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Evaluation of the early term healing effects of resveratrol on corneal wounds in rats

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Abstract: The aim of this study was to determine the effects of resveratrol (RES) treatment on corneal wound healing. Randomly divided into 2 groups were 26-week-old male Wistar albino rats. Group 1 was the control group (C) and group 2 was the RES group. A 2–3-mm-long vertical incision was made centrally in the right cornea of each rat to the descemet membrane using loupe magnification (2.5X) and the wounds were sutured using 10/0 nylon material. In the RES group, the rats were fed 30 mg/kg/day RES via oral gavage; the C and RES groups were provided food and water ad libitum for only 11 days. The matrix metalloproteinase-9 (MMP-9), nitric oxide, and malondialdehyde (MDA) levels, and antioxidant status (AOS) were measured. On day 11, the rats were euthanized and examined histologically. The score of 3 for connective tissue proliferation, in both the C and RES groups, indicated rates of 11.1% and 0.0%, respectively ($P < 0.05$). The score of 3 for inflammatory cell reaction, in both the C and RES groups, indicated rates of 11.1% and 0.0%, respectively ($P < 0.05$). The MDA results were as follows: days 0 and 11, 1.61 ± 0.316 and 2.854 ± 0.572 for the C and RES groups, respectively. The increase in group C was statistically significant ($P < 0.001$). The MMP-9 level was 1.115 ± 0.197 and 2.842 ± 0.368, respectively, in the 2 groups. The increase in the RES group was statistically significant ($P < 0.01$). According to the AOS, the intergroup difference was statistically significant ($P < 0.05$). As a result, RES inhibited vascularization of the corneal wound and retained the transparency of the corneal tissue. It is therefore suggested that extended studies and follow-up times are needed to better evaluate the outcomes of RES on the healing corneal wounds.

Key words: Rat, resveratrol, corneal wound, MMP-9

1. Introduction

Superficial abrasions, which occur from a posttraumatic tear in the corneal epithelium, are treated as superficial corneal ulcers, and deep abrasions are treated as deep ulcers. The treatment of corneal lacerations depends on the depth of the wound. Superficial lacerations heal quickly, as do superficial ulcers. The cleft in lacerations that are thicker than one-half of the cornea is sutured with a 7/0–10/0 absorbable or nonabsorbable suture. Full-thickness corneal lacerations are treated using surgical procedures. The prognosis of corneal lacerations depends on the severity and depth of the wound. The prognosis of the rupture after blunt trauma is worse than that of lacerations with sharp tools [1].

Resveratrol (RES) was first identified in 1963 as the active compound of the dried roots of *Poligonum cuspidatum*, also known as Kojoto in Japan. The first studies were conducted by Langcake and Pryce in 1976 using grape vines (*Vitis vinifera*). This plant has been used since ancient times in both Japanese and Chinese traditional medicine for the treatment of suppurative dermatitis, gonorrhea, favus, tinea pedis, and hyperlipidemia. Its chemical structure contains 3,4',5-trihydroxystilbene, and trans and cis isomers of the molecule known as 3,4',5-stilbestrol [2,3]. Among its many biological effects, the antiinflammatory function of RES is due to its ability to inhibit cyclooxygenase [4]. RES is a wine polyphenol and inhibits platelet aggregation [2].

The aim of this study was to investigate the effects of RES on healing early-term corneal wounds in rats.

2. Materials and methods

First, 26-week-old male Wistar albino rats (250–300 g) were used in this study and randomly divided into 2 groups, as the control (C) and resveratrol (RES) groups (n = 10 each). The rats were housed in individual cages at the Afyon Kocatepe University Experimental Animals Research and Application Center in Turkey after having undergone the necessary health protocols (permission number: AKÜHADYEK 49533702/118, reference number: 110-16, July 9th, 2016). The rats were fed rat feed and water ad libitum. No special conditions were used.

2.1. Anesthesia and surgery protocols

The rats were anesthetized intramuscularly with xylazine HCl (Rompun, 5–10 mg/kg; Bayer, Germany) and 25–30

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mg/kg of ketamine HCl (Ketasol 10%; Richter Pharma AG-Austria) in both groups. For 5 days postoperatively, tobramycin (Tobrased 0.3% eye drop) was applied topically as an antibiotic, twice a day, in the morning and the evening. The rats in the RES group received 30 mg/kg of RES (100 mg/capsule, Solgar) for 11 days by gastric gavage. The rats were placed on the operation table in the left-lateral position and the corneal wounds were created on the right eyes. A 2–3-mm-long vertical incision was made centrally in the right cornea of each rat to the descemet membrane using a scalpel, at a magnification loupe of 4.5, in both groups.

After creating the corneal wound, 2 drops of 0.3% tobramycin, an antibiotic from the aminoglycoside group, were applied to the right eye of each rat in both groups, and the wound was sutured with 10/0 nylon (Alcon Company, Geneva, Switzerland) using the simple interrupted suture method, after which 1 drop of 0.3% tobramycin was reapplied. Tarsorrhaphy was performed on the right eyelid of each rat in both groups using a 6/0 Vicryl-Ethicon) polyglactin 910 suture. Tobramycin was applied only to the right eye of each rat for 5 days. Photographs of the corneal wounds were taken on day 0 and 11 days after surgery.

2.2. Histopathological examination

The eyes of the animals were removed and fixed in a 10% neutral buffer containing formaldehyde solution after euthanasia and they were sliced and transferred onto cassettes for tissue processing at the end of 48 h. After alcohol and xylene dilutions, the tissues were processed and blocked in paraffin. The blocks were sliced to 4–5 µm with a microtome and transferred onto glass slides. The sections were stained with H&E and examined under a light microscope. The histopathological changes in the corneal wound line were scored using a modification of the method proposed by Sancak and Bozkurt [5]. Connective tissue proliferation (CTP), inflammatory cell reaction (ICR), and epithelial cell regeneration (ECR) were investigated. A grading system was used to evaluate the depth of the corneal lesion and conduct intragroup comparisons. The detected connective tissue changes were scored on a scale from 0 to 3 (0 = none, 1 = mild, 2 = moderate, and 3 = severe). ICR within the region was also scored using the same scale, as follows: 0 = no ICR, 1 = focal and mild presence, 2 = prevalent but mild presence and focal but severe presence, and 3 = prevalent and severe presence. ECR, another important parameter, was also scored on a scale from 0 to 3, as follows: 0 = very good epithelialization that was close to normal, 1 = full ECR but a hyperplastic incision zone, 2 = unattached incision zone but a distinct epithelial hyperplasia, and 3 = unattached incision zone and the lack of epithelial hyperplasia.

2.3. Biochemical examination

Blood samples were collected from all of the rats on days 0 and 11. The matrix metalloproteinase-9 (MMP-9), nitric oxide (NO_x), and malondialdehyde (MDA) levels, and antioxidant status (AOS) in the blood were determined for biochemical evaluation.

2.3.1. MMP-9 levels in the serum

To determine the MMP-9 levels, a commercial rat kit using the double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) method was used (Shanghai YL Biotech Co. Ltd., Shanghai, China; catalogue number YLA0585RA) with rat MMP-9/gelatinase B. The absorbance values at the end of the experiment were measured at 450 nm and given in ng/mL.

2.3.1.1. Measurement protocols

The following protocols were used for all of the measurements:

1. The samples and all of the reagents were kept at room temperature for 1 h.

2. The standard and samples were added to microplate wells, except for the blank well, and incubated at 37 °C for 60 min.

3. The microplate was rinsed 5 times with wash buffer in the ELISA washer.

4. Chromogen A and B were added to all of the wells, including the blank well, and incubated at 37 °C for 10 min.

5. After adding the stop solution, the absorbances of the standard and samples were measured against the blank at 450 nm.

6. In accordance with the calibration curve that was plotted using the absorbances of the standards, the MMP-9 levels were determined in ng/mL.

2.3.2. NO measurement

The NO_x in the serum samples was measured using a modification of the method proposed by Miranda et al. [6], in which nitrite + nitrate was used as the indicator of NO_x.

2.3.2.1. Testing procedure

The serum samples were mixed with 10% trichloroacetic acid (TCA) that was 3 times their volume and centrifuged to deproteinize them before measuring. The mixture was centrifuged at 4 °C and 12,000 rpm for 15 min. Next, 100 µL of the supernatant were transferred to the ELISA plate and 100 µL of vanadium III chloride ($VaCl_3$) (400 mg of $VaCl_3$ was completed to 50 mL with 1 M of HCl and stirred), 50 µL of sulfanilamide (200 mg of sulfanilamide was dissolved in 5% HCl in a volume of 10 mL) and 50 µL of 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride (NEED) solution were added. The ELISA plate was incubated at 37 °C for 30 min. The mixture was read at 540 nm using the ELISA reader. The amount of NO was calculated in accordance with the $NaNO_3$ standard curve previously plotted for various densities.

2.3.3. MDA levels in the serum

The MDA levels in the samples were determined using a modification of the double boiling-based method proposed by Draper and Hadley [7]. In the first round of boiling, bound MDA was released from the proteins, and the proteins were precipitated. In the second round of boiling, the total MDA reacted with the TBA and the absorbance of the colored complex was measured at 532 nm. Using the molar absorption coefficient of the MDA, its concentration was calculated.

2.3.4 Antioxidant status

First, 2 experimental tubes were prepared, so that each tube contained either the control or specimen. Into both tubes, 2.5 mL of 10% TCA solution was added, and 0.5 mL of sample and 0.5 mL of distilled water were added to the sample and control tubes, respectively. The tubes were sealed and kept in a boiling water bath for 15 min. Both tubes were then cooled under cold water and centrifuged at 3000 rpm for 10 min. Next, 2 mL of each supernatant were transferred to a different tube and 1 mL of 0.675% TCA solution was added to the tubes. The tubes were tightly sealed, resubmerged into the boiling water bath for 15 min, and then cooled under cold water. The absorbance of the specimen tube was measured against a blind sample at 532 nm using spectrophotometry. Using the extinction coefficient of the MDA-TBA complex at 532 nm, the MDA level in the serum was calculated in terms of nmol/mL.

The AOS of the samples was determined using a commercial ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA; catalogue number 709001).

2.3.5. Statistical analysis

The results were given as the mean ± standard deviation. According to the histopathological examination, the chi square test was used in the statistical evaluation of the corneal healing criteria comprising the CTP, ICR, and ECR parameters of the corneas obtained from the C and RES groups. Moreover, the Kruskal-Wallis test was conducted and the statistical differences were analyzed. In the serum measurements, the statistical calculations were conducted for only the MMP-9 levels using the Duncan alfa test. $P < 0.05$ was accepted as significantly significant.

3. Results

The histopathological findings obtained after the animals were euthanized at the end of 11 days, and the NO, MDA, and MMP-9 levels and AOS were measured to determine the blood biochemistry of both the corneal wounds in the C group that received tobramycin and the RES group that received tobramycin + RES, and the results obtained from the statistical analyses were evaluated and are provided in Table 1.

3.1. Biochemical analysis

The results of the NO_x, MDA, AOS, and MMP-9 assessments in the blood samples collected from the C and RES groups are provided in Table 1.

The change in the blood serum NO levels in the RES group was significantly lower than that in the C group ($P < 0.05$). The serum MDA levels in the RES group were significantly lower than those in the C group ($P < 0.05$). The AOS levels in the RES group were significantly higher than those in the C group ($P < 0.05$). The MMP-9 levels in the RES group were significantly lower than those in the C group ($P < 0.05$) (Table 1).

3.2. Histopathological examination

The cornea of 1 of the rats was not evaluated because of failing to excise an appropriate section; therefore, only 9 corneas from each group were examined.

The light microscopy examination revealed CTP, vascularization, ICR, and completed epithelization zones around the incision zone in the C group (Figure 1).

The scorings of CTP (Table 2), ICR (Table 3), and ECR (Table 4) were represented within the pathological examinations of the right eyes of the RES group rats at 11 days after the operation.

The examination using light microscopy revealed that epithelization was completed but mild epithelial hyperplasia was still present, and the inflammatory changes and fibrosis had decreased to a negligible degree in the RES group (Figure 2).

3.3. Postoperative examination

Postoperative findings revealed that infection did not develop until the day the animals in both groups were euthanized, except for in 1 of the rats. In the C group,

Table 1. NO_x, MDA, AOS, and MMP-9 levels in the groups.

Group	NO (nmol/mL)	MDA (nmol/mL)	AOS (mmol/L)	MMP-9 (ng/mL)
Control	8.067 ± 1.44^{6ab}	2.854 ± 0.571^{ab}	0.254 ± 0.052^{a}	2.842 ± 0.368^{ab}
Resveratrol	7.643 ± 1.787^{b}	2.362 ± 0.513^{b}	0.284 ± 0.063^{b}	2.437 ± 0.323^{b}

NO, nitric oxide; MDA, malondialdehyde; AOS, antioxidant status; MMP-9, matrix metalloproteinase-9. Different letters in the same column indicated statistical significance ($P < 0.05$).

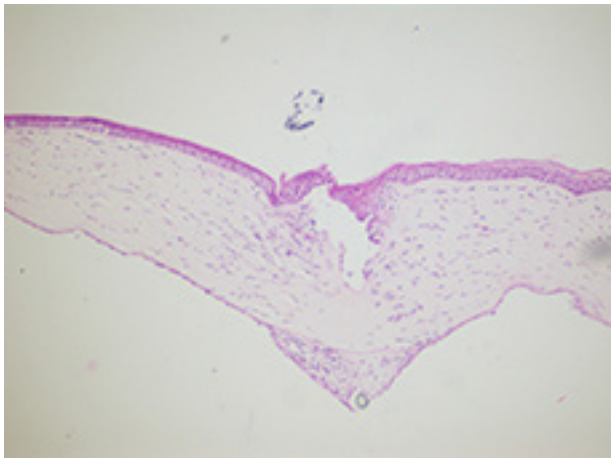

Figure 1. Histopathological appearance of the tissue in the control group.

Table 2. Connective tissue proliferation (n = 9).

Score	0	1	2	3
Control group	11.1%	33.3%	44.4%	11.1%
Resveratrol group	11.1%	33.3%	55.6%	0.0%

Chi square test P > 0.05.

Table 3. Inflammatory cell infiltration (n = 9).

Score	0	1	2	3
Control group	11.1%	22.2%	55.6%	11.1%
Resveratrol group	11.1%	44.4%	44.4%	0.0%

Chi square test P > 0.05.

Table 4. Epithelial cell regeneration (n = 9).

Score	0	1	2	3
Control group	22.2%	22.2%	55.6%	0.0%
Resveratrol group	22.2%	33.3%	44.4%	0.0%

Chi square test P > 0.05.

the corneal wound recovered completely (Figure 3). Corneal edema developed in both groups after suture, but opacification was lower and the corneal edema more distinctly disappeared in the RES group than in the C group (Figure 4). Moreover, neovascularization occurred in specimen 5 in the RES group (Figure 5).

4. Discussion

Superficial abrasions, which occur from a posttraumatic tear of the corneal epithelium, are treated as superficial corneal ulcers, and deep abrasions are treated as deep ulcers. The treatment of corneal lacerations depends on their depth. Superficial lacerations heal quickly, as do superficial ulcers. The cleft in lacerations with a thickness greater than one-half of the corneal thickness is sutured with a 7/0–10/0 absorbable or nonabsorbable nylon thread. Full-thickness corneal lacerations require a surgical procedure. The prognosis of corneal lacerations depends on the severity and depth of the wound. A rupture after blunt trauma is worse than a laceration with a sharp instrument [1,8].

The keratocytes in the region, and fibrocytes and macrophages coming from limbal surrounding cells, contribute greatly to vascular healing in corneal stromal damage. These cells create fibrin, collagen, and cell irregularities and damage the corneal transparency, leading to opacity [9,10]. Surgical equipment and methods are known to affect the treatment prognosis of a corneal laceration [1,8].

In this study, corneal opacity was observed in 2 rats in the C group before day 6. On the other hand, while corneal edema disappeared in the RES group on day 11, except in 1 case, it was detected in the C group in same period. Brakenhielm et al. [11] investigated the effects of natural red wine on wound healing and found that orally administered RES delayed wound healing as a result of suppressing the angiogenesis (at a concentration of 5.7 µg/mL); however, the current study showed that a high dose of RES reduced oxidative stress, while not delaying wound healing.

RES is a wine polyphenol that inhibits platelet aggregation through the preferred inhibition of cyclooxygenase 1, which synthesizes thromboxane A_2, an inducer of platelet aggregation and vasoconstriction. Studies have shown that RES potentially inhibits the oxidation of low-density lipoprotein particles, either by chelating copper or directly removing free radicals [2,12]. It also suppresses angiogenesis in animals [11].

RES is a hydroxystilbenzene and is responsible for the inhibition of arachidonate, mitogen-activated protein kinase activation, and protein kinase-C, and for the degranulation of mast cells [13]. RES inhibits lipid peroxidation, chelates copper, works as a free radical scavenger, disrupts the synthesis of eicosanoids, promotes thrombocyte inhibition, and exhibits antiinflammatory, anticancer, and estrogenic activities [14].

Li et al. [15] revealed that RES prevented oxidative damage in a lens epithelial cell culture. Zheng et al. [16]

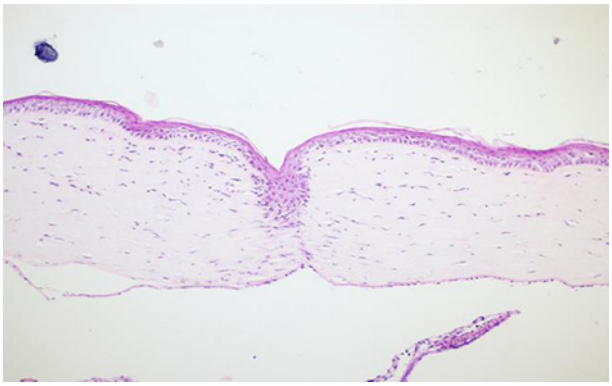

Figure 2. Histopathological appearance of the tissue of the corneal tissue in the resveratrol group.

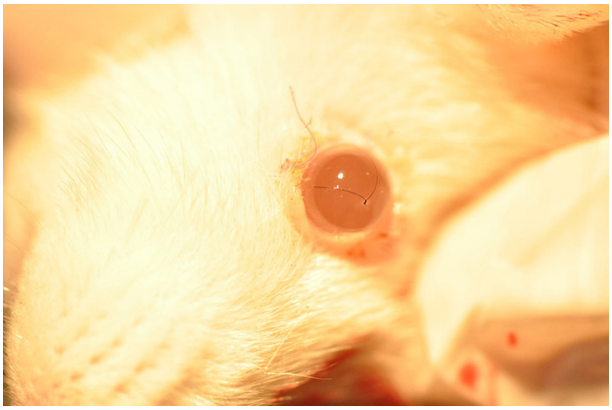

Figure 3. Appearance of the wound line on day 11 in the control group (complete recovery).

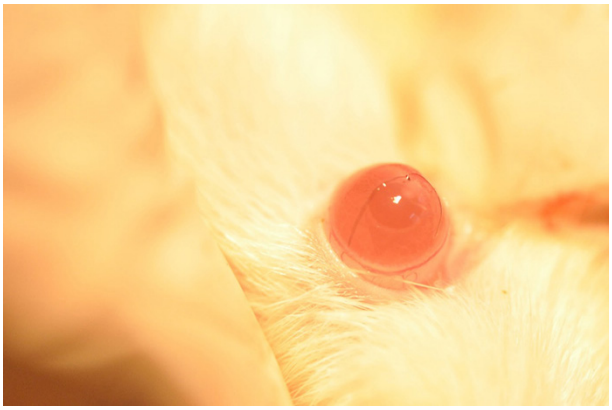

Figure 4. Appearance of the recovering cornea wound in the resveratrol group.

Figure 5. Appearance of the neovascularization in case 5 in the resveratrol group.

found that RES prevented oxidative stress against H_2O_2 in human lens epithelial cells by increasing catalase and superoxide dismutase-1 levels, and heme oxygenase-1 expressions.

In this study, both the NO and MDA levels were significantly lower in the RES group, and the AOS was significantly higher in the RES group on day 11. It is worth noting that an AOS of 0.284 ± 0.033 mmol/L was the same on both days 0 and 11. This result was also supported by the data found in the literature and viewed as evidence of the free-radical scavenging activity and positive effects of RES on wound healing. The biochemical findings were also supported by the histopathological findings.

Under normal conditions, MMPs are produced in the connective tissue for continuous embryonic development, tissue morphogenesis, and growth. This production is highly important in trophoblast implantation, the menstrual cycle, ovulation, repair of tissue damage, and wound healing [17,18]. MMP-9 is part of the gelatinase group of enzymes that are mainly responsible for the digestion of gelatin and the denaturation of collagen, and are either lacking or at low levels in normal mammalian tissues. The expression of MMP usually increases in the repair and remodeling processes, and in diseases [19]. Inflammatory cytokines and MMPs increase in the presence of various inflammatory-process stimuli, such as wound healing and stress. Compared with iso-osmatic saline, using hyperosmotic saline as a proinflammatory stressor in a mouse model increased the expression and production of interleukin-1β, tumor necrosis factor-α, and MMP-9 by the cornea on the ocular surface and conjunctival epithelia [19].

Inflammation, reepithelization, angiogenesis, granulation tissue formation, and matrix deposition cause a response in different cell types (fibroblasts, inflammatory cells, and endothelial cells), which is regulated by cell–cell and cell–matrix interactions [17,20,21]. MMPs have

multiple roles in the regulation of tissue repair and are mediators with proteolytic activity [17].

The inflammatory phase is characterized by the transfer of neutrophils and monocytes to the wound region. A rapid reaction occurs in the wounded tissue through tissue factors and the release of chemo-attractants. Wounded tissue causes venous stasis. High levels of MMP-9 are released due to an accumulation of plasma components (fibrin and acute wound fluid). In this phase, MMP-9 stored in macrophages and neutrophil granules is released into the microenvironment [22]. In the proliferation phase, MMP-9 is excessively expressed on the sides of a healing wound [23]. Studies on the remodeling phase have shown that fibroblast-mediated collagen and excessively released MMPs played important roles in wound closure [24,25].

MMPs-1, 2, 8, and 9 play a role in teardrops; MMPs-1, 9, 10, 12, 13, and 14 play a role in corneal epithelium; MMPs-1, 2, 3, and 14 play a role in stroma, and MMPs-2 and 9 play a role in corneal endothelium [17].

In this study, the MMP-9 levels in the RES group were significantly low according to the measurements on day 11. This was supported by the clinical and histopathological findings and interpreted as an indicator of complete wound healing and decreased MMP-9 release, leading to its lower levels.

Neovascularization is the formation of new blood vessels causing decreased cornea transparency and sight loss [26]. Edema, vascularization, cicatrix formation, pigmentation, cellular infiltration, and inflammation-dependent corneal degeneration are normal reactions to corneal damage [27] Neovascularization developed in 1 specimen from each group (specimen 5 in the RES group and specimen 2 in the C group).

Epithelial hyperplasia can be observed after damage of the corneal epithelium, and restoration to normal epithelial thickness is possible only after months, or even years [28]. The deficiency of lysozyme and lactoferrin, which are tear proteins secreted by the lacrimal gland, reduces defense mechanisms and increases sensitivity to infections [29].

In the pathological examination, the pathological scorings of CTP, ICR, and ECR were calculated for both groups. In the RES group, a score of 3 was too low to be statistically significant. The ICR score of 3 was also not statistically significant in that group. There were no statistically significant differences between the groups in any of the ECT scores.

Considering the histopathological results, although there were no statistically significant differences between the RES and C groups, in the RES group, the score 3 percentage of CTP was 0%, while the score 1 and score 3 percentages of ICR were 44.4 and 0%, respectively. The score 1 of ECR was 33.3%.

The results revealed that, according to the evaluation of the biochemical and histopathological findings, RES significantly affected the recovery of the transparent appearance of the cornea, and thus, successful corneal healing; however, more comprehensive studies involving long-term monitoring are needed.

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Conflict of interest

The authors declare no conflicts of interest.

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