Evaluation of resveratrol organogels prepared by micro-irradiation: fibroblast proliferation through in vitro wound healing

Objective: Resveratrol (RSV) has therapeutic potential with several biological activity. The aim of this study was to develop organogel formulations of RSV and to investigate the proliferation and migration via in vitro wound model of primary normal human skin fibroblasts (NHDF).

Methods: The optimum RSV concentration was determined by MTT assay. Three different types of polyethylene glycol (200, 400 and 600) were used to prepare Carbopol 940 based organogels by micro-irradiation method. Differential scanning microscopy (DSC) and rheological analyses were conducted. Proliferation activity and migration of fibroblast cells were determined with the Giemsa staining.

Results: The percentage of migration rate obtained with RSV-PEG-400 organogel was the highest, as 59.7%. The values obtained with RSV-PEG-200 and RSV-PEG-600 were 36.6% and 48.7%, respectively.

Conclusion: This study showed that RSV organogels produced by micro-irradiation method could be a model for active molecules that have low water solubility for different applications as pharmaceuticals, and cosmeceuticals.

Keywords: Resveratrol; Organogel; Polyethylene glycol; Proliferation; Wound healing; CytoSelect™.

Özet

Amaç: Resveratrol (RSV) pek çok biyolojik aktivite ve terapotik potansiyele sahip bir moleküldür. Çalışmanın amacı RSV içeren organojel formülasyonları geliştirmek ve NHDF (Normal Human Dermal Fibroblast) hücreleri ile oluşturulan in vitro yara modellere proliferasyon ve migrasyon üzerindeki etkinliğini belirlemektir.

Yöntemler: Organojel içeriğindeki en uygun RSV konsentrasyonu MTT testi ile belirlenmiştir. Üç farklı tip polietilen glikol (200, 400 ve 600) ve Carbopol 940 kullanılarak Mikrodalga yöntemi ile hazırlanan resveratrol içeren organojellerin fibroblast proliferasyonuna etkisinin in vitro yara iyileşme modeliyle değerlendirilmesi

Sonuçlar: En yüksek migasyon oranı PEG-400 ile oluşturulan RSV içeren organojellerde %59.7 oranında...
belirlenmiştir. RSV-PEG-200 ve RSV-PEG-600 ise sırasıyla 36.6% ve 48.7% olarak belirlenmiştir.

Tartışma: Bu çalışma mikrodalga yöntemi ile geliştirilen RSV içeren organojel formülleri çözünürlüğü düşük aktif molekülleri farmasötik ve kozmetik gibi farklı uygulama alanlarında kullanıma için model olarak kullanılabilir.

Anahtar kelimeler: Resveratrol; Organojel; Polietilen glikol; Proliferasyon; Yara modeli; CytoSelect™.

Introduction

Resveratrol (RSV) (3,5,4′-trihydroxystilbene) is a phytoalexin, a small polyphenol found in various berries, nuts, and other plant sources which synthesized response to injury, ultraviolet rays and microbial infection in plants. It has therapeutic potential for fungal diseases, various skin inflammation, has a positive impact on proliferation of fibroblast cells, and improvement of the skin structure. In the recent years, RSV has gained attention with several findings implicated as a potent sirtuin 1 (SIRT1) activator capable of mimicking the effects of calorie restriction, and regulating longevity in lower organisms. Age-related metabolic diseases are currently a topic of intense investigation [1]. However, RSV is sensitive to environmental factors and has low aqueous solubility (<0.01 mol/L), stability and bioavailability. Thus, improving its absorption through the skin via preparing an optimized formulation is one of the most studied subject areas at the present [1–3]. Organogels are apolar solvent-based systems formed by thermal methods, which act to increase the penetration of active substances through the skin. It is a valuable alternative especially for lipophilic substances. They are optically clear and biocompatible [4, 5].

For that reason, for the first time in this study RSV organogel formulations were developed by using three different PEG types (200, 400 and 600) to produce carbomer based organogels. Micro-irradiation method was employed. This method has been developed recently by Gokce et al. [5] to avoid spending heat by conventional means for saving time and energy. After the preparation of organogels by this novel technique, RSV was applied at different concentrations on primary normal human skin fibroblasts (NHDF) cells and optimum concentration was determined by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)] assay. The effects on proliferation and migration were investigated by in vitro wound healing model of NHDF cells and results were evaluated by a software system.

Materials and methods

Materials

RSV (96%) (Chemos GmbH, Germany), Carbopol 940 (C940) (Lubrizol, Belgium), PEG-200, 400 and 600 (Fluca, Italy), DMEM Ham’s F12, fetal bovine serum (FBS), penicillin/streptomycin, dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS) (Lonza, Visp, Switzerland), cell stain solutions and Giemsa stain CytoSelect™ (Cell Biolabs, San Diego, CA, USA) were purchased. All the other chemicals used were analytical grade.

Organogel preparation

C940, was added to 25 mL of PEG 200, 400 and 600, separately at concentrations of 1%, 2%, 3%, 4% and 5%. The mixture was agitated at 24,000 rpm, for 5 min by a high speed homogenizer (Ultra-Turrax Ika T25). The mixture was poured into glass Petri dishes and exposed to micro-irradiation (Arcelik MD574; 1200 W/1 h) for 2 min. 0.05 M RSV was added to samples before homogenization for preparation of drug-incorporated formulations [5] to form RSV-PEG-200, RSV-PEG-400 and RSV-PEG-600 coded organogels.

Rheological analyses

Rheological analyses were performed using a thermally controlled rheometer (Haake Mars Modular Advanced Rheometer Systems, Germany). The cone/plate geometry with 60 mm diameter and 1° angle was used, and the gap was 105 mm. Oscillation stress sweep test was conducted for determination of the linear viscoelastic regime. The flow measurements were performed on the produced gels at the 0.05–10 Pa range of stress at 25°C. Frequency sweep tests were conducted at 25°C with a step-wise increasing frequency range between 0.05 Hz and 50 Hz frequency, at 1 Pa stress, in the field of linear viscoelasticity.

DSC analyses

DSC analyses were performed with (Perkin Elmer DSC 8000, USA) to determine melting points and understand the crystallization behavior. The samples were sealed in aluminum pans under nitrogen air atmosphere
at a flow rate of 20 mL/min and evaluated in 25–300°C temperature range.

**In vitro cell culture studies**

**Maintenance of NHDF cells**

NHDF cells were cultivated in Dulbecco’s modified Eagle’s medium F12 (DMEM/F12), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL of penicillin and 100 μg/mL of streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. The cells were passaged twice a week and were used in the test actively proliferating cells at the exponential phase.

**MTT cell proliferation assay**

Cell viability was determined after 24, 48 and 72 h with MTT assay. The best proliferating RSV concentration was determined after calculating level of cell viability. For this purpose, NHDF cells (8 × 10⁵ cells/well) were cultivated in DMEM-Ham’s/F12 medium into 96-well plates for 24 h at 37°C in a humidified atmosphere of 5% CO₂. After 24 h the cells were treated with different concentrations of RSV (0.1–0.006 M) and incubated for 24, 48 and 72 h. MTT (2.5 mg/mL) stock solution was added as 25 μL at the end of each incubation period and incubated for 4 h at 37°C. Stock solution was prepared by dissolving RSV in ethanol:water mixture (1:1 v/v), and then dilutions were adjusted by PBS up to required concentrations. Dissolved formazan crystals in DMSO was measured at 570 nm (reference filter 630 nm) with a UV visible spectrophotometer.

Percentages of surviving cells in each culture were determined by the following formula:

\[
\% \text{ Viable cells} = \frac{\text{absorbance of treated cells} - \text{absorbance of blank}}{\text{absorbance of control} - \text{absorbance of blank}} \times 100.
\]

**CytoSelect™ in vitro wound healing model**

The “wound” was created using the CytoSelect™ wound healing kit (Cell Biolabs, San Diego, CA, USA) according to the provided protocol. For this purpose, NHDF cells (8 × 10⁵ cells/well) in DMEM-Ham’s/F12 medium were cultured with insert using a 24-well plate. Two percent C940 including organogels in PEG 200/400/600 were diluted with PBS to give a 1.3 × 10⁻² M of RSV concentration. The same dilution process was conducted for RSV free organogels. The control group was treated with DMEM Ham’s/F12 only. Three parallel samples were prepared for each group. The cells were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂. The migration of NHDF was observed using an inverted microscope (Olympus, Japan) at 24, 48 and 72 h.

**Giemsa cell staining**

Cells were fixed by removing media and adding 0.5 mL of fixing solution to each well. Following cell incubation for 10 min at room temperature, each well was carefully washed three times with PBS. Then 400 μL of cell Giemsa stain solution was added to each well and incubated with the cells for an additional 15 min at room temperature. After that, wells were carefully washed three times with PBS and the migration of NHDF cells were photographed under inverted microscope at 24, 48 and 72 h using an image analyzing system (Olympus, Japan).

**Determination of wound healing**

We discern between two open area concepts by TScratch®: open image area and open wound area. TScratch® is a software tool developed to automatically analyze wound healing assays (scratch assays) [6].

The percent closure was determined by with Equations 1, 2 and 3 below:

\[
\text{Percent closure} = \frac{\text{Migrated cell surface area}}{\text{Total surface area}} \times 100. \tag{1}
\]

Total surface area = 0.9 mm × length. \tag{2}

Wound area was calculated as below (Equation 3) to determine the migration rate of cells.

\[
\text{Migration rate} = \frac{\text{Length of cell migration (nm)}}{\text{Migration time (h)}}. \tag{3}
\]

**Statistical analyses**

Statistical analysis was conducted by ANOVA followed by Tukey’s test for comparisons between groups. 0.05 was taken as the p value, to indicate statistical significance (p < 0.05).
Results

The RSV organogels of C940 in different types of PEG were successfully prepared by high-speed homogenization followed by microwave-assisted heating for the first time in this study.

The rheological properties of the prepared gel formulations were examined and all of them performed the characteristics of non-Newtonian fluid, as expected. All gel formulations performed pseudoplastic flow properties and as seen from the viscosity profiles of gels were shear thinning systems (Figure 1). Polymer chain indicates of the flow when the threshold is stronger than the interaction of the shear stress [7, 8]. This behavior also provides the ability to disperse when the pressure is applied to the gels. C940 concentration affected the strength of the produced organogels. The increment in PEG chain and molecular weight resulted as an increase in the resistance to flow. Obtained viscosity values in the same C940 concentration were in order as;

PEG 600 > PEG 400 > PEG 200. PEGs are prepared by polymerization of ethylene oxide and are available in molecular weights between 300 g/mol and 10,000,000 g/mol. The physical properties change due to molecular weight [9].

Due to high lipophilicity of RSV no crystallization occurred in produced organogels. DSC analyses of RSV showed that RSV was completely dissolved in the base of organogels. As shown in Figure 2, the melting process for RSV takes place at 265.8°C and no peak observed in the gel formulations of RSV which proved that RSV was dissolved in gel base during preparation. Different concentration of RSV (0.1, 0.054, 0.027, 0.013 and 0.006 M) was used to determine the proliferation of NHDF cells. Cell viabilities (%) were determined by MTT assay for 24, 48 and 72 h (Figure 3). According to obtained data, RSV significantly increased NHDF cell proliferation at $1.3 \times 10^{-2}$ M concentration for 48 h.

In this study, the prepared organogels with PEG-200/400/600 were diluted and applied on NHDF cells.

![Figure 1: Viscosity vs. shear rate profiles of organogels prepared with different ratios (1%–5%) of C940 in (A) PEG 200 (P2), (B) PEG 400 (P4), (C) PEG 600 (P6) at room temperature.](image-url)
The maximum effective concentration of RSV on proliferation after the MTT assay results was found as $1.3 \times 10^{-2}$ M and this concentration was used for preparation of the organogels. The suitable concentrations of C940 in different PEG-types for application were determined as 2%. The formulations were sticky and were not suitable for spreading at higher concentrations. Figures 4–7 show the effect of organogels prepared with 2% C940 in different PEG types on NHDF cell proliferation and migration at 48 h on wound healing. It was observed that NHDF cells migrate to wound gap from both sides by organogels prepared with PEG 400. Also according to microscopic analyses, percent closure of cells with organogels with RSV was more than RSV free organogels.

PEG-200/400/600 derivatives have different chain lengths and it was thought that the wound closure rate differed due to the different molecular weights of PEGs in different proportions. Migration rate of control group after Giemsa staining was calculated by using TScratch® as 27.7%. The highest migration and wound area closure rate belonged to RSV-PEG-400 organogels with a value of 59.7% (Figure 8). The area of migration were calculated as 35.5% for RSV-PEG-200 and it was not statistically different than control ($p > 0.05$). RSV-PEG-600 had a closing rate as 48.6% and it was lower than RSV alone, and RSV-PEG-200 ($p < 0.05$). On the contrary RSV alone and RSV-PEG-400 were not different with high healing rates. As can be seen from Figures 1–8, RSV makes a significant contribution for the closure of wounds in comparison to control and free organogels.
Large tissue losses that are originated from wounds causes critical problems worldwide. Researchers try to develop new strategies to favor wound healing. The ‘topical’ conventional approaches in dermal tissue repair are being insufficient in the problems encountered during the treatment [5, 9–16].

RSV can be a novel strategy for dermal tissue repair. In the present study, RSV organogels were prepared with C940 using different types of PEG by micro-irradiation method. After the optimization studies, the formulations were evaluated by in vitro cell wound models. Organogels are vehicles to deliver wide variety of agents through the skin owing to possess both the properties of oil and aqueous based formulations [10].

Wound healing is the tissue response and it is the result of regeneration process. Normal human dermal fibroblasts (NHDF) can be used for wound healing studies and dermatological research to investigate diseases like scleroderma, fibrosarcoma, fibrosis, xeroderma pigmentosum and histiocytoma. NHDF is isolated from the dermis of juvenile foreskin or adult skin from different locations like the face, the breasts, the abdomen and the thighs [11–14].

This study showed that the proliferation activity and the migration of fibroblast cells were significantly increased by organogels comprising RSV. In RSV-PEG-200
organogels, the cells showed a tendency to migrate however their intensity was not sufficient enough to close the wound area. In RSV-PEG-600 organogels the cells were intense and packed. However their movements were less dynamic and their migration rates were slower than RSV-PEG-400 organogels. The optimum results were obtained from RSV-PEG-400 organogels with almost 60% of wound closure. The closure % could be listed in order as: RSV-PEG-400 > RSV-PEG-600 > RSV-PEG-200. PEGs are reported to be sensitive to degradation and the produced byproducts can be toxic to mammalian cells [12]. The micro-irradiation method applied in this study did not produce any toxic combination that would alter the healing period of NHDF cells due to the proliferation observed from all organogel formulations.

Figure 6: Images of Giemsa staining the effect of NHDF cell proliferation and migration obtained from organogels prepared with PEG 600 at 48 h on the in vitro wound healing model: (A) RSV free organogel, (B) RSV-PEG-600 organogel. The results were analyzed with TScratch® based MatLAB® programmes (Zurich, Switzerland).

Figure 7: Images of Giemsa staining the effect of NHDF cell proliferation and migration obtained with RSV at 48 h on the in vitro wound healing model: (A) RSV free NHDF cells, (B) NHDF cells with RSV solution. The results were analyzed with TScratch® based MatLAB® programmes (Zurich, Switzerland).

Figure 8: Wound closure % of fibroblast cells after treatment with RSV alone and RSV-PEG organogels in comparison to control group. The results were analyzed with TScratch® based MatLAB® programmes (Zurich, Switzerland).
Wound healing effects of organogels on NHDF were determined on wound model using Matlab-based program TScratch® showed that RSV and PEG types had significant effects on cell proliferation and migration. RSV, enhanced fibroblast migration and proliferation when compared to control or RSV free organogels. It was determined that migration and wound closure rate has been improved by the efficacy of the formulation prepared with PEG 400.

In conclusion, it can be stated that organogels of C940 in different PEG types can be successfully produced by micro-irradiation method which can be available for pharmaceuticals, cosmeceuticals and nutraceuticals. The developed organogel prepared with RSV represents a potential alternative for wound healing.

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