Preparation of Fucoidan-Chitosan Hydrogel and Its Application as Burn Healing Accelerator on Rabbits

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Treatment of dermal wounds with macromolecular agents such as natural polymers is one of the research areas of the biomaterial science. Fucoidan is a sulphated polysaccharide which is commonly obtained from seaweeds. The great number of studies on the different pharmacological properties of fucoidan is present, but there is limited information about using of fucoidan in the treatment of dermal burns. The aim of this study was to prepare fucoidan-chitosan hydrogels and to investigate their treatment efficiency on dermal burns. Hydrogels were prepared by swelling the polymers in acidic solution and their swelling, mechanical (hardness, cohesiveness and adhesiveness) and bioadhesive properties were investigated. The viscosity and water absorption capacity of formulations increased with increase in the polymer concentration. In contrast to the cohesiveness results, the adhesiveness of hydrogels increased with the polymer concentration. The bioadhesion was changed between 0.012—0.142 mJ·cm^{-2} and enhanced with addition of fucoidan into gel formulations. It was formed dermal burns on seven adult male New Zealand white rabbits and the optimum gel formulation applied on the wounds. Control and treatment group biopsy samples were taken on days 7, 14 and 21 and each burn wound site was evaluated histopathologically. No edema was seen in tested groups except control after 3 d treatment. After 7 d treatment, fibroplasia and scar were fixed on wounds treated with fucoidan-chitosan gel and fucoidan solution. The best regeneration on dermal papillary formation and the fastest closure of the wounds were observed in fucoidan-chitosan hydrogels after 14 d treatment.

Key words fucoidan; chitosan; burn healing; hydrogel; bioadhesion; texture analyzer

Burn healing includes a specific biological process related to the general phenomenon of growth and regeneration and the primary objective in burn care is the promotion of rapid wound healing with the best functional results. The principal function of a burn dressing is to provide an optimum healing milieu for natural healing and desirable burn dressing may, therefore, be characterized on the basis of its performance such as; (a) provision of adequate gaseous exchange, (b) provision and maintenance of a moist environment, (c) protection from infections and contamination, (d) absorption of wound fluids and (e) painless and easy removal.[1,2]

Hydrogels are ideal biopolymeric pharmaceutical forms for the treatment of skin wounds. They have low interfacial tension, high molecular and oxygen permeability, good moisturizing and mechanical properties that resemble physiological soft tissue.[3,4] For this reasons, polysaccharides, *e.g.* chitosan, are having hydrogel forming properties have been considered to be advantageous in its application as a wound dressing material.[5] Chitosan, [α (1→4) 2-amino-2-deoxy-β-D-glucan], a unique polysaccharide derived from deacetylation of chitin, has been used in wound treatment owing to its good biocompatibility, biodegradability and accelerated granulation.[6—8] On the other hand, fucoidan is a sulphated polyfucose polysaccharide and has attracted considerable biotechnological research interest since the discovery that it possessed anti-coagulant activity similar to that of heparin and also reported to possess other properties including antithrombotic, anti-inflammatory, anti-tumoral and anti-viral effects.[9,10] Many of these effects are thought to be due to its interaction with growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β). Fucoidan may, therefore, be able to modulate growth factor-dependent pathways in the cell biology of tissue repair.[11] Although a great number of studies on different pharmacological properties of fucoidan and chitosan are present, there is little information on the fucoidan-based system used in burn healing and its only limited with cell culture.[12]

The aim of this study was to prepare fucoidan-chitosan hydrogel and to investigate its treatment efficiency on dermal burns on rabbits.

MATERIALS AND METHODS

Materials Chitosan (MW 250 kDa, deacetylation degree ≥90%, Pronova A/S, Norway; MW 400 kDa, deacetylation degree ≥60%, Fluka, Germany; MW 750 kDa, deacetylation degree ≥75%, Sigma, U.S.A.), Fucoidan (from *Fucus vesiculosus*) and lactic acid (85% m/v) purchased from Sigma, U.S.A. All other reagents used were of analytical grade.

Preparation of Hydrogels The composition of gel formulations was given in Table 1. Fucoidan was dissolved in 1% m/v lactic acid solution by mechanical shaking at 300 rpm for 1 h. Then chitosan was left to swell in the fucoidan solution overnight to prepare hydrogels.[13] Formulations were kept at 4 °C until experiment.

Viscosity Measurement The viscosity of hydrogels was determined by using a rotational viscosimeter (Brookfield DV-II+, U.S.A.) at 25 °C. The results were expressed as a mean of three measurements.

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Table 1. Composition, Viscosity and Water Absorption Capacity of Hydrogel Formulations

Codes	Chitosan concentration (%)	Fucoidan concentration (%)	Chitosan origin	Viscosity values (cPs±S.D.)	Water absorption capacity (g±S.D.)
A1	1.50	0.50	Sigma	3612±31.8	0.80±0.04
A2	1.75	0.50	Sigma	10230±64.0	0.88±0.03
A3	2.00	0.50	Sigma	18217±15.7	0.99±0.03
A4	2.00	—	Sigma	15991±99.0	0.90±0.03
B1	2.00	0.25	Sigma	15312±22.6	1.03±0.03
B2	2.00	0.75	Sigma	22338±46.3	1.07±0.04
C1	2.00	0.50	Fluka (M.W.)	10330±18.8	0.85±0.03
C2	2.00	0.50	Protan 243	801±18.7	0.60±0.02

Swelling Study The swelling studies were carried out gravimetrically in pH 7.4 phosphate buffer (PBS) according to the method of Noble *et al.*[14] One gram hydrogel was put in a petri dish and 10 ml of PBS (pH 7.4) was added until the hydrogels reached the constant weight at 25 °C. At predetermined time intervals, the hydrogels blotted with the filter paper to remove excess water and reweighed. Each experiment was performed in triplicate. The swelling ratio (D_s) was calculated using Eq. 1.

$$D_s = W_t / W_0 \quad (1)$$

where W_t is the weight of hydrated gel at time t, W_0 is the initial weight of the gel.

Mechanical Properties of Hydrogel Formulations The mechanical properties of hydrogel formulations were determined using a software-controlled penetrometer, TA-XT*Plus* Texture Analyzer (Stable Micro Systems, U.K.), with a 5 kg load cell at 37±0.5 °C. Each formulation was transferred into universal bottle (25 ml) to a fixed height of 8 cm and kept in the ultrasonic water bath to remove air bubbles for 20 min and the temperature was adjusted to 37 °C. The Perspex probe of 10 mm diameter was compressed twice into each formulation at a defined rate of 2 mm·s^{-1} to a depth of 15 mm. A delay period of 15 s was allowed between the two compressions. Data collection and calculation were performed using the *Texture Exponent 4.0.4.0* software package of the instrument. From the resultant force–time plot, mechanical parameters such as hardness, adhesiveness and cohesiveness of the hydrogel formulations were defined.[15]

Hardness was defined as the force required to attain a given deformation or as the maximum peak force during the first compression cycle. Adhesiveness was defined as the negative force area for the first compression cycle and represented the work required to overcome the attractive forces between the surface of the gel and the surface of the probe. Cohesiveness defined the ratio of the area under the force–time curve produced on the second compression cycle to that produced on the first compression cycle, where successive compressions were separated by a defined recovery period. Each experiment was carried out six times.

***In Vitro* Bioadhesion Studies** TA-XT*Plus* Texture Analyzer equipped with a 5 kg load cell was used for mucoadhesion test.[16] Freshly excised chicken back skin was used as a model tissue after removing all fats and debris. A section of skin was attached to the lower end of the probe ($P_{0.5}$ Perspex, θ: 12.5 mm) of the instrument with cyanoacrylate glue. The hydrogels were packed into the 30 mm diameter tubes and centrifuged to remove the air in the hydrogels and to create a smooth surface to contact with the skin. The tests were conducted at 37 °C. The probe holding the skin was lowered on to the surface of the gel with a constant speed of 0.1 mm·s^{-1} and contact force of 0.5 N applied. After keeping in contact for 120 s, the probe was then moved vertically upwards at a constant speed of 0.1 mm·s^{-1}. The area under the curve (*AUC*) was calculated from force–distance plot as the work of adhesion using *Texture Exponent 4.0.4.0* software package of the instrument. The formulation given below was used to calculate the work of adhesion per cm^2 (Eq. 2). Each experiment was carried out in triplicate.

$$\text{work of adhesion (mJ}\cdot\text{cm}^{-2}) = \frac{AUC}{\pi r^2} \quad (2)$$

πr^2 = the mucosal surface being in contact with gel

Skin Burn Wounds Experimental design and treatment of animals were approved by the Animal Care Committee of Selçuk University. Seven male New Zealand white rabbits (4.2±0.3 kg) were used for the evaluation of dermal burn wounds. The back of the rabbits was depilated and ketamine (25 mg/kg) and xylazine (1 mg/kg) were injected intramuscularly into the rabbits to induce sedation before a heated aluminium stamp was applied. The electrically heated stamp was maintained at a temperature of 80 °C and applied for 14 s to form a dermal burn wound (burn area: 3.8 cm^2) described by Knabl *et al.*[17] Burn wound depth was assessed by histopathological determination of the depth of damage to the following skin elements; hair follicule (epithelial cells), connective tissue collagen (a change in collagen staining), nerves and smooth muscles (mesenchymal cells) and blood vessels (endothelial cells). The burn depth was measured starting from the epithelium basal layer. It was confirmed that all animals had deep dermal burns. Each rabbit has four burn wounds; the first was as control and hence did not receive any treatment, the second was treated with chitosan hydrogel (A4) (2 ml/3 d, CJ), the third was treated with fucoidan-chitosan hydrogels (B2) (2 ml/3 d, FCJ) and the last group is treated with fucoidan solution of 0.75% m/v (2 ml/3 d, FS) for 21 d. Formulations were applied onto the wet fascia of the wound area after escharectomy. The biopsy samples were taken on days 7, 14 and 21 from six rabbits and the degree of healing was evaluated macroscopically and histopathologically. One rabbit was separated to take photographs of the wound areas on days 7, 14, 21.

Histopathological Examination The biopsy of skin samples (0.5×1.5 cm^2) were fixed in a 10% buffered formaldehyde solution then embedded in paraffin block and sectioned 4 μm increments. The sections were made perpendi-

cular to the anterior-posterior axis and perpendicular to the surface of the wounds. The sections were positioned on a slide and stained with haematoxylin-eosin and Masson's trichrome reagents. The measurement of "wound epithelial elongation" was carried out on the line between two irregular zones for as many as needed depending on the microscopic length on the epithelial line in the wound epithelial elongation region and they were added each other to get the final elongation. The measurement for wound epithelial thickness was done at 5 different locations excluding rete pegs on the line in the wound epithelial elongation section. The number of rete pegs suggesting migrating of cells from epidermal appendages was counted in the wound epithelial elongation section. Results were given as a mean of six rabbits.

Acute inflammatory cells (polymorphonuclear leukocytes), chronic inflammatory cells (mononuclear leukocytes), fibroblast and collagen proliferation were evaluated separately. Each item was graded by two pathologists according to a semi quantitative approach as absent (0), mild (1), moderate (2) and severe (3) without the knowledge of the specimen groups.

AgNOR Staining and Quantification Study The nucleolar organiser regions (NORs) stained by silver and the argyrophilic NOR-associated proteins are called AgNORs respectively. The samples were prepared according to an earlier study.[18] The 4 μm section of samples were cut from the paraffin block, dewaxed with xylene and hydrated. The silver staining solution (0.3 ml) containing one part by volume of 2% m/v gelatine in 1% m/v formic acid and two parts of 25% m/v aqueous silver nitrate solution was immediately poured on each slide. After staining (20 min), the solution was poured off and the slides were washed with bidistilled water, placed for 10 min in a 5% m/v sodium thiosulphate solution, rinsed with bidistilled water and dried. The AgNOR proteins appeared as well-defined black dots that counted in 50 cells of each sample. The analysis was performed in triplicate for each batch of hydrogels.

Statistical Analysis *In vitro* data obtained from each experiment was subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparisons test. Differences between the groups were tested for significance by the chi-squared test (χ^2) for the *in vivo* studies. $p<0.05$ was considered to be indicative of significance.

RESULTS AND DISCUSSION

The Characterization of Fucoidan-Chitosan Hydrogels Hydrogels are well-accepted formulations for the treatment of wounds and burns due to their ability to absorbe exudates, to keep moisture on the wound surface, and to permeate water vapor and oxygen in a controlled way.[19,20] Hydrogels provide a cover on the wound in the absence of injured skin integrity. An ideal gel formulation used for burn healing should be easily applicable and retained for a long time on the wound.[21,22] Therefore, a gel should show appropriate viscosity. In our study, the viscosity of the fucoidan-chitosan hydrogels which affected by molecular weight of chitosan and concentration of polymer ($p<0.05$) were ranged between 801 and 22.338 cPs (Table 1) and obtained results were consistent with the literature.[23] Preliminary animal studies demonstrated that gels with a viscosity less than 10000 cPs retained for a short period on the skin whereas higher viscosity values did not change the retaining time (data not shown). Thus hydrogels having viscosity more than 10000 cPs was considered to be more appropriate for *in vivo* studies.

During the pathophysiologic prognosis of a burn, especially 2nd and 3rd degree, substantial increase of exudates including fibroblasts and dead cells is observed.[24] Increasing amounts of exudates slow both wound contraction and eschar tissue formation down and could initiate microbial infections. Suitable gel formulations should absorb the wound exudates and also keep the moisture on wound surface during treatment.[25,26] Therefore, absorption capacity and swelling rate of the gels are crucial factors.[6—8] Fucoidan-chitosan gels differ in their water absorption capacity depending on polymer concentration and molecular weight of chitosan. Addition of fucoidan, more hydrophilic than chitosan, increased both the water absorption capacity and the swelling rate of the gels ($p<0.05$) (Fig. 1). Higher fucoidan concentration did not significantly change the total water absorption capacity of the gel ($p>0.05$), but increased the swelling rate ($p<0.05$) (Fig. 1). Similar findings were obtained with chitosan. Increase in the molecular weight of chitosan accelerated the swelling rate of gels and thus formulation prepared with less than 250 kDa chitosan demonstrated a low swelling rate com-

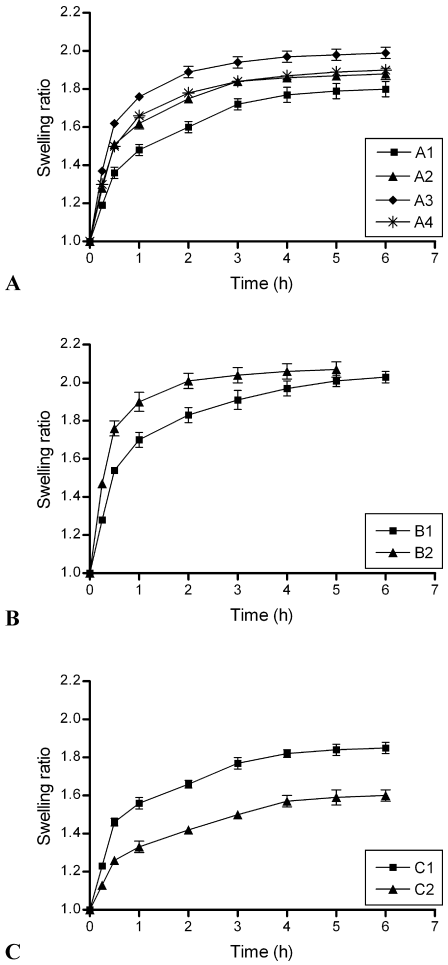

Fig. 1. The Effects of (A) Chitosan, (B) Fucoidan Concentrations and (C) Molecular Weight of Chitosan on the Swelling of Hydrogel Formulations ($n=3$)

Bars indicate standard deviation (S.D.).

pared to others (Fig. 1c). Results were in accordance with the literature.[27,28] Water absorption of hydrogels containing copolymers takes place in two stages, namely, infiltration of water inside the porous polymeric network and swelling of the gel.[27,28] Water absorption rate and capacity of the fucoidan-chitosan gel was found to be higher then chitosan formulation due to hydrophilic nature of fucoidan. In the view of *in vivo* preliminary study data, gel formulations prepared with higher concentrations of chitosan and fucoidan were found to possess ideal characteristics such as absorbing exudates and keeping the moisture on the wound surface during the treatment period.

The semi-solid formulations should have appropriate mechanical properties to boost clinical efficiency. Chitosan (positively charged) and fucoidan (negatively charged), which were used in this study, form a hydrophobic polyelectrolyte complex in the gel. It was reported that polyelectrolyte complexes enhance the mechanical properties of hydrogels such as spreadibility, hardness and cohesiveness due to the ionic interaction between oppositely charged polymers.[29] Polyelectrolyte complexes were also found to be more effective systems than either negatively charged polymer or positively charged polymer alone as wound dressing.[13] In this study, the mechanical properties such as hardness, cohesiveness and adhesiveness of gel formulations were investigated using texture profile analysis.[15,30—32]

The hardness test was performed to measure the required force to produce deformation of hydrogels. Low gel hardness ensures that the minimum work is required for removal of gels from the container and the applicability onto the desired site. On the other hand, value decreases the retention time of gel formulation on the wound and therefore, a hydrogel formulation should have an appropriate hardness value for the effective treatment of wounds.[31,32]

It was reported that the hardness of gel formulations were significantly affected by the molecular weight and concentration of the polymer.[15] Tan *et al.* showed that the gel hardness was dependent on the concentrations of hydroxyethylcellulose, polyvinyl pyrrolidone and polycarbophil in formulations.[31] In our study, we found that the hardness values of hydrogels increased significantly by 5 times (A1: 22.7±0.6 mN; A3: 104.0±4.6 mN), due to the increase in the chitosan concentration from 1.5 to 2% ($p<0.05$) (Fig. 2). It was also observed that the hardness of the gel formulations containing chitosan with low (C1: 14.7±0.6 mN) and medium (C2: 12.7±0.6 mN) molecular weight were 8—9 times lower than the gels including high molecular weight one (A3) ($p<0.05$) (Fig. 2). The addition of increasing amounts of fucoidan changed the hardness of gels significantly ($p<0.05$). Hydrogel hardness was gradually increased with addition of fucoidan due to the formation of polyelectrolyte complex between the protonated amine groups of chitosan and sulphate groups of fucoidan by electrostatic interaction (B1: 40.3±1.2 mN; B2: 122.0±1.0 mN). In a previous study, addition of anionic polymer such as carboxymethyl cellulose improved the mechanical strength of chitosan hydrogels.[33] Cevher *et al.* showed that the hardness value less than 396 mN was found to be suitable for the application onto mucosal ephitelium.[32] Similar results were obtained in our study and it was determined that the hardness of prepared gels were in the acceptable range based on previous studies.[31,32]

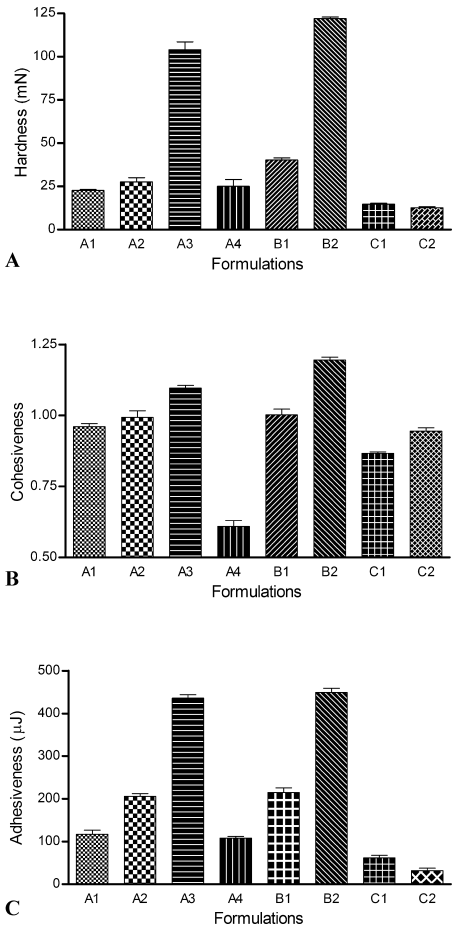

Fig. 2. The Mechanical Properties of Hydrogel Formulations

(A) Hardness, (B) cohesiveness and (C) adhesiveness ($n=6$). Bars indicate standard deviation (S.D.).

The cohesiveness is important to determine the reconstruction ability of the gel after application.[34,35] The high cohesiveness value increases the performance of the product at the application site by providing full structural recovery following gel application.[15,32] In this study, the cohesiveness (A1: 0.960±0.011; A3: 1.096±0.014) increased upon increasing the chitosan concentration ($p<0.05$) (Fig. 2). The addition of fucoidan at increasing amounts enhanced the cohesiveness of the hydrogels (B1: 1.002±0.020; B2: 1.195±0.010). While no significant difference was found between the cohesiveness of gel formulations including low (C1: 0.932±0.012) and medium (C2: 0.944±0.014) molecular weight chitosan ($p>0.05$), the cohesiveness of gel formulations containing high molecular weight chitosan (A3) differed from the remaining formulations significantly ($p<0.05$). According to obtained data, it was seen that A3 and B2 formulations containing the mixture of the highest amounts of chitosan and fucoidan showed highest and acceptable cohesiveness value.

In order to shorten the treatment period and improve patient compliance, gels should retain on the application site for a desired time.[15,31] Therefore, the adhesiveness plays an important role on healing. The concentration and molecular weight of chitosan influenced the adhesiveness of the resultant gel. The adhesiveness increased upon increasing the chitosan concentration from 1.5% (A1: 117±10 μJ) to 2% (A3:

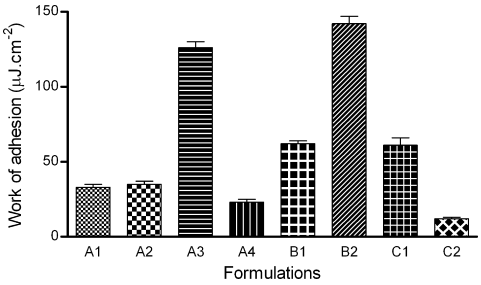

Fig. 3. Bioadhesion Properties of Hydrogel Formulations ($n=3$)
Bars indicate standard deviation (S.D.).

436±8 μJ) ($p<0.05$) and the molecular weight from 250 kDa (C2: 32±6 μJ) to 750 kDa (A3) ($p<0.05$). Addition of fucoidan was found to enhance the adhesiveness. The gel formulation containing 0.75% fucoidan (B2: 449±10 μJ) were at least twice more adhesive than that containing 0.25% fucoidan (B1: 215±11 μJ) when compared to hydrogels prepared with the same amount of chitosan ($p<0.05$). In comparison to other formulations, gels containing high amount of chitosan and fucoidan (A3 and B2) exhibited the greatest adhesion value (Fig. 2).

Mucoadhesion Studies The mucoadhesion test was performed to measure the adhesive strength of gel formulations to the wound area. The works of adhesion results obtained from mucoadhesion test were given in Fig. 3. The findings showed similarity with the adhesiveness results obtained from texture profile analysis. Factors such as the molecular weight and concentration of the polymer used in the gel can influence the mucoadhesive performance of the formulation.[32] Increasing the chitosan concentration from 1.5% (A1: 33±2 $\mu J \cdot cm^{-2}$) to 2% (A3: 126±4 $\mu J \cdot cm^{-2}$) enhanced the mucoadhesion ability (Table 1, Fig. 3). The molecular weight of chitosan and increasing amount of fucoidan influenced the mucoadhesion ability of formulations (C2: 12±1 $\mu J \cdot cm^{-2}$; A3). For example, the work of mucoadhesion of the hydrogels containing 2% chitosan alone (A4), 2% chitosan plus 0.25% fucoidan (B1) and 2% chitosan plus 0.75% fucoidan (B2) were 23±2 $\mu J \cdot cm^{-2}$, 62±2 $\mu J \cdot cm^{-2}$ and 142±5 $\mu J \cdot cm^{-2}$, respectively ($p<0.05$). A3 and B2 formulations exhibited the highest mucoadhesion in all formulations analysed (Fig. 3).

***In Vivo* Wound Healing Study** According to obtained *in vitro* data related to swelling, mechanical and bioadhesion studies, B2 formulation was chosen for the treatment of experimental burns on rabbits. The histological changes in groups during treatment were given in Table 2. The scheme of histopathological evaluation of the wound area is shown in Fig. 4. According to findings, haemorrhage and edema were observed only in the control group during healing period. The antibacterial and anti-inflammatory effects of fucoidan and chitosan, as stated in previous literature,[36,37] could be the reason of the absence of edema in treatment groups (Table 2). The number of polymorphonuclear leukocyte (PMNL) cells were higher in the control group than those in treatment groups up to day 21 and the great number of the PMNLs proved that the inflammation continuous during this period, as shown in Table 2 and Fig. 5. PMNLs were replaced with mononuclear leukocyte (MNL) cells (Table 2) in the fucoidan-chitosan hydrogel group and hair growing was observed on the healing zone on days 14 and 21 (Figs. 5, 6). Fast epithelial regeneration and great number of MNLs increase the fibroblast and collagen reproduction in the wound area through the secretion of growth hormones and cytokines during healing period.[38—40]

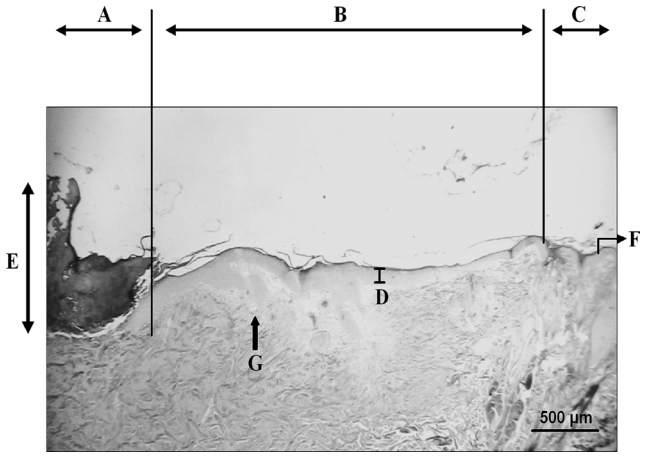

Fig. 4. The Scheme of the Microscopic Evaluation of the Wound Area
A: the eschar area of the wound, B: the wound epithelial elongation, C: non-burned epithelial area, D: wound epithelium thickness (the healing area of the wound), E: the eschar thickness of the wound, F: non-burned epithelium thickness, G: the papillary structures as called rete pegs or finger like projections.

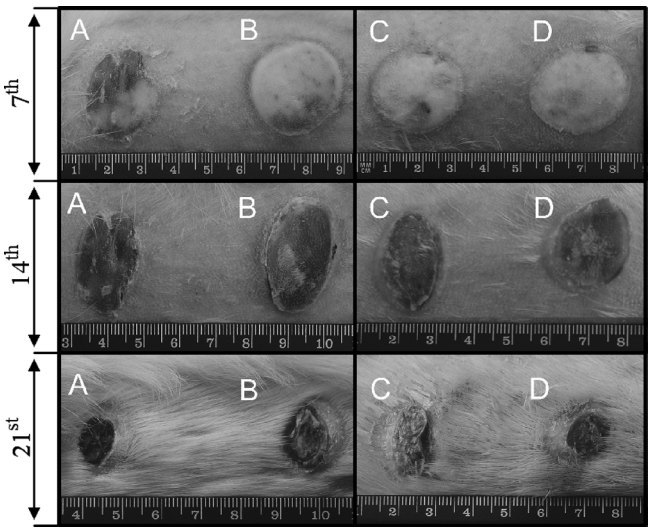

Fig. 5. The Photographs of the Wounds Area on Days 7, 14, and 21
A: control wounds, B: treated with chitosan hydrogel, C: treated with fucoidan-chitosan hydrogel and D: treated with fucoidan solution.

There was no significant difference between groups in the length of wound epithelium within the first 7 d ($p>0.05$), however, the longest new epithelium formation was observed in the group treated with fucoidan-chitosan hydrogel on days 14 (2358±40 μm) ($p<0.05$) and 21 (5566±555 μm) ($p<0.05$), as shown in Table 3. Wound epithelium thickness values in the fucoidan-chitosan hydrogel group, which was measured as 162±10 μm, 261±16 μm and 189±23 μm on days 7, 14 and 21 respectively, was shown to be significantly higher during healing period when compared to other groups (Table 3) ($p<0.05$). The increase in the epithelium thickness could be explained by migration of fibroblasts into healing zone as well as increasing in collagen synthesis in the wound

Table 2. The Histological Changes Graded from Absent to Severe in the Four Groups on Days 7, 14 and 21

Histological alteration	7th day Number (percentage)				14th day Number (percentage)				21st day Number (percentage)			
	Control group	Chitosan hydrogel group	Fucoidan-chitosan hydrogel group	Fucoidan-solution group	Control group	Chitosan hydrogel group	Fucoidan-chitosan hydrogel group	Fucoidan-solution group	Control group	Chitosan hydrogel group	Fucoidan-chitosan hydrogel group	Fucoidan-solution group
Bleeding												
Grade[a)]												
0	0 (0.0)	2 (33.3)	0 (0.0)	1 (16.7)	0 (0.0)	4 (66.7)	6 (100.0)	4 (66.7)	3 (50.0)	5 (83.3)	6 (100.0)	6 (100.0)
1	0 (0.0)	4 (66.7)	6 (100.0)	4 (66.7)	5 (83.3)	2 (33.3)	0 (0.0)	2 (33.3)	2 (33.3)	1 (16.7)	0 (0.0)	0 (0.0)
2	3 (50.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
3	3 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Fibroblast proliferation												
Grade												
0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (83.3)	0 (0.0)
1	6 (100.0)	4 (66.7)	0 (0.0)	0 (0.0)	4 (66.7)	4 (66.7)	0 (0.0)	1 (16.7)	4 (66.7)	0 (0.0)	1 (16.7)	6 (100.0)
2	0 (0.0)	2 (33.3)	6 (100.0)	6(100.0)	2 (33.3)	2 (33.3)	1 (16.7)	5 (83.3)	1 (16.7)	6 (100.0)	0 (0.0)	0 (0.0)
3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (83.3)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Collagen formation												
Grade												
0	5 (83.3)	3 (50.0)	0 (0.0)	1 (16.7)	4 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (66.7)	0 (0.0)
1	1 (16.7)	3 (50.0)	6 (100.0)	5 (83.3)	2 (33.3)	5 (83.3)	0 (0.0)	3 (50.0)	3 (50.0)	0 (0.0)	2 (33.3)	4 (66.7)
2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	3 (50.0)	3 (50.0)	3 (50.0)	5 (83.3)	0 (0.0)	2 (33.3)
3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (50.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)
Mononuclear leukocyte												
Grade												
0	5 (83.3)	4 (66.7)	2 (33.3)	5 (83.3)	4 (66.7)	2 (33.3)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	6 (100.0)	4 (66.7)
1	1 (16.7)	2 (33.3)	2 (33.3)	1 (16.7)	2 (33.3)	4 (66.7)	2 (33.3)	4 (66.7)	6 (100.0)	6 (100.0)	0 (0.0)	2 (33.3)
2	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Polymorphonuclear leukocyte												
Grade												
0	0 (0.0)	0 (33.3)	0 (33.3)	2 (33.3)	0 (0.0)	5 (83.3)	6 (100.0)	4 (66.7)	0 (0.0)	5 (83.3)	6 (100.0)	4 (66.7)
1	2 (33.3)	4 (66.7)	4 (66.7)	4 (66.7)	3 (50.0)	1 (16.7)	0 (0.0)	2 (33.3)	5 (83.3)	1 (16.7)	0 (0.0)	2 (33.3)
2	2 (33.3)	2 (33.3)	2 (33.3)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
3	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

a) 0: absent, 1: mild, 2: moderate, 3: severe.

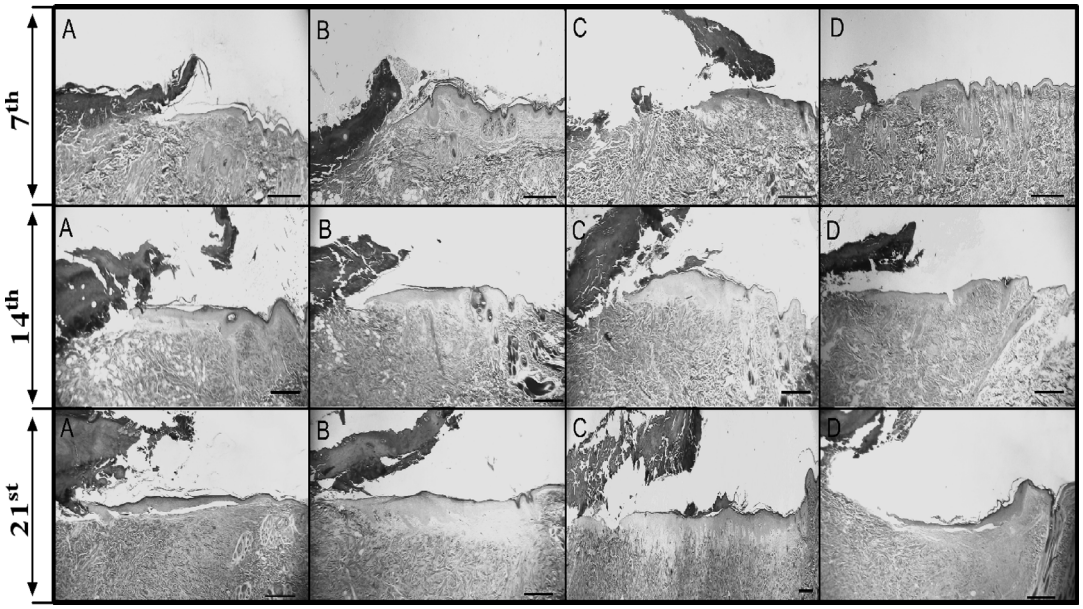

Fig. 6. The Histopathological Photographs of the Burn Epithelial Tissues Stained with Haematoxylin and Eosin on Days 7, 14, and 21
A: control wounds, B: treated with chitosan hydrogel, C: treated with fucoidan-chitosan hydrogel and D: treated with fucoidan solution, the bars are 500 μm.

Table 3. Wound Epithelial Elongation and Thickness Values on Days 7, 14 and 21 after Treatment (n=6)

Days	Wound epithelial elongation (μm±S.E.)				Wound epithelium thickness (μm±S.E.)			
	Control group	Chitosan hydrogel group	Fucoidan-chitosan hydrogel group	Fucoidan solution group	Control group	Chitosan hydrogel group	Fucoidan-chitosan hydrogel group	Fucoidan solution group
7	1302±90	1300±109	1333±93	1455±64	111±10	96±2	162±10	121±8
14	1950±82	1941±107	2358±40	2086±134	154±5	136±3	261±16	187±9
21	3533±196	3666±152	5566±555	3586±149	134±8	162±21	189±23	146±10

Table 4. The Number of Rete Pegs and NORs on Days 7, 14 and 21 after Treatment (n=6)

Days	Number of rete pegs±S.E.				Number of NORs±S.E.			
	Control group	Chitosan hydrogel group	Fucoidan-chitosan hydrogel group	Fucoidan solution group	Control group	Chitosan hydrogel group	Fucoidan-chitosan hydrogel group	Fucoidan solution group
7	3.4±0.5	3.5±0.2	4.7±0.2	3.1±0.4	2.7±0.2	2.9±0.1	3.2±0	2.9±0.2
14	3.9±0.2	3.5±0.2	4.5±0.2	6.1±0.2	2.6±0.2	4.2±0.1	8.5±0.1	3.6±0.5
21	3.8±0.4	4.5±0.5	8.3±2.8	4.6±0.7	2.6±0.3	4.3±0.1	1.8±0.1	3.5±0.8

area as previously reported.[41,42] It was observed that fibroblast and collagen amounts in the group treated with fucoidan-chitosan hydrogel had increased significantly during healing period (Table 2).

Contraction rate in the fucoidan-chitosan hydrogel group showed significant acceleration especially between days 14 and 21, whereas the smaller closure on the wound surface was achieved in other groups. Macroscopic and histopathologic findings indicated that the healing period of the wounds treated with fucoidan-chitosan hydrogel was completed on day 21, but other groups not (Tables 2 and 3, Figs. 5 and 6). It was observed a synergic effect between fucoidan and chitosan for the wound healing due to results obtained from previous studies have shown that fucoidan stimulates the fibro blast and epithelial cell growth and increases the secretion of TGF-β_1 accelerating the wound healing[11] and chitosan accelerates re-epithelization by accelerating infiltration on the wound area and providing fibroplasias.[8,43] Therefore, the higher treatment efficiency observed with fucoidan-chitosan hydrogel compared to other groups was suggested to be the result of the synergic effect of these two polymers.

At the dermal-epidermal junction, the counter of the bottom of the epidermis is irregular with numerous projections known as rete pegs or finger like structures (Fig. 4). These projections help to anchor the epidermis to the dermis.[44,45] Our results demonstrated the number of rete pegs in the

group treated with fucoidan-chitosan hydrogel was higher than the other groups during treatment period ($p<0.05$) and reached highest level (8.3 ± 2.8) on day 21 (Table 4, Fig. 6). The highest number of the nuclear organized regions (NOR) in the epithelial cell nucleus, stating the division and proliferation of cells, were seen in the fucoidan-chitosan group on day 14 (8.5 ± 0.1) when compared to other groups ($p<0.05$) (Table 4) and this finding was consistent with the results of previous studies.[44,45] As a result, it was proved that the fucoidan-chitosan hydrogel was found highly effective to increase the healing and re-epithelization of the wound by day 21.

CONCLUSION

The *in vitro* and *in vivo* studies investigating the efficacy of hydrogels in the treatment of dermal burns in rabbit model have shown that the application of fucoidan-chitosan hydrogel on the burn wound induces significant wound contraction and healing. Thus, the fucoidan-chitosan hydrogels may be suitable as a wound substitute and can be used in wound healing.

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