a 5-ml syringe attached to a blunt 27 G stainless steel needle. Syringe was attached to a pump (Fanavaran, Iran) flowing at a constant rate of 1 ml/h. The electrostatic spinner used was equipped with an adjustable DC power supply (Fannavaran, Iran) with an applied voltage of 20 kV at RT. Electrospinning was carried out at ambient temperature and relative humidity of 40 ± 5%. The electrostatically spun nanofibers were removed from the metal collector wrapped and placed under vacuum prior to usage.

Fourier transform infrared spectroscopy

The interaction between the polymer and active molecules were studied using Fourier transform infrared spectroscopy (FTIR). Samples were placed on KBr holder and spectras were recorded at RT on a Perkin Elmer (Spectrum Two) in the wavelength region 500–4000 cm^{-1}. Spectrum data were collected on Spectrum software version 10.03.02.

Scanning electron microscopy

The surface morphology of nanofibers was evaluated using a scanning electron microscope (Zeiss) at an accelerating voltage of 22.0 kV after coating with gold for 10 s. Nanofiber morphology and fiber diameter were carried out using image analysis software. Average fiber diameters were determined by measuring 30 random fibers from the scanning electron microscopy (SEM) micrographs.

Solution viscosity measurement

Viscosity of the polymeric solutions was measured using a viscometer at RT (Fungilab, Evol series, Spain). A 2-min acquisition time was set and Spindle of L_3 type was used with a speed of 200 r.p.m. Data are represented as mean ± standard deviation three individual measurements.

Stability studies

Storage stability of the ascorbic acid and caffeine-loaded electrospun nanofiber were carried out in RT and in the fridge temperature (FT) for 45 days. Aqueous ascorbic acid and caffeine samples were used as control samples. Drug content was quantified by spectrophotometer (n = 3).

In vitro drug release

The actual ascorbic acid and caffeine content of nanofibers were quantified by dissolving each sample in solvent and measuring their concentration using a UV-vis spectrophotometer. The release kinetic of ascorbic acid and caffeine from electrospun nanofibers were measured using the total immersion method. Briefly a known amount of nanofiber mat (10 mg) was suspended in 10 ml of phosphate-buffered saline (PBS) (pH 7.4) placed in shaker-incubator (GFL-3031 Rontgen, Germany) at 25°C and 32°C (shaker speed 100 r.p.m.). The caffeine used in this study was freely soluble in the dissolution media and therefore the sink condition was met. At time intervals, a 1 ml of sample was collected and the same amount of fresh medium was replaced to maintain the sink condition. Release studies were performed in triplicates. Cumulative release profile was performed on all samples in the temperatures studied. Due to the overlap of maximum absorption wavelength, the drug release from samples containing both ascorbic acid and caffeine was not performed.

UV-Vis spectroscopy

UV absorption measurements were carried out at RTs on a UV-vis spectrophotometer (Cecil, England) at 265 and 270 nm for ascorbic acid and caffeine, respectively. Standard solutions (2–0.05 μg/ml concentrations) were prepared in water and the R^2 value of the calibration curve for the standard solutions was 0.9992 and 0.9997, respectively.

Disc diffusion test

To evaluate the antifungal activity of ascorbic acid and caffeine on *Candida albicans*, agar disc diffusion tests were carried out. Samples were cut into small circular pieces (6 mm). All samples (as listed in Table 1) were placed under UV light for half an hour prior test. The sterile plates were filled with Mueller-Hinton agar medium. Concentration of the fungal suspension was adjusted to a turbidity of 0.5 McFarland standard units prior being dispersed on the surface of the medium. After inoculation, samples were placed on the surface and incubated at 36 ± 1°C for 24 h. Inhibition zones were measured in millimeters (mm) using a ruler.

Animals & handling

Healthy adult male Wistar rats (180–200 g) were obtained from Pasteur institute, Tehran, Iran. All animals were housed in standard cages with ventilation, temperature control and 12 h light–dark cycle. Animals had free access to standard rodent diet and water. Animals were adapted to the laboratory conditions for 2 weeks prior to the study. All animals were maintained and handled in accordance with the standard ethical guideline and welfare of experimental animal. The protocol was approved by the local ethical committee of Urmia University of Medical Sciences.

Excision wound model

The open excision-type wound 1 × 1 cm^2 was created on the back region (thoraco–lumber) under xylazine (5 mg/kg) + ketamine (40 mg/kg) anesthesia. After recovery animals were randomly allocated into five groups as follows and were housed individually in disinfected cages (n = 4):

- Control: open wound (nothing was applied to the wound area);
- Pure nanofiber mat without any drug;
- Nanofiber mat containing ascorbic acid;
- Nanofiber mat containing caffeine;

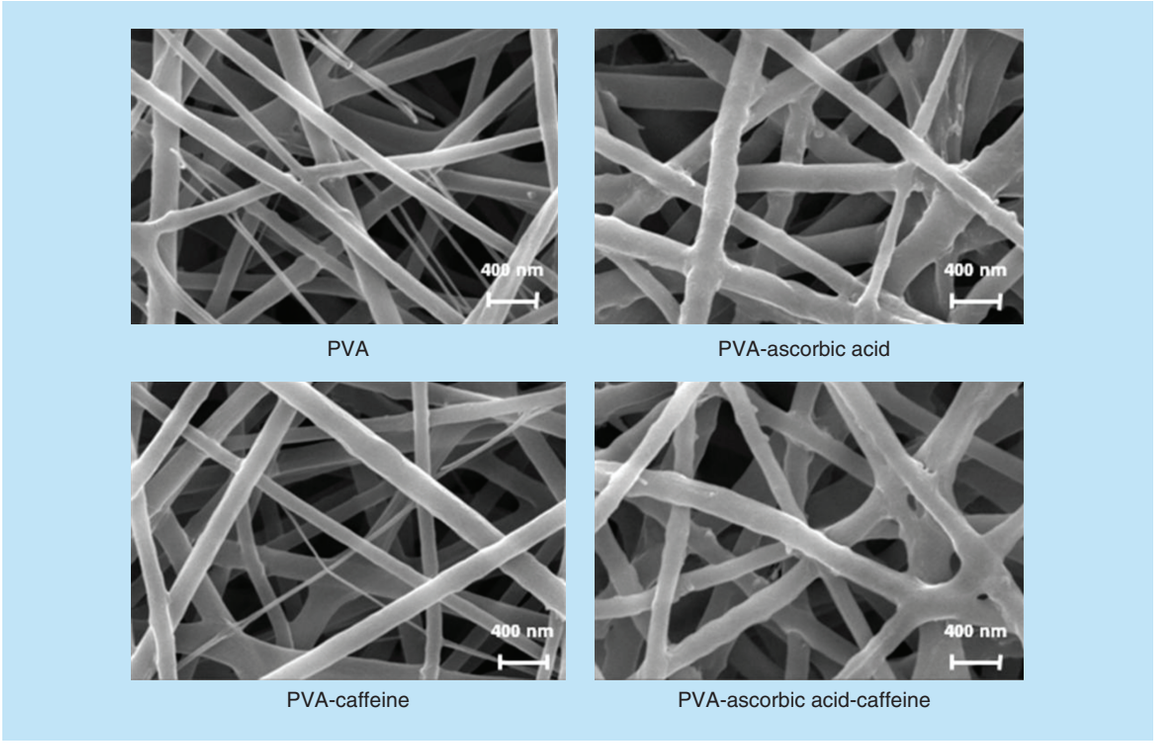

Figure 1. Scanning electron microscopy images of electrospun nanofibers.
PVA: Poly vinyl alcohol.

- Nanofiber mat containing ascorbic acid + caffeine.

All dressings were applied to the wound area once daily for 2 weeks.

Wound contraction measurement

Wound contraction (%) was determined by measuring wound area soon after wounding and every other day for 2 weeks. Animals were held firmly and the area (mm^2) was measured precisely using a ruler. Measurements were expressed as percentage of wound contraction. Wound contraction was expressed using formula as follows [15]:

Tissue processes & histopathological analysis

At the end of the treatment period, biopsies of the skin were collected under anesthesia. Healed skin including epidermis, dermis and hypodermis were fixed in 10% neutral buffered formalin for histopathological evaluation. Samples were embedded in paraffin for slice sections (5-µm thick). Histopathological changes at the wound site were evaluated using hematoxylin and eosin (H&E) staining. Samples were visualized under a light microscope (Oxion) at 4, 10 and 40× magnification and photographed with Image Focus Alpha.

Statistical analysis

All presented data are expressed as mean ± SD. Statistical analysis was performed using one way ANOVA and $p < 0.05$ was considered statistically significant ($n = 4$).

Results

SEM

The SEM micrograph of electrospun nanofibers are demonstrated in Figure 1. All formulations were capable of forming homogeneous nanofibers without the formation of beads. Some defects were observed in samples containing ascorbic acid.

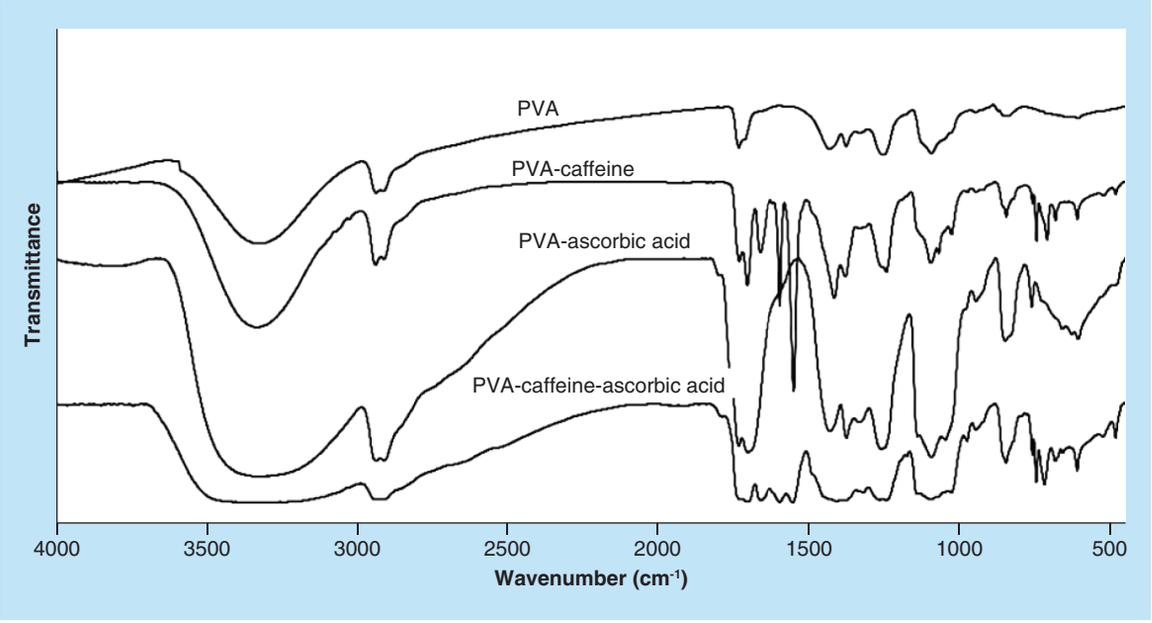

Figure 2. Fourier transform infrared spectroscopy spectra of poly vinyl alcohol nanofibers with caffeine and ascorbic acid.
PVA: Poly vinyl alcohol.

Viscosity measurement

PVA solution had a viscosity of 510.5 ± 11.3 cP. Addition of ascorbic acid and caffeine slightly increased solution viscosity and increased fiber diameter (Table 1). The results of viscosity measurements are supported by the fiber diameter measurements and morphology.

FTIR analysis

Infrared spectra of ascorbic acid and caffeine loaded nanofibers are shown in Figure 2. For pure PVA, a band around 3307 cm^{-1} is attributed to the O-H stretching vibration of the hydroxyl group. Sharp band at 2937 and 1431 cm^{-1} attribute to the C-H stretching band. Sharp peak at 1093 cm^{-1} represents the C-O stretching band of the PVA backbone. The characteristic peaks for functional groups of caffeine are shown in 1732 cm^{-1} (C = O stretching of the amide group), 1596 cm^{-1} (-CH_3 stretching) and 758 cm^{-1} (vibration in the skeleton of pyrimidine ring). In the samples containing ascorbic acid, a broad band in the 3320 cm^{-1} frequency may be assigned to the overlapping -OH stretching bands of ascorbic acid and PVA backbone. C = C stretching of ascorbic acid is seen at 1670 cm^{-1} [7,16,17]. In samples containing combination ascorbic acid and caffeine, the –OH stretching of the PVA molecule shows a shift to higher frequency (3341 cm^{-1}). Results indicate good compatibility and successful encapsulation of ascorbic acid and caffeine inside the PVA fibers.

Stability studies

Storage stability of ascorbic acid and caffeine in the developed formulations was assessed for 45 days at two different temperatures. Control samples were sealed tightly to protect from light and prevent additional drug degradation. At the end of the 45 day storage, control samples underwent almost 40% degradation at RT, however storage at fridge temperature reduced drug degradation and slightly increased drug stability. Drug content in nanofiber formulations was almost 80% at both temperatures. This indicates that incorporation of ascorbic acid and caffeine in nanofibers help to retain drug stability. Thus, it should be mentioned that the effect of pH as an influencing factor is not investigated on drug stability but will be considered in the future work.

In vitro drug release

Cumulative release profile of ascorbic acid and caffeine at 25°C and 32°C is shown in Figure 3. The release profile

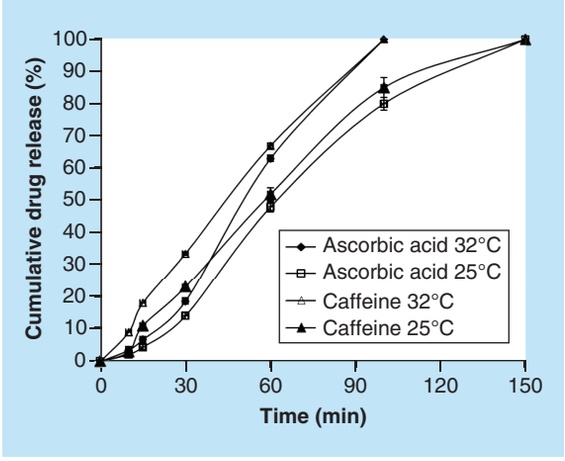

Figure 3. Cumulative release profile of ascorbic acid and caffeine from poly vinyl alcohol nanofibers at 32°C and 25°C. (Data are means ± SD of three independent experiments).

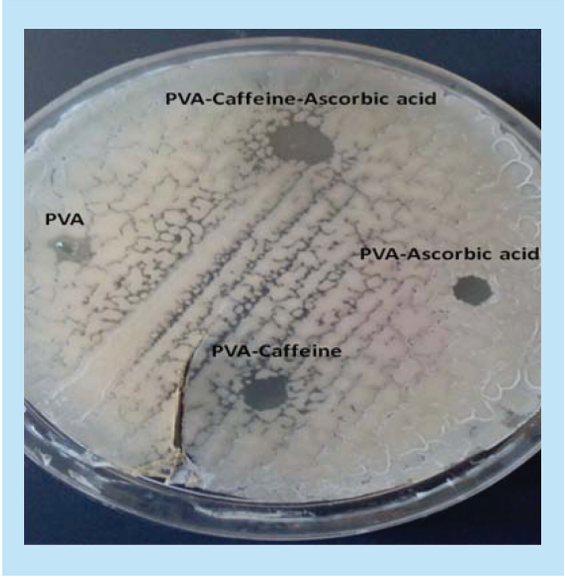

Figure 4. Fungal growth inhibition zone of formulations.
PVA: Poly vinyl alcohol.

indicates a burst manner release in water. At 32°C, fibers released almost 63% ± 2.5 and 66% ± 2.7 of the incorporated ascorbic acid and caffeine in 1 h, respectively. A slower drug release was observed at RT.

Disc diffusion test

Fungal growth inhibition zone of all formulations are shown in Figure 4. The test disks (6 mm) are applied on to the inoculated surface and the disk absorbs water from the medium and slowly disintegrates to release its drug content. As a result, at the end of 24-h incubation period, the disc has disintegrated. Depending on the type of molecule loaded onto the nanofiber mat, nanofiber mats may or may not have antibacterial/antifungal effect. However, in most cases the plain nanofiber mat does not show any antibacterial/antifungal effect. Pure PVA nanofibers did not display any inhibition zone [18]. Fungal growth was inhibited by samples containing caffeine (6.7 mm). The inhibition zone diameter increased to 16.7 mm for samples containing both caffeine and ascorbic acid where the antifungal effect was enhanced.

Wound contraction studies

In order to evaluate the topical healing properties of ascorbic acid and caffeine, rats were treated daily with drug-loaded nanofiber mats. Figure 5 depicts the percentage of wound contraction with different formulations over a 15 day period. Based on the results presented, it could be suggested that ascorbic acid and caffeine-loaded nanofiber mats provide maximum wound-healing activity and skin regeneration compared with the other groups.

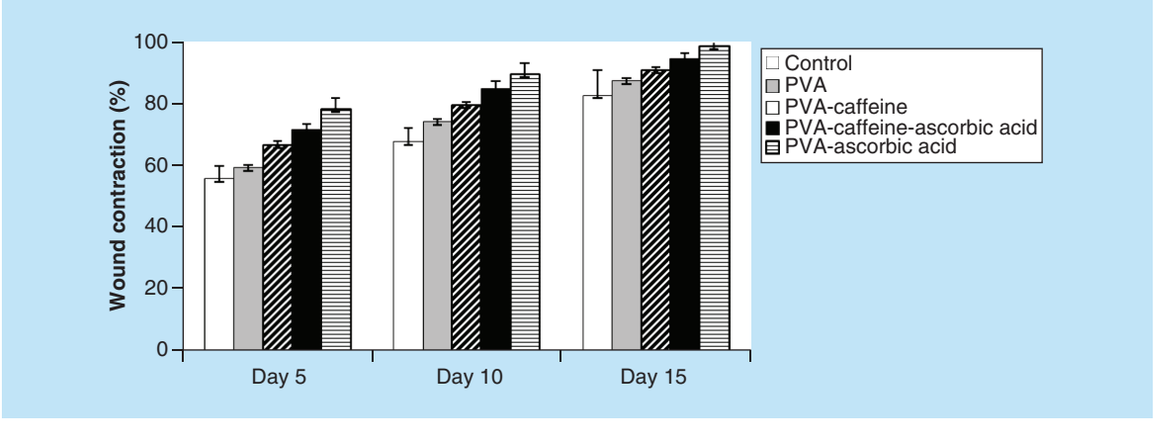

Figure 5. Wound contraction (%) in the five studied groups. Data are expressed as mean ± SD (n = 4).
PVA: Poly vinyl alcohol.

Results indicate that healing rate with ascorbic acid-containing formulations was higher than those of caffeine or control groups. The wound closure was 78.5, 90 and 98% after 5, 10 and 15 days of surgery, respectively, for skin treated with ascorbic acid. The prepared dressings adhered to the skin and dissolved gradually while releasing their drug content. The scar area was difficult to evaluate due to the formation of crust on the wound site.

Histopathological findings

H&E stained wound sections (40×) of the five study groups on day 15 postwounding are shown in Figure 6. Characteristic of an ideal wound dress is to restore the skin structure and regenerate all the skin appendages. Wound sections of the control group and the PVA alone group were dominated by large amounts of fibroblasts (Figure 6). Fibroblast in the group treated with pure PVA was less observed indicating tissue repair. The wound sections of the ascorbic acid-treated group showed re-epitheliaziation and high type III collagen deposition. Acceleration in the epidermal regeneration and wound closure is observed in the ascorbic acid-treated group. Images indicate significant increase in the formation of blood vessels and angiogenesis in caffeine-treated group. The ascorbic acid dressing provides satisfactory healing ratio and increased the skin width. A more stratified epidermal layer was seen in the ascorbic acid-treated group after 4 weeks of treatment with well-defined dermal–epidermal junctions.

Discussion

Successful fabrication of ascorbic acid and caffeine-loaded nanofibers where achieved via electrospinning method. SEM images of the as spun fibers indicate that the addition of caffeine reduced the repulsive forces and improved fiber production. The average diameter of electrospun nanofibers ranged from 215 to 390 nm. Addition of ascorbic acid slightly increased fiber diameter. Caffeine loaded-nanofiber diameter ranged from approximately 123–325 nm. This could be due to the surface active effect of caffeine [19]. Results of the FTIR analysis show good compatibility and successful encapsulation of ascorbic acid and caffeine inside the PVA fibers. The stability tests indicate significant enhancement in drug stability of the electrospun nanofibers when compared with the aqueous samples.

Drug-release studies signify a biphasic pattern at both temperatures over 150 h study period. Polyvinyl alcohol loaded caffeine/riboflavin nanofibers have been previously developed as fast dissolving oral drug-delivery system. Drug-release pattern indicate a burst release pattern where 100% of the embedded drug was released within 60 s [16]. Fast dissolving paracetamol and caffeine-loaded nanofibers show almost 80% drug released within the first 6 min [20]. Surface active effect of caffeine has been reported previously. It was found that caffeine formed an adsorption layer at the air/liquid interface [19]. This could help to explain the rapid degradation of the caffeine loaded PVA nanofibers. The slow onset of caffeine release rate observed in our work may be due to the application of its salt form or variation in the molecular weight of the polymer used. Rapid release of some drugs may be due to the hydrogen binding or polymer chain branching caused with the addition of certain drugs or additives that help to enhance the degradation rate of the delivery system.

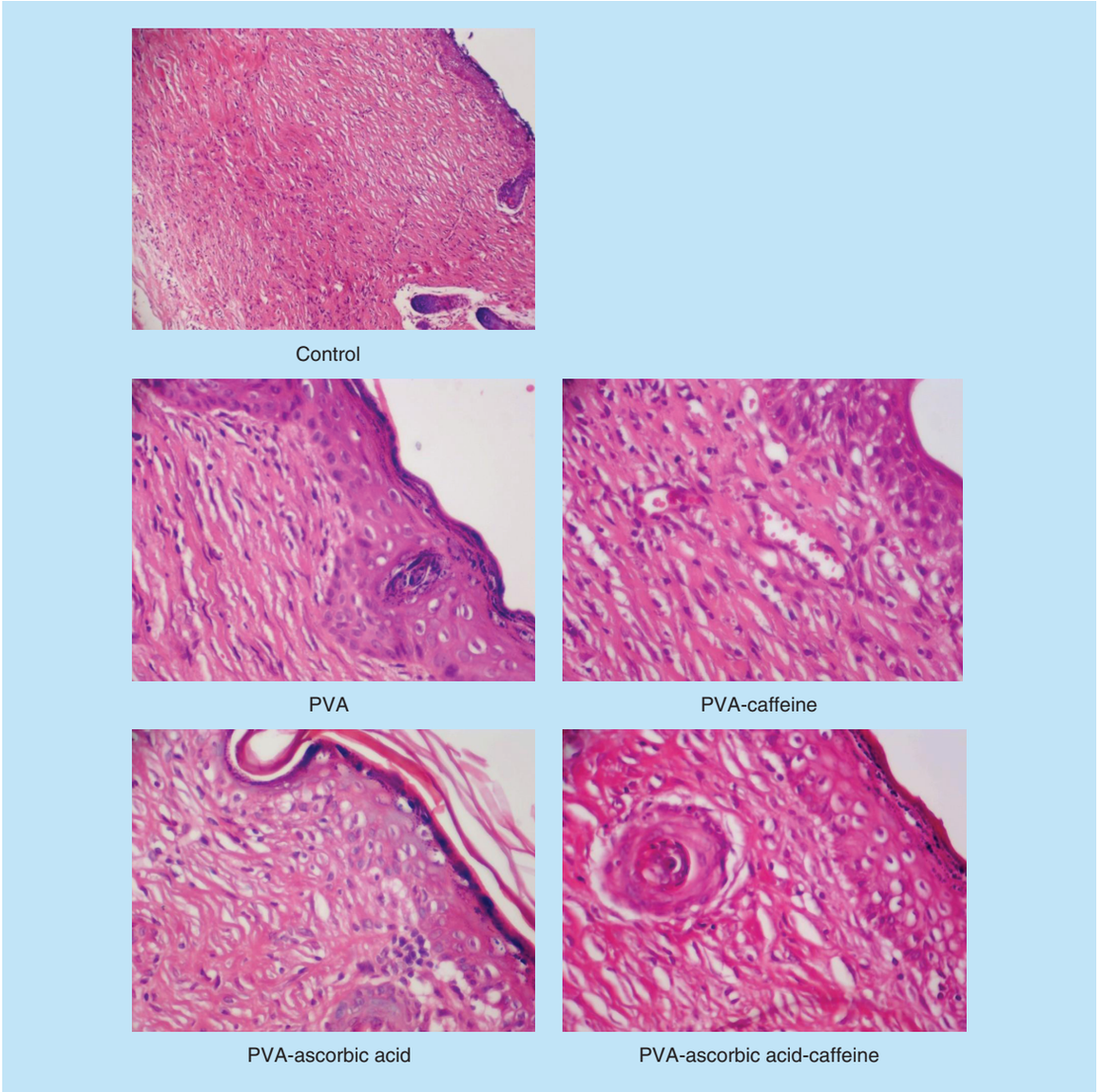

Figure 6. Image of hematoxylin and eosin staining of the histopathological sections of skin tissue on day 15 postwounding (40× magnification).
PVA: Poly vinyl alcohol.

Ascorbic acid and lecithin-loaded nanofibers were fabricated with polyethylene oxide to prevent oxidative damage and help protect red blood cell rupture in implant devices. Binary release of ascorbic acid and lecithin in distilled water over 110 h indicate a gradual dissolution of the nanofiber and slow onset of drug release [21].

Antifungal activity of the electrospun nanofibers were performed. Fungal growth inhibition zone measurements show enhancement in the antifungal activity of caffeine and ascorbic acid. The mechanism that result in this property may probably be due to the charge density effect and antioxidant activity of ascorbic acid. Previous findings have reported the intrinsic antifungal and antibacterial effect of caffeine and its derivatives. Antibacterial activity of a plant derivative polyphenol may be enhanced in the presence of ascorbic acid [22–24].

These results indicate that the wound dressing containing both ascorbic acid and caffeine show slightly better results as compared with the ascorbic acid or caffeine-treated groups in restoring the structural properties of the skin in terms of collagen deposition or blood vessel formation and wound closer rate. Further evaluation warrants better understanding of the effect of topical ascorbic acid and caffeine in wound treatment.

Skin permeation of coffee silk skin extract-loaded nano lipid carriers has been studied via Franz diffusion cells on pig ear skin. Incorporation of caffeine in lipid extract was suggested for better penetration into skin cells, however, results did not find any significant difference in the skin permeation of caffeine without lipid carriers. Caffeine is a hydrophilic molecule and previous studies have demonstrated the difficulty in the skin permeation of this molecule [13].

The effect of ascorbic acid on wound healing has been studied before. Post-treatment of intraperitoneal injection of ascorbic acid at a dose of 250 mg/Kg was found to accelerate wound healing after exposure to whole body radiation. Results indicate a statistically significant decline in healing time of wounds exposed to 2 and 4 Gy irradiations treated with ascorbic acid. There was no significant change in healing rate of the wounds exposed to 6 and 8 Gy irradiation. Ascorbic acid inhibited the radiation-induced reduction in collagen deposition [9].

Collagen based dressing containing antibiotic was developed for its healing effect and wound reduction in rat model. Histological assessment of wound indicates that collagen deposition was significantly higher in the treatment group and a well defined dermis was observed on day 9 of the treatment [25]. Oral administration of ascorbic acid at a daily dose of 1000 mg significantly enhanced wound closure and caused reduction in wound size of patient with poor wound healing after surgical intervention.

Caffeine has inhibitory effect on wound healing and epithelialization and impedes keratinocyte proliferation and migration in a dose-dependent manner [26]. However other studies concluded that caffeine can enhance cell proliferation [27,28].

Findings of the present work and the results of the previously reported data indicate that topical application of ascorbic acid may help to enhance wound healing to a great extent. Topical application of caffeine on excisional wound although did not cause significant results but helped to increase vascular proliferation and long term treatment may lead to new and important findings.

Conclusion

A wound-healing drug-delivery system was developed from electrospun nanofiber containing ascorbic acid and caffeine. Physicochemical characteristics of the as spun nanofiber films were investigated using SEM and FTIR. *In vitro* drug-release studies were performed at room temperature and fridge condition of 25°C and 32°C, respectively. Wound-healing activity of the nanofiber mats was tested *in vivo* using rat model with skin excision. Fibroblasts and blood vessels were increased in caffeine dressings compared with the ascorbic acid group. Animals treated with ascorbic acid showed well formed thick granulation tissue as well as collagen deposition and very few fibroblast cells. This may indicate a high rate of skin remodeling and construction using ascorbic acid-containing dressings. The findings of the present work suggest the benefits of topical ascorbic acid for its high wound-healing effects.

Future perspective

Future work concerns deeper analysis on the effect of wound pH on drug stability and wound-healing rate. We further plan to explore existing drug molecules with wound-healing characteristics, which at the same time could also help to prevent infection at the site of action. Fabrication of such materials into electrospun nanofiber scaffolds may promote wound healing and skin regeneration.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All animals were maintained and handled in accordance with the standard ethical guideline and welfare of experimental animal. The protocol was approved by the local ethical committee of Urmia University of Medical Sciences. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Formulation & characterization of nanofiber

- Ascorbic acid and caffeine-loaded poly vinyl alcohol nanofibers were formulated and visualized via scanning electron microscopy.
- Nanofiber diameter decreased with the addition of caffeine. This was thought to be due to the surface active effect of the molecule as previously reported.
- Fourier transform infrared spectroscopy analysis showed good compatibility and successful encapsulation of ascorbic acid and caffeine inside the poly vinyl alcohol fibers.
- Stability tests show that ascorbic acid and caffeine encapsulated in nanofibers were significantly more stable than the control.
- Drug release shows a biphasic pattern over the 150 h study period.
- Ascorbic acid synergistically enhanced the antifungal activity of caffeine against *Candida albicans*.

Wound healing & histological studies

- Excision wound were created on the back of rat models and wound closer rates were studied on day 5, 10 and 15.
- Wound closer rate did not show significant difference among the studied groups. This was thought to be due to the formation of crust on the wound which may interfere with measurements.
- Wounds treated with ascorbic acid-loaded nanofiber showed well-formed collagen deposition, while the group treated with caffeine had large amounts of blood vessels.

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