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Evaluation of burn wound healing activity of thermosensitive gel and PLGA nanoparticle formulation of quercetin in Wistar albino rats

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ABSTRACT

Modern burn treatments with gene therapy, stem cells and healing factors are highly cost and not always possible to access these treatments. For this reason, new effective treatment approaches should have easy accessibility. Recent studies have shown that quercetin, a flavonoid, has a wound-healing effect. However, quercetin has low water solubility and cannot uptake into cells sufficiently. To overcome these disadvantages quercetin nanoparticle and thermosensitive gel formulations appear to be potential alternatives. In this study, to compare the efficacy of quercetin-loaded PLGA nanoparticle with quercetin-loaded pluronic thermosensitive gel formulations, nanoparticle and thermosensitive gel formulations were developed, characterized and evaluated in the burn model. Biochemical analysis has been performed to investigate the effects of formulations on such as growth factors and some cytokines that are effective in wound healing process. Finally, histopathological analyzes has been performed to evaluate the presence and density of granulation tissue, inflammation and fibrosis. Encapsulation efficiency (EE) of nanoparticles was found 25 ± 5%. It is indicating that during the preparation of PLGA nanoparticles, there is more quercetin lost than preparation process of gel. Also quercetin release from PLGA nanoparticles is limited when compared with gel formulation of quercetin. PLGA nanoparticles and thermosensitive gel formulations are effective when compared with quercetin solution and Silverdin®. Considering the cost of PLGA polymer, EE and efficacy results it was concluded that pluronic thermosensitive gel formulations could be a cost-effective option for burn wound therapy.

1. Introduction

Exposure to certain physical or chemical factors such as heat, radiation, and chemical substance contamination, leads to tissue damage which is defined as burn [1]. Regarding the data of the World Health Organization (WHO) in 2018, approximately 180.000 fatal burn injuries occur annually [2]. The annual cost of treatment for a burn is estimated to exceed U.S$1 billion [3]. Since the 1960s silver compounds, which have been widely used in the topical treatment of burn wounds, have had systemic toxic effects such as dermatitis, allergy, hemolysis, formation of immune complexes, and methemoglobinemia [4–6]. On the other hand, modern burn treatments with gene therapy, stem cells, and healing factors are costly and not always possible to access these treatments [7,8]. For this reason, new effective treatment approaches should have low toxicity and easy accessibility.

Flavonoids are naturally sourced components with anti-inflammatory effects, antioxidant and antimicrobial activities. Quercetin (3,5,7,3′,4′-pentahydroxyflavone), one of these flavonoids, is widely distributed in many plants [9,10]. In addition to its anticancer, neuroprotective and antioxidant, anti-inflammatory, anti-cytotoxicity effects, recent studies have shown that quercetin has a wound-healing effect on many types of wounds [11–13]. C. mollissima shell ethanol extract (ECMS) containing quercetin showed anti-inflammatory and wound-healing effects by inhibiting TNF-a and IL-6 secretions in

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Table 1
Nanoparticle formulations and results.

Formulation Code	Amount of Quercetin (mg)	Amount of PLGA (mg)	Particle Size (nm)	Zeta Potential (mV)	Polydispersity Index	Encapsulation Efficiency (%)
F1	**3**	**30**	216 ± 10.4	−20 ± 4	0.10 ± 0.04	17 ± 11
F2	**2**	**30**	205.7 ± 8.5	−17.7 ± 3.5	0.10 ± 0.02	25 ± 5
F3	**1**	**30**	215.7 ± 15.5	−18 ± 5.2	0.09 ± 0.01	21 ± 5

macrophages [14]. Quercetin reduces fibrosis over the wound area without delaying wound closure. Over the injured area, quercetin increases αV integrin associated with fibroblast proliferation and decreases β1 integrin associated with fibroblast migration and fibrosis [15,16]. Quercetin scavenges free radicals such as hydroxyl ions and superoxide anions, exhibits antioxidant properties by preventing oxidative cellular damage and oxidant injury [17,18]. Despite all these effects, quercetin has low water solubility and cannot penetrate into cells sufficiently. To overcome these disadvantages quercetin nanoparticle and thermosensitive gel formulations appear to be potential alternatives [19–25]. Even though nanoparticle or gel formulations containing quercetin have been developed until today, these studies are limited due to the lack of comparison in terms of production difficulties and effectiveness.

In this study, to evaluate cost-effective option for burn wound therapy, quercetin-loaded PLGA nanoparticle and quercetin-loaded pluronic thermosensitive gel formulations were developed, characterized and applicated in the burn model. Biochemical analysis has been performed to investigate the effects of formulations on such as growth factors and some cytokines that are effective in wound healing process. Finally, histopathological analyzes has been performed to evaluate the presence and density of granulation tissue, inflammation and fibrosis.

2. Materials and methods

2.1. Animals

82 adults male Wistar albino rats weighing 220–250 g (8 weeks old) used in the study were obtained from Selcuk University Experimental Medicine Research and Application Center (SUDAM). Rats were housed on a 12-h dark/light cycle. Standard water and feed were given ad libitum. For the burn wound methodology, rats were kept alone in cages throughout the study. This study was carried out in accordance with ethical rules and was approved by the Animal Experiments Ethics Committee of Selcuk University Experimental Medicine Research and Application Center (Protocol number: 2020–4).

2.2. Chemical agents

Ketamine used in the study was obtained from Pfizer pharmaceutical company (Turkey), Xylazine (Rompun) from Bayer (Turkey) and silver sulfadiazine (Silverdin®) from Deva pharmaceutical manufacturer (Turkey). Quercetin, PLGA (50:50 lactide-glycolideratio) and polyvinyl alcohol (PVA) (MW 30.000–70.000) were purchased from Sigma Aldrich (USA). Acetone (99.5≤, ACS grade) was purchased from Carlo Erba (Turkey). Pluronic F127 was purchased from BASF (Turkey).

2.3. Preparation and characterization of quercetin-loaded nanoparticles

PLGA nanoparticles were prepared by the nanoprecipitation method [26,27]. Various formulation trials were carried out to load quercetin into nanoparticles with the highest efficiency. PLGA polymer and quercetin were dissolved in acetone in the amounts indicated in Table 1. The organic phase was added dropwise to a solution containing 10 ml of 1% PVA stirred at 1.100 rpm. Acetone was removed with a rotary evaporator. The nanoparticle suspension was centrifuged at 13.500 rpm for 15 min to remove uncharged quercetin and PVA, and the supernatants were discarded and the nanoparticles were collected. The particle

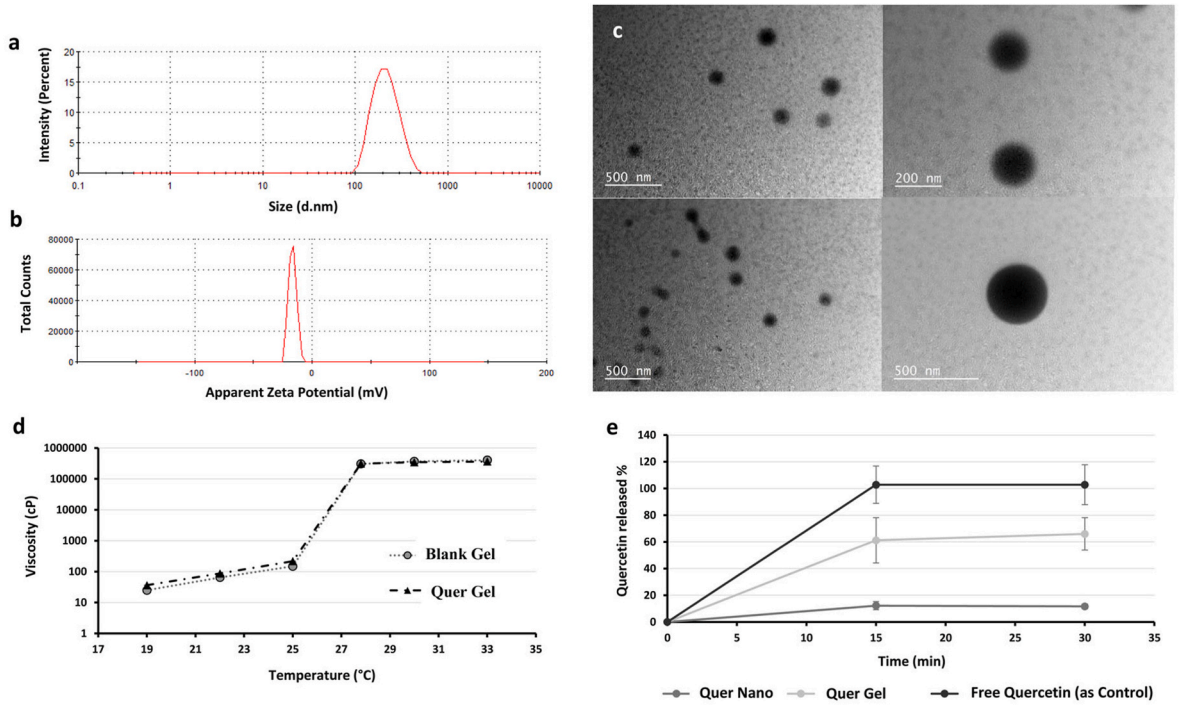

Fig. 1. **a:** Particle size distribution of Quercetin Loaded PLGA Nanoparticles **b:** Apparent Zeta Potential of Quercetin Loaded PLGA Nanoparticles **c:** TEM images of Quercetin Loaded PLGA Nanoparticles **d:** Viscosity against Temperature Evaluation of Thermosensitive Gels **e:** Quercetin release graph from nanoparticle and gel.

sizes and zeta potentials of the obtained nanoparticles were measured with Zetasizer Nano ZS (Malvern Instruments, UK). To determine the encapsulation efficiency, 1 mg of nanoparticles were dissolved in DMSO and the amount of quercetin was measured by UV spectrophotometry (Shimadzu UV-1800 spectrophotometer, Japan) at 378 Wavelengths. The morphological features of the selected formulation were determined by the transmission electron microscopy (TEM) method, and the quercetin release study from nanoparticles was carried out at 37 ± 1 °C with continuous stirring at 150 rpm, providing a sink condition in pH 7.4,1% Tween 80 containing PBS [28]. Briefly, 0.1 mg quercetin or nanoparticles containing the same amount of drug were added into release medium. At certain time intervals, the samples were analyzed with UV spectrophotometer and their release rates were plotted against time.

The encapsulation efficiency was calculated by the following formula:

Encapsulation efficiency (%) = Amount of quercetin loaded into nanoparticles / Amount of quercetin initially added to the formulation × 100

2.4. Preparation and characterization of quercetin thermosensitive gel

Pluronic thermosensitive gels were prepared by the cold method [23]. First of all, gels were prepared by using different ratios of Pluronic F127 and the ideal percentage was determined as 18%. Then, free quercetin-loaded thermosensitive gel was prepared by adding of 0.5 mg quercetin solved in 20 μl DMSO into the gel (final concentration: 0.5 mg/mL). Gelation temperatures were measured as described by Timur et al. [23]. Briefly, 10 mL of gel was added to a 20 mL clear vial at 4 °C with a magnetic stirrer. After this vial was placed in the water bath, continuous mixing of the magnetic fish was ensured at 150 rpm. The temperature of the water bath was then increased by 1 °C per minute. The point at which the magnetic fish stopped spinning was determined as the gelation temperature (Tg). The viscosity of the gel was measured with the Brookfield, DV2TRV Viscometer (Essex, UK) by the cone plate method by increasing the temperature at the temperatures indicated in Fig. 1d. The quercentin release from thermosensitive gel formulation was evaluated with similar method of nanoparticles. Briefly, gel formulations containing 0.1 mg quercetin were incubated at 37 ± 1 °C with continuous stirring at 150 rpm, providing a sink condition in pH 7.4,1% Tween 80 containing PBS. At certain time intervals, the samples were analyzed with UV spectrophotometer.

When quercetin-loaded PLGA nanoparticles were compared with quercetin-loaded pluronic thermosensitive gel formulations, EE of nanoparticles was found 25 ± 5%. It is indicating that during the preparation of PLGA nanoparticles, there is more quercetin lost than preparation process of gel. There is no loss of active substance in the preparation of the gel. Also quercetin release from PLGA nanoparticles is limited when compared with gel formulation of quercetin. Considering the cost of PLGA polymer and EE of both formulation. It was concluded that pluronic thermosensitive gel formulations seems advantageous because of its easier preparation, no loss of active substance during drug loading and lower cost. The difference in follow-up effectiveness studies will be evaluated together with these findings at the end of the study.

2.5. Burn model

In vivo second-degree burn model in animals was created similar to previous studies [29]. Briefly, after rats were anesthetized with ketamine and xylazine, the left area of their back was shaved and the shaved back skin was contacted with a hot cylinder filled with boiled water at 95 °C for 15 s to induce a second-degree burn model. A second-degree burn model was created in rats as a result of direct exposure to hot water. Except for Group1 (Control without burn), all rat groups were subjected to this burn model. The treatment protocol was initiated 24 h after the burn model was induced [30]. Since the created burn model caused dehydration after the burn model was created, 5 mL of saline was administered to all rats subcutaneously (s.c) and a drop of saline was dropped into their eyes. Wound images were photographed on the 1st, and 14th days to evaluate macroscopic wound healing rates and lesion sizes. Then tissues were stored at −80 °C for biochemical and histopathological analysis.

2.6. Experimental design

Animals were randomly divided into 8 groups:

- **Group-1: Control without Burn (Cntrlwo Burn)**

  The group with no wound and no treatment.

- **Group-2: Control only Sovent (Cntrl only Solvent)**

  The group with a burn and no treatment (75% DMSO).

- **Group-3: Blank PLGA Nanoparticles (Blank Nano)**

  Group with a burn and blank PLGA nanoparticles were applied.

- **Group-4: Blank Thermosensitive Gel (Blank Gel)**

  Group with a burn and blank thermosensitive gel was applied.

- **Group-5: Reference Treatment Silver Sulfadiazine Cream (Silverdin®)**

  Group with a burn and 1% silver sulfadiazine (Silverdin) was applied.

- **Group-6: Quercetin Solution (Quer Solution)**

  The group with a burn and the quercetin solution (in 75% DMSO) was applied at a dose of 0.5 mg/mL.

- **Group-7: Quercetin Loaded PLGA Nanoparticles (Quer Nano)**

  The group with a burn and quercetin-containing PLGA nanoparticles (nano-quercetin) were applied, with a quercetin dose of 0.5 mg/mL.

- **Group-8: Quercetin Loaded Thermosensitive Gel (Quer Gel)**

  The group with a burn and free quercetin-loaded thermosensitive gel was applied, with a quercetin dose of 0.5 mg/mL.

All prepared formulations were applied topically to the burned back area, once a day for 14 days, in a volume to cover the burned area, in accordance with the method.

2.7. Biochemical analysis

For biochemical analysis, tissues were taken from the burned areas of the rats on the 14th day. It was stored at −80 °C until the day of analysis. Tissues' weight was recorded. All the samples put out to room temperature on the day of analysis. It was homogenized to 1/10 with PBS (0.01 M, pH = 7.4, Sigma catalog no: P4417). Homogenization was performed with Heidolph brand Silent Crusher mechanic homogenizer (Schwabach/Germany). Homogenates were centrifuged at 5000 g for 10 min and obtained supernatants were portioned to analyze concentrations of TGF-β, TNF-α, IL-6 and IL-1β. All the analyzes were performed in accordance with the commercial test kit procedure, with Rayto Microplate Elisa washer (RT-2600; Shenzhen, China) and BMG Labtech (Ortenberg, Germany) Elisa reader.

Interleukin 1β analysis was performed with the BT-Lab trade Elisa kit (Catalog no: E0119Ra). The sensitivity of the assay was 10.23 pg/mL, the standard curve range was 20–8000 pg/mL, the intra-assay CV value was <8%, and the inter-assay CV value was <10%. Values were calculated as pg/mL.

Interleukin 6 analysis was performed with the BT-Lab trade Elisa kit (Catalog no: E0135Ra). The sensitivity of the assay was 0.052 ng/L, the standard curve range was 0.1–40 ng/L, the intra-assay CV value was <8%, and the inter-assay CV value was <10%. Values were calculated as ng/L.

Tumor Necrosis factor-α (TNF-α) analysis was performed with BTLab brand Elisa kit (Catalog no: E0764Ra). The sensitivity of the assay was 2.51 ng/L, the standard curve range was 5–1000 ng/L, the intra-assay CV value was <8%, and the inter-assay CV value was <10%. Values were calculated as ng/L.

Transforming growth factor (TGF-β) analysis was performed with the BT-Lab trade Elisa kit (Catalog no: E1688Ra). The sensitivity of the assay was 4.38 ng/L, the standard curve range was 8.12–2000 ng/L, the intra-assay CV value was <8%, and the interd-assay CV value was <10%. Values were calculated as ng/L.

All data obtained were calculated by proportioning the gram tissue values and the units of analyzes were expressed as pg/g tissue for IL-1β, and ng/g tissue for IL-6, TNF-α, TGF-β.

2.8. Histopathological analysis

On histopathological analysis, tissues obtained on 14th day were fixed in 10% neutral-buffered formalin for 24 h and then embedded in paraffin. Sections of 4 μm thick were taken on slides with microtome and then stained with both Hematoxylin-eosin and Masson trichrome stains to evaluate fibrosis. On Leica BX53 microscope, the following parameters were assessed: The presence and thickness of the regenerated epithelium and granulation tissue, the presence and intensity of inflammation and fibrosis.

The thickness of epithelium and granulation tissue were calculated by using computer assisted image analysis program. Inflammation and fibrosis scores were given as: None, 0; mild, 1; moderate, 2; severe,3.

2.9. Statistical analysis

Comparisons of more than two groups were performed using ANOVA followed by Tukey's post hoc test. For comparing two groups, student's t-test was performed. Data are demonstrated as the mean ± standard deviation.

3. Results and discussion

3.1. Preparation and characterization of quercetin loaded nanoparticles

The nano precipitation method is a frequently used method for the preparation of nanoparticles loaded with hydrophobic drugs [31]. Although the particle size generally varies depending on the amount of polymer in organic phase, spherical monodisperse particles of the size between 50 and 300 can be obtained with nanoprecipitation method [27,32]. In this study, nanoparticles were prepared by nanoprecipitation method considering the hydrophobic property of quercetin. Since the effects of process parameters on the characteristics of nanoparticles have been studied in detail in our previous study [27], in this study, amount of quercetin was defined as critical factor and a study was carried out to determine the highest encapsulation efficiency. F1, F2, F3 formulation trials were carried out by adding 3, 2, 1 mg of quercetin, respectively, into the fixed amount of polymer. The average particle sizes of the F1, F2 and F3 nanoparticle formulations prepared by the nanoprecipitation method are 216, 205 and 215 nm, respectively, and the PDI values of the nanoparticles are 0.1, 0.1 and 0.09, respectively (Table 1). Obtained particle sizes were found in defined range in previous studies with low PDI values [27,33]. The amount of quercetin loaded into the nanoparticles did not cause a significant effect on the nanoparticle size. All PLGA nanoparticles have negative surface potential (between −17.7 and −20) and there is no significant zeta potential difference between formulations (Table 1). TEM micrographs are shown in Fig. 1c to show the morphology of the nanoparticles. All nanoparticles are spherical and the particle size of the nanoparticles is consistent with DLS measurements. Among the prepared formulations, the F2 formulation showed the highest encapsulation efficiency with 25 ± 5%, and there is no linear relationship between the amount of quercetin used during nanoparticle preparation and the encapsulation efficiency (Table 1). In addition, the F2 formulation has the smallest nanoparticle size with a size of 205.7 ± 8.5 nm. For these reasons, the F2 formulation was chosen as the optimum formulation and further studies were continued with this formulation. The release profile of quercetin-loaded nanoparticles is shown in Fig. 1e. In a study, it is concluded that approximately 100% of the free quercetin used as a control was released in the first 15 min, whereas approximately 12% of the quercetin loaded into the nanoparticles was released. Also similar burst release from quercetin loaded PLGA nanoparticles was demonstrated [25]. This showed that controlled release of quercetin could be achieved with nanoparticles. However, the amount of quercetin released from the nanoparticles was limited to about 12%. This may be due to the limited corrosion of nanoparticles as a result of the loading of quercetin, a hydrophobic drug, onto PLGA, which is also a hydrophobic polymer. While this limited drug release may provide an advantage in delivering the drug to deeper tissue, it may also be a disadvantage if the free drug limits its curative effect on the surface.

3.2. Preparation and characterization of quercetin thermosensitive gel

In the preparation of the thermosensitive gel formulation, a thermosensitive gel formulation was prepared by using plunoric F127, which is widely used for this purpose [23]. The gelation temperatures of the formulations containing and without quercetin were found to be 27.5 °C and 28 °C, respectively. The results of the gelation temperature, the temperature at which the liquid phase turns into a gel, are consistent with the results of the viscosity studies. In this study, an in-sugar gelling system was used to facilitate the application of the formulation to burns and wounds, to ensure the reproducibility of the application, to increase the residence time on the wound and to create a dressing for the wound. The formulation gels at a temperature above room temperature and below human body temperature. In this way, it becomes easier to apply the formulation to wounds and burns.

The change in the viscosity of the prepared gel against the increase in temperature was investigated and as seen in Fig. 1d, the viscosity of the gel increased as the temperature increased. The viscosity of the empty gel at 25 °C was found to be 147.63 Poise, and the viscosity of the gel containing quercetin was 216.53 Poise. At 33 °C, the viscosity of the empty gel increased to 403,200 Poise, while the viscosity of the quercetin-loaded gel increased to 366,100 Poise. These results show us that free quercetin has no effect on the thermosensitivity of Pluronic F127. There is no step in the preparation of the gel that will cause drug loss. The release profile of the quercetin-loaded gel is shown in Fig. 1e. While approximately 100% of the free quercetin used as a control was released in the first 15 min, approximately 65% of the quercetin loaded in the gel was released. Compared to the nanoparticle formulation, the higher drug release will provide the advantage of higher effectiveness on the wound surface.

3.3. Biochemical results

It is important and immediate to consider the physiopathology of the burn for an effective treatment. The healing of burn wounds depends on many factors, such as the degree of the burn, its cause, the general condition of the patient, and associated co-morbidities [34]. Wound healing is a biological process that starts with inflammation and cell

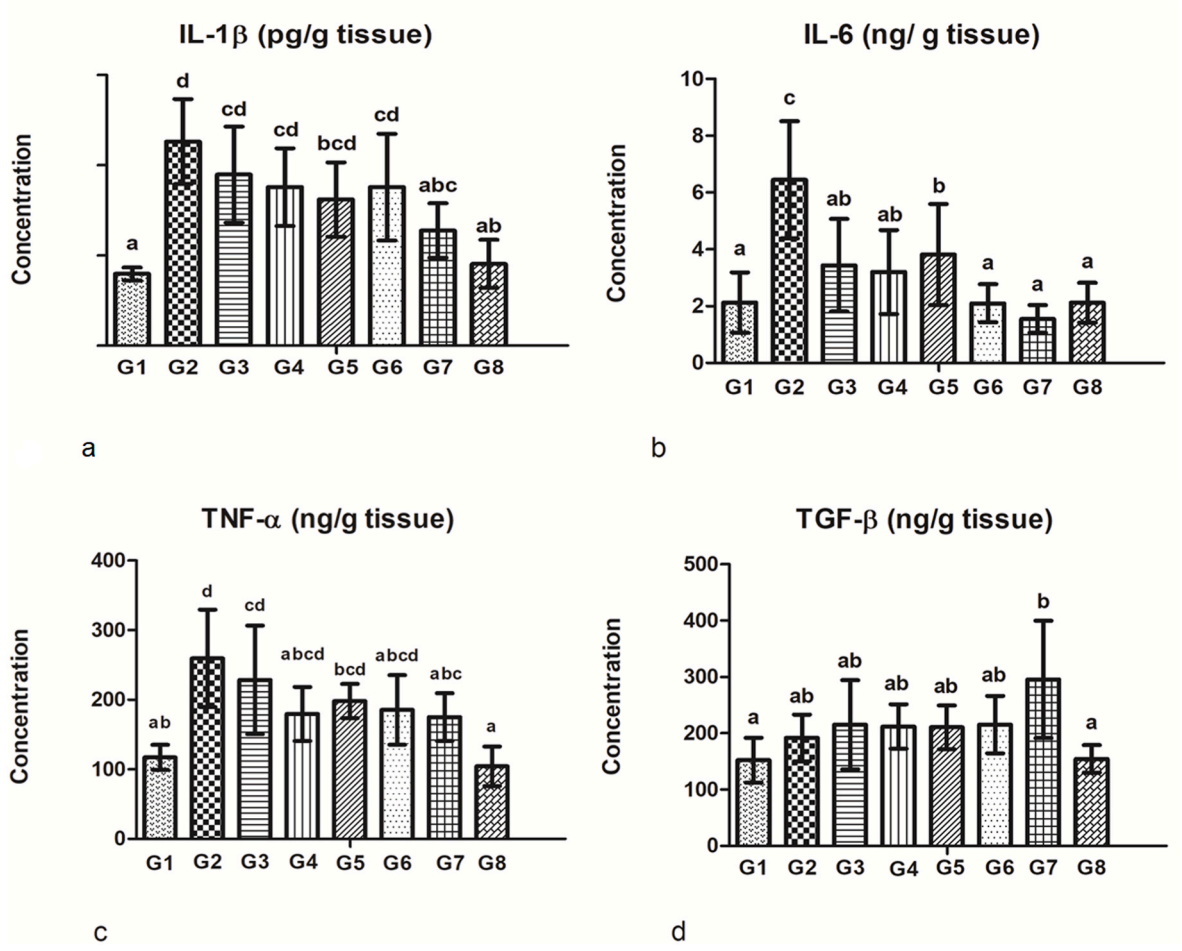

Fig. 2. a: Comparison of IL-1β Levels of Study Groups, **b:** Comparison of IL-6 Levels of Study Groups, **c:** Comparison of TNF-α Levels of Study Groups, **d:** Comparison of TGF-β Levels of Study Groups. a,b,c,d: The difference between the means between groups with different letters in the same column is important. ($P < 0.05$). (G1: Cntrl wo Burn, G2: Cntrl only Solvent, G3: Blank Nano, G4: Blank Gel, G5: Silverdin®, G6: Quer Solution, G7: Quer Nano, G8: Quer Gel).

proliferation at the cellular level, in which various cell groups serve together [16]. In this process, it is a success to regain the damaged functional structure by the integration of factors characterized with cell division, chemotaxis, angiogenesis, extracellular matrix protein synthesis, and scar formation. In the performing of these functions, inflammatory cells, fibroblasts, keratinocytes, cytokines involved in intercellular communication, and chemotaxis is required for the release of mediators such as growth factors [35].

The local response to the sudden increase in body surface temperature begins with vasodilation in the blood vessels in the burned area and with the activation of the concurrent pathways to lower the temperature. That is, local physio pathological changes in the burn wound are an inflammatory process characterized by heat-induced protein denaturation and the results with the release of inflammatory mediators. Cytokines and other inflammatory mediators released from the burn area cause systemic effects. The inflammatory response consists of a short period of vasoconstriction followed by periods of vasodilation and hyperemia that increase blood flow to the injured area. In the inflammatory phase of wound healing, the vasoconstriction mechanism, which is activated to reduce blood loss in damaged vessels, activates the coagulation cascade. Platelets activate clotting factors, while thrombin stimulates platelets [36]. Thus, chemotactic and vasoactive regulators released into the environment cause short-term arteriolar vasoconstriction. This process is followed by active vasodilation [37]. Inflammatory response is an important step in the wound healing process, even though excessive inflammatory response delays wound healing. The inflammatory cascade triggered by thermal burn causes the production of dangerous pro-inflammatory such as Interleukins (IL-1, IL-6), Tumor Necrotic Factor-alpha (TNF-α), Prostaglandin E2 (PGE2) and Transforming Growth Factor (TGF-β) [38].

In the proliferative phase of wound healing, fibroblasts migrate through the extracellular matrix towards the wound with the effect of growth factors [39]. In the proliferative phase, the primary stimulator of the new blood vessels is vascular endothelial growth factor (VEGF). Macrophages play a key role in angiogenesis by secreting TNF-α and fibroblast growth factor (FGF). Therefore, VEGF is known to be the most effective factor among all growth factors that trigger wound angiogenesis, which is important in the skin regeneration process [40]. Growth factors are powerful promoters of wound healing. These factors accelerate incisional wound repair through different mechanisms. Transforming growth factor-beta (TGF-beta), which is identified a chemotactic factor, take roles in stimulating granulation tissue and increasing synthesis of extracellular matrix. A single topical dose of TGF-beta has been shown to increase the wound breaking strength in normal tissue repair besides in models of impaired wound repair which is with severe monocytopenia [41].

Furthermore, a biological mechanism of wound healing includes cellular interactions of fibroblast, keratinocyte, immune and endothelial cells which means cell proliferation, inflammation, adhesion and neovascularization. Therefore, TGF-β1 and VEGF-α is effective in the formation of biomolecules during the wound healing process. It is well known that cell growth and differentiation, polypeptide production are effected more by TGF-β than other growth factors in the wound healing process [40]. In addition, macrophages are major cellular source of

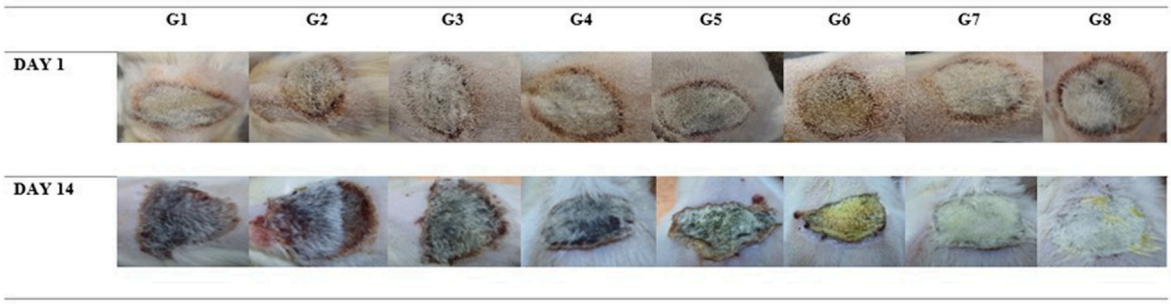

Fig. 3. The effects of formulations on the evolution of a wound. Macroscopic wound healing examples on days 1 and 14. (G1: Cntrl wo Burn, G2: Cntrl only Solvent, G3: Blank Nano, G4: Blank Gel, G5: Silverdin®, G6: Quer Solution, G7: Quer Nano, G8: Quer Gel).

TNF-α and IL-6. These cytokines mediates the acute phase responses to injury and achieve maximum levels at the 12th and 24th hours following the burn trauma. So that, the process of post burn complications such as sepsis and immune dysfunction are mediated majorly by TNF-α and IL-6 [42].

Based on this information, we aimed to compare the efficacy of quercetin-loaded PLGA nanoparticle and thermosensitive gel formulations as a burn wound treatment. These formulations were evaluated biochemically in terms of inflammation and proliferation biomarkers. Also, we performed a pathological point of view with data of inflammation and fibrosis scores and the thickness of epithelium and granulation tissues.

3.3.1. Evaluation of Interleukin-1 Beta's (IL-1β) values

In the present study, IL-1β levels (pg/g tissue) were found as795.48 ± 71.33 in Group 1, 2262.45 ± 470.47 in Group 2, 1896.91 ± 532.96 in Group3, 1757.82 ± 431.11 in Group 4, 1621.04 ± 411.16 in Group 5, 1757.60 ± 590.15 in Group 6, 1275.61 ± 301.89 in Group 7 and906.46 ± 268.20 in Group 8, respectively (Mean ± SD). As seen in Fig. 2a, it was determined that the IL-1β levels of the Group 7 and Group 8 groups reached to the level of the Group 1, and the differences between the groups were not significant. The difference between Group 7 and Group 8 with the Group 2 was statistically significant. While the difference between the Group 8 and Group 3, Group4, Group6 was statistically significant, no statistically significant difference was observed between the Group5 and Group 8.

The highest level of IL-1β levels (pg/g tissue) were in control group (with DMSO) and group of the quercetin solution (in 75% DMSO) was administered at a dose of 0.5 mg/mL. These results show us that treatment with form of quercetin with DMSO solvent is still in period of late inflammation phase. As it known, the inflammation phase begins at least 72 h after the injury. The proliferation phase begins on the third day after injury and continues for about 2 weeks [43,44]. Therefore, high inflammation biomarkers levels at 14 days of the wound healing indicates that the progression to the proliferative phase are delayed, and the tissue has not still completed the inflammatory processes and has not developed enough tissue immunity. According to levels of group 6,7 and 8; we commented as treatment with free quercetin-loaded thermosensitive gel has lower level of IL-1β and this level were similar with the healthy control group. But, treatment with quercetin loaded with DMSO group showed higher inflammation than quercetin-loaded thermosensitive gel and quercetin-containing PLGA nanoparticles groups. Thus, we can conclude that treatment with the form of quercetin-thermosensitive gel and PLGA nanoparticles are more effective than the treatment with DMSO solvent form. When we evaluate the reference group with all quercetin treated group according to IL-1β levels, we found IL-1β concentrations were higher in references group than the groups treated with quercetin-thermosensitive gel (Group8) and PLGA nanoparticles (Group7). The differences between the groups were not statistically different, but comparing the Group 5 with healthy group there was a significant difference. Therefore, we conclude that the group treated with Silver Sulfadiazine Cream did not reach to the level of healthy control group as the opposite to the findings of the group treated with form of quercetin-thermosensitive gel (Group8) and PLGA nanoparticles (Group 7) reach to the healthy values.

When we consider IL-6 and IL-1βdata as a whole, we concluded that the groups treated with the forms of quercetin-thermosensitive gel (Group 8) and PLGA nanoparticles (Group 7) are more effective in wound healing regarding inflammation status. As is well known, neutrophil counts decrease after 2–3 days in the absence of infection. However, in the late phase of inflammation IL-1β affects the lymphocytes to migrate as a last cell to the wound area [43,44]. This means that the inflammatory phase of wound healing still continues and the healing process takes longer when compared to quercetin supplemented groups (Group 7 and Group 8) with references group (Fig. 2a).

3.3.2. Evaluation of Interleukin-6 (IL-6)'s values

IL-6 levels (ng/g tissue) were 2.13 ± 1.06 in Group 1, 6.45 ± 2.06 in Group 2, 3.44 ± 1.63 in Group 3, 3.20 ± 1.48 in Group 4, 3.82 ± 1.78 in Group 5, 2.10 ± 0.67 in Group 6, 1.55 ± 0.49 in Group 7 and 2.12 ± 0.70 in Group 8, respectively (Mean ± SD) in the present study. IL-6 levels in the Group 6, Group 7 and Group 8 showed a similar value to our healthy control (Group 1) group. Il-6 levels were a significantly higher than all the remaining groups. The statistical difference between the Silverdin treatment group (Group 5), which is defined as references group, and the Group 1, Group 6, Group 7 and G8 is remarkable different from each other (Fig. 2b).

As it seen in Fig. 2b; IL-6 levels of the groups with burn and treatment of Quercetin + DMSO (Group 6), quercetin-containing PLGA nanoparticles (Group 7), free quercetin-loaded thermosensitive gel (Group 8) were not differ from the control group. These findings show that inflammation decreased in all groups given different forms of quercetin to the level of healthy, therefore, we consider that the effects of each forms of quercetin on healing were similar according to evaluation of IL-6 levels. Moreover, in reference group which is included treatment with Silver Sulfadiazine Cream showed higher IL-6 levels comparing with the other all quercetin forms treated group. We consider that late phase of burn healing wound is still incomplete for references group although the process of the late inflammation phase should be completed up to two weeks. So that, this is an indication that the treatment with Silver Sulfadiazine Cream is not as effective as the groups of treatment of the quercetin. The other point of our findings is that; both groups with treated of non-quercetin-loaded thermosensitive gel and PLGA nanoparticles without quercetin loaded showed higher levels of IL-6 than the Group 6,7 and 8. The differences between the groups were not statically important. Control group with 75% DMSO which is defined as group2 showed the highest level of IL-6 levels. All these findings showed that the quercetin loaded groups treatment is more effective comparing than the all other groups. Moreover, we can come to conclusion with the findings of biochemical inflammatory marker IL-6 levels (Fig. 2b) and recovery images (Fig. 3), the treatment with quercetin loaded show that the immune barrier formation process against microorganisms has been

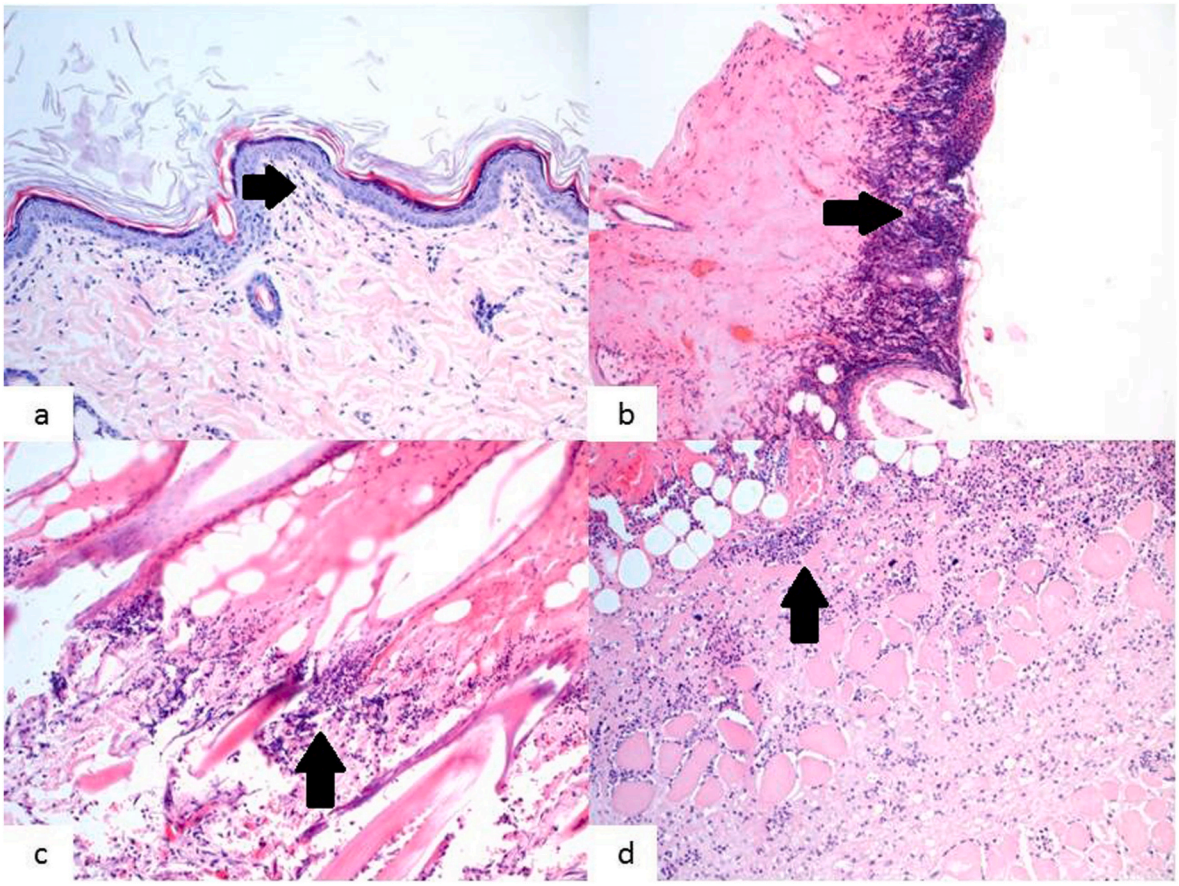

Fig. 4. Mild (score 1) inflammation in healthy control group (Group 1) (a), severe (score 3) inflammation in Group 2 and Group 3 (b and c), moderate to severe (score 2 to 3) inflammation in Group 7 (d). (HEX200) (G1: Cntrl wo Burn, G2: Cntrl only Solvent, G3: Blank Nano, G4: Blank Gel, G5: Silverdin®, G6: Quer Solution, G7: Quer Nano, G8: Quer Gel).

completed in normal process.

3.3.3. Evaluation of Tumor Necrosis factor Alfa (TNF-α)'s values

TNF-αlevels (ng/g tissue) were as 117.28 ± 17.84 in Group 1, 259.63 ± 70.03 in Group 2, 228.45 ± 77.95 in Group 3, 179.56 ± 38.85 in Group 4, 198.13 ± 24.56 in Group 5, 185.49 ± 49.90 in Group 6, 175.22 ± 34.27 in Group 7 and 104.55 ± 28.29 in Group 8, respectively (Mean ± SD). As seen in Fig. 2c, the levels of TNF-α were reduced in Group 7 and Group 8 treatment groups with the similar levels in Group 1, healthy control group. Again, the difference between Group 7 and Group 8 and the Group 2 was statistically significant. Whereas the difference between the Group8 group and the Group3, Group5 groups was significant, no statistically significant difference was found in the Group7 treatment group.

In our experimental groups, at the 14 th day of the samples taken, the lowest levels were found in quercetin thermosensitive gel treated group. This level was not differing from healthy group. Furthermore, in evaluation of values of all quercetin supplemented group and reference group, it is clear that form of quercetin-thermosensitive gel has robust anti-inflammatory effects. Furthermore, we can emphasize that in accordance of wound healing processes evaluated in terms of daily biological status, it was determined that wound healing was faster in the quercetin supplemented group which can be seen in both Figs. 3 and 5.

As it well known, TNF-α and IL-1 especially with TGF-β1 and TGF-β2 secreted from inflammatory cells and extracellular fragments, stimulate the migration, proliferation of the fibroblasts to the wound area and collagen production from the 3rd day after injury [45]. Therefore, we postulate that during the 14 days treatment period, the quercetin-thermosensitive gel showed its effect in all processes in the inflammation phase towards healing wound and had a healing effect due to have its' lowest in TNF-α.

3.3.4. Analysis of Transforming Growth Factor beta (TGF-β)

In experimental groups, TGF-β levels (ng/g tissue) were as 152.21 ± 39.87 in Group 1, 191.80 ± 41.48 in Group 2, 215.16 ± 19.18 in Group 3, 212.06 ± 39.40 in Group 4, 210.52 ± 39.01 in Group 5, 215.35 ± 50.91 in Group 6, 295.51 ± 104.15 in Group 7 and 154.57 ± 24.52 in Group 8, respectively (Mean ± SD). When we evaluate the TGF-β levels according to Fig. 2d, there is no significant difference between the levels of Group 1 and Group 8, but a statistically significant difference is observed between the Group 7 and both groups. TGF-β, which is one of the most important growth factors of the proliferation phase of wound healing, stimulates inflammation, angiogenesis, re-epitalization and connective tissue regeneration and collagen synthesis [38,40]. The phase that starts at the 2–3 weeks after burn damage are defined as the maturation of the burn wound healing. In this phase, collagen and elastin are produced in the scar tissue area. TGF-β takes a role in this phase [46].

In our study, the highest levels were found in Group 7 who were treated with quercetin loaded- PLGA nanoparticles. When we evaluate whole result which is shown in Fig. 2d, quercetin-thermosensitive gel form of quercetin has close levels with the healthy group. In group 7 we think that the maturation phase is still keep on. We think that this may be due to the fact that only 12% of the quercetin can be released from nanoparticles. Since most of the quercetin remains in nanoparticles, the importance of penetrating state becomes evident. Thus, a large part of the drug is not released. The mentioned process can be interpreted as the late or long effect of the quercetin with this form in the treatment of

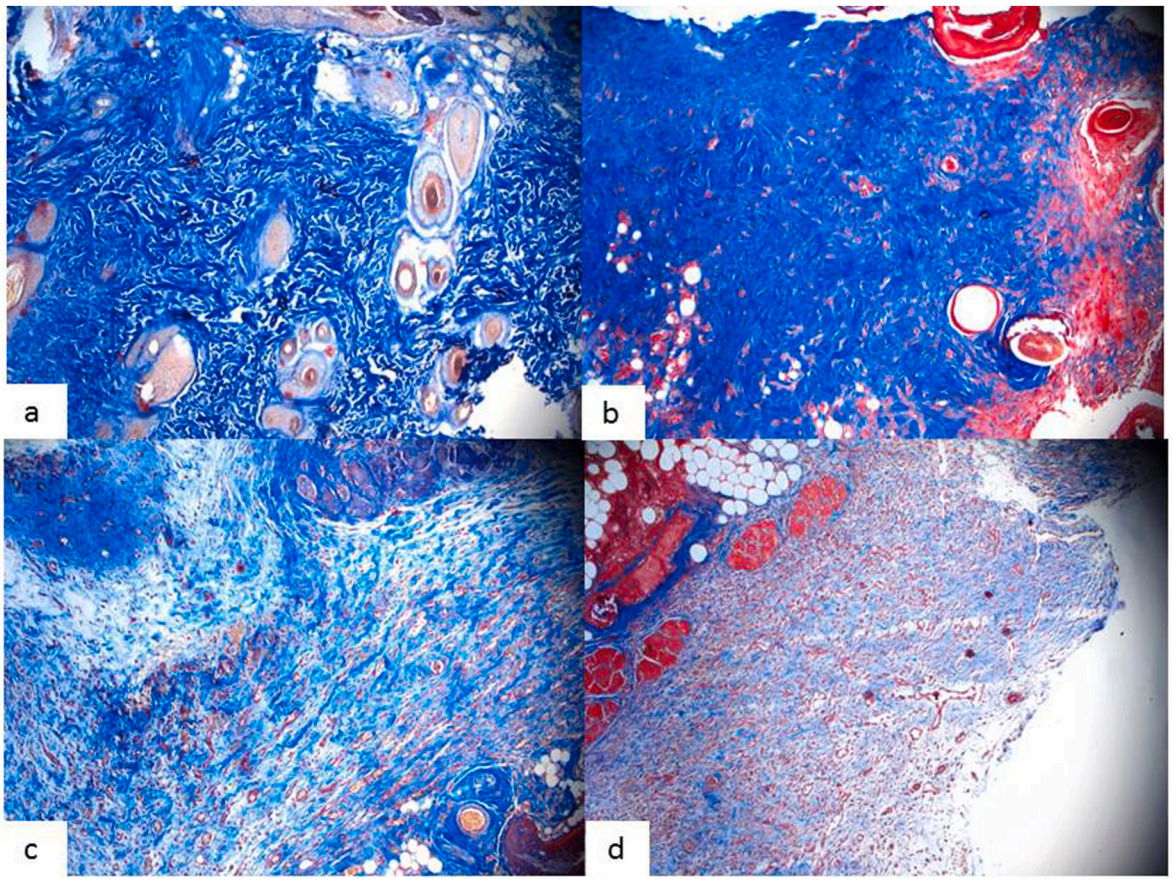

Fig. 5. Moderate (score 2) fibrosis in Group 2 and Group 6 (a and b) and mild (score 1) fibrosis in Group 7 and Group 8 (c and d). (Masson trichrome X100) (G1: Cntrl wo Burn, G2: Cntrl only Solvent, G3: Blank Nano, G4: Blank Gel, G5: Silverdin®, G6: Quer Solution, G7: Quer Nano, G8: Quer Gel).

burns (Fig. 3).

As a result of our study, when all biochemical parameters are evaluated regarding 14 day of treatment, it can be said that the effects of all groups treated with quercetin on wound healing are more effective than the reference treatment, and quercetin-thermosensitive gel form of quercetin has a superior anti-inflammatory effects to the DMSO solvent and PLGA nanoparticles form of quercetin.

Furthermore, another important finding of our study is pathological examinations which makes our study more comprehensive.

3.4. Histopathological results

In the present study, inflammation scores were determined as 0,83 ± 0,4 in Group1, 3,00 ± 0,00 in Group2, 3,00 ± 0,00 in Group3, 2,75 ± 0,50 in Group 4, 3,00 ± 0,00 in Group 5, 2,80 ± 0,44 in Group 6, 2,60 ± 0,54 in Group 7 and 3,00 ± 0,00 in Group 8, respectively (Mean ± SD) (Fig. 4). Inflammation score was lowest in healthy control group (G1) and the difference between Group 1 and other groups was statistically significant, however, there was no statistically significant difference between any of the treatment groups. Within the treatment groups, the lowest inflammation score was in Group7, quercetin-containing PLGA nanoparticles group. Despite the lack of statistical significance, this result was consistent with biochemical findings and shows the anti-inflammatory effect of quercetin-containing PLGA nanoparticles.

Fibrosis scores were as 0,00 ± 0,00 in Group1, 2,00 ± 0,81 in Group2, 1,50 ± 0,57 in Group3, 1,00 ± 0,00 in Group 4, 1,75 ± 0,50 in Group 5, 2,20 ± 0,44 in Group 6, 1,00 ± 0,00 in Group 7 and 1,00 ± 0,00 in Group 8, respectively (Mean ± SD) (Fig. 5). Fibrosis score was lowest in healthy control group (Group 1) and the difference between Group 2 and Group 6 groups and Group 4, Group 7 and Group 8 groups was statistically significant. Control group with 75% DMSO (Group 2) and quercetin loaded with DMSO group (Group 6) revealed higher fibrosis scores compared to quercetin-containing PLGA nanoparticles (Group 7) and free quercetin-loaded thermosensitive gel (Group 8).

TGF-β is involved in a variety of wound healing processes, including inflammation, angiogenesis stimulation, fibroblast proliferation, collagen synthesis and deposition, and remodelling of the new extracellular matrix [47,48]. Fibrosis is expected to be more prevalent in groups with higher levels of TGF-β. The results regarding fibrosis were compatible with biochemical results of the present study and the fibrosis scores were lower in quercetin-containing PLGA nanoparticles (Group 7) and free quercetin-loaded thermosensitive gel (Group 8). This finding reveals that these formulations can reduce formation of fibrosis in wound healing process. Consequently, we can emphasize that treatment with quercetin-thermosensitive gel and PLGA nanoparticles is more effective than treatment with DMSO solvent.

The other histopathological parameter evaluated in the present study was thickness of the regenerated epithelium. The thickness of the regenerated epithelium was calculated as 0,00 ± 0,00 in Group 1, 1283,75 ± 178,57 in Group 2, 1047,12 ± 171,26 in Group 3, 1356,97 ± 144,10 in Group 4, 1072,85 ± 311,09 in Group 5, 1220,96 ± 283,65 in Group 6, 1450,02 ± 380,80 in Group 7, 1328,50 ± 193,56 in Group 8, respectively (μm ± μm) (Fig. 6). The difference between Group 1 and the treatment groups was significant, however, within the treatment groups, no statistically significant difference was determined.

The last parameter included in the study was thickness of the granulation tissue. It was calculated as 0,00 ± 0,00 in Group 1, 1220,05 ± 340,06 in Group 2, 699,37 ± 272,76 in Group 3, 1210,12 ± 354,98 in Group 4, 1698,22 ± 737,23 in Group 5, 992,50 ± 143,46 in Group 6, 2114,28 ± 541,11 in Group 7, 1933,88 ± 436,22 in Group 8,

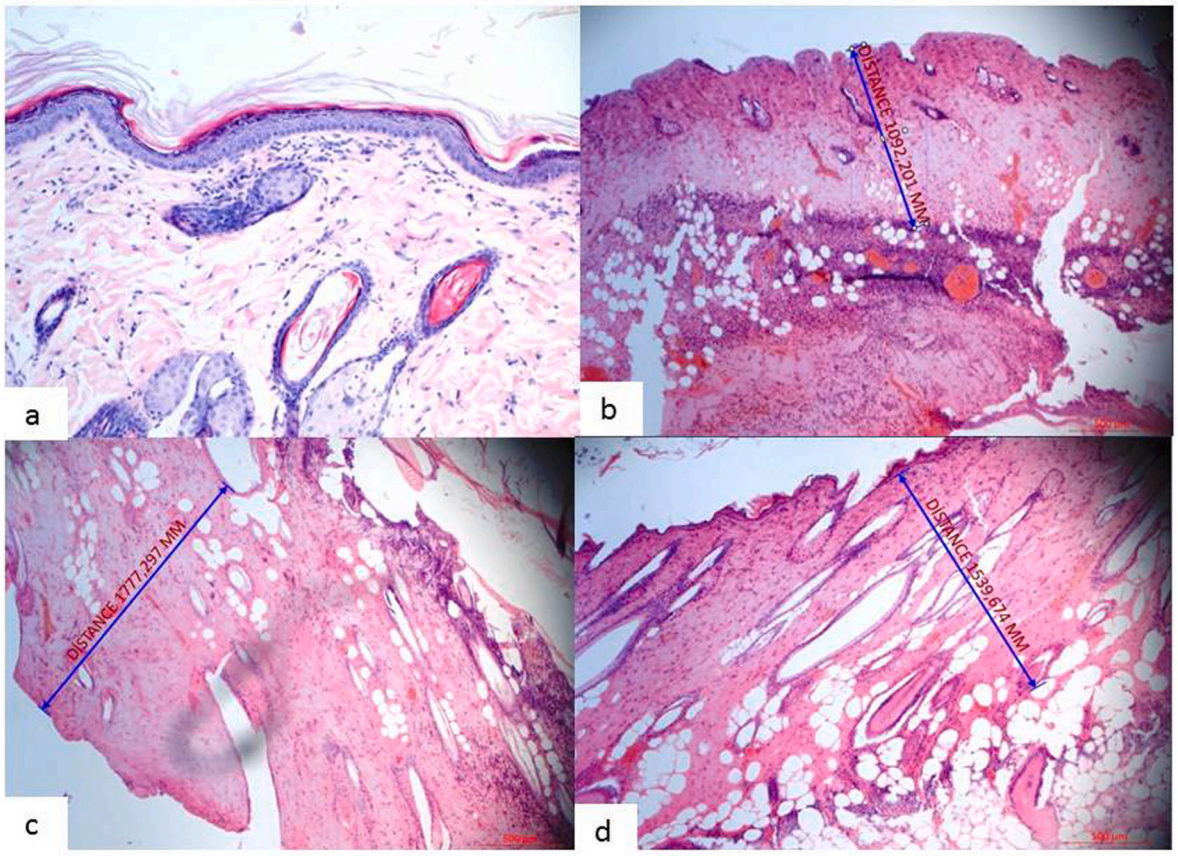

Fig. 6. Intact epithelium without regeneration in healthy control group (Group 1) (a), thickness of the regenerated epithelium in Group 3, Group 7 and Group 8 (b, c and d). (HEX100) (G1: Cntrl wo Burn, G2: Cntrl only Solvent, G3: Blank Nano, G4: Blank Gel, G5: Silverdin®, G6: Quer Solution, G7: Quer Nano, G8: Quer Gel).

respectively (μm ± μm) (Fig. 7). There was statistically significant difference between the groups. Higher values were determined in quercetin-containing PLGA nanoparticles (Group 7) and free quercetin-loaded thermosensitive gel (Group 8) groups compared to other groups.

TGF-β helps with wound healing and re-epithelialization. TGF-β1 is rapidly upregulated and secreted by keratinocytes, platelets, monocytes, macrophages, and fibroblasts following acute injury. TGF-β1 is required for the initiation of inflammation and the formation of granulation tissue [48]. In the present study, the highest levels of TGF- β were found in Group 7, quercetin-containing PLGA nanoparticles, biochemically. This was supported by increased granulation tissue thickness in Group 7, which was statistically significant. Thus, we can conclude that quercetin-containing PLGA nanoparticles can provide a better wound healing process by increasing granulation tissue thickness. Even though statistically no significant were found, epithelial thickness was highest in Group 7, which might be an indicator of the association with biochemical TGF- β elevation in this group.

4. Conclusion

As a result, this study demonstrated that during the preparation of PLGA nanoparticles, there is more quercetin loss than pluronic thermosensitive gel. Also quercetin release from PLGA nanoparticles is limited when compared with gel formulation of quercetin. PLGA nanoparticles and thermosensitive gel formulations are effective when compared with quercetin solution and silverdin. When our biochemical and pathological examinations are evaluated in whole, we demonstrated that PLGA nanoparticles and quercetin thermosensitive gel formulations can be effective in the treatment of burn wounds according to the levels of IL-6, IL-1β, TNF-α, and TGF-β parameters. Besides, this finding is supported by histopathologic results including lower inflammation and fibrosis scores, as well as higher granulation tissue thickness values obtained with these formulations. Considering the cost of PLGA polymer, it was concluded that pluronic thermosensitive gel formulations could be a more cost-effective option for burn wound therapy. Research on the creation of novel treatments for this circumstance is essential because burns remain one of the most serious health problems in existence today. So that, it may be possible to obtain new and effective treatments with current drug technology. Since the conditions that can cause both physical and psychological problems in burns, there are basic focuses such as completely removing or minimizing tissue damage and providing rapid recovery in the patient. Our study includes different formulation types that incorporate technologies used to develop new drugs. In our study, we supported the effects and advantages of these formulations with both biochemical and histopathological data. In this respect, present study will give directions for the future studies.

Authors' contributions

Nihal Cetin: Conceptualization, Investigation, Methodology, Writing- Original draft preparation, Visualization, Formal analysis, Project administration. Esma Menevse: Biochemical analysis, Writing-Original draft preparation, Review & Editing, Supervision. Zeliha Esin Celik: Histopathologic analysis, Writing- Original draft preparation, Visualization, Cengizhan Ceylan: Metodology, Formal analysis, Investigation. Seyma Tetik Rama: Metodology, Investigation. Yakup Gultekin: Methodology, Investigation. Tamer Tekin: Methodology, Investigation. Adem Sahin: Conceptualization, Investigation, Methodology, Writing - Review & Editing.

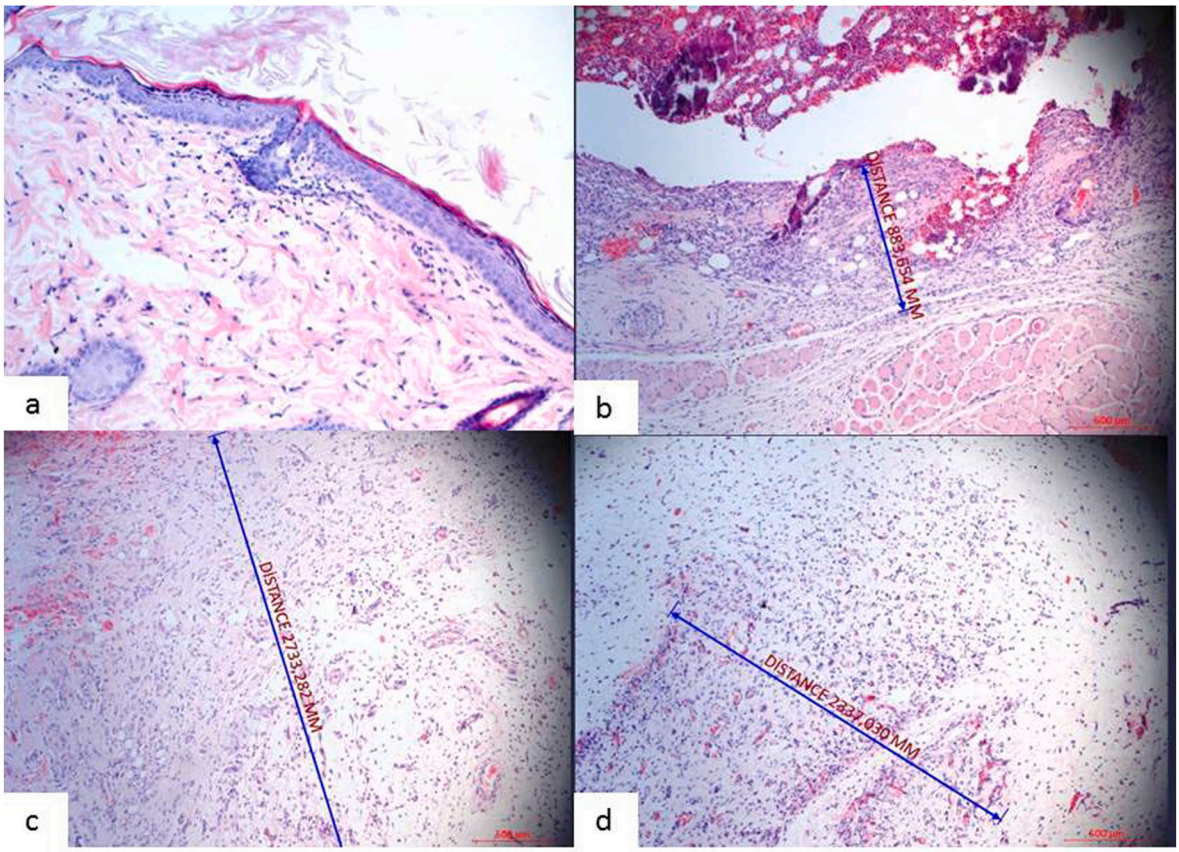

Fig. 7. No granulation tissue formation in healthy control group (Group 1) (a), thickness of the granulation tissue in Group 3, Group 7 and Group 8 (b,c and d). (HEX100)(G1: Cntrl wo Burn, G2: Cntrl only Solvent, G3: Blank Nano, G4: Blank Gel, G5: Silverdin®, G6: Quer Solution, G7: Quer Nano, G8: Quer Gel).

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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