

## Research Article

# Partial Effect of Resveratrol on Wound Healing: Study on Wistar Albino Rats

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### Abstract

**Objectives:** Resveratrol (RSV) is a polyphenol compound found naturally in fruits. Resveratrol's antioxidant, anti-aging, cardioprotective, anti-inflammatory, and anti-carcinogenic effects have been shown. This study aims to determine the effects of RSV on perfusion, neovascularization, inflammation, and granulation stages of wound healing in addition to its antioxidant capacity.

**Methods:** 16 rats were divided into two groups as the RSV and control. Six excisions were made in each rat. Three excisions in each rat were sutured and the other 3 were left as open wounds. Topical RSV was applied to wounds of rats in the RSV group. Blood perfusion of the wounds in both groups was measured using the PeriScan PIM 3 System Laser Doppler Blood Perfusion Imager. Punch biopsies were taken for histopathological examinations and the evaluation of antioxidant capacity.

**Results:** GPX level of the closed wounds of RSV group was higher compared to the control group. There was no statistically significant difference in SOD and MDA levels between the groups. Neovascularization was registered higher in the open wounds of RSV group.

**Conclusion:** RSV may be effective in wound healing. The results may shed light on new therapeutic approaches that can be used in the reversal of oxidative stress in wound-causing diseases.

**Keywords:** ELISA, Granulation, Resveratrol, Superoxide Dismutase, Wound

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Disruption of cellular, functional, or anatomical continuity in living tissue is called a wound, and this condition can result from chemical, physical, microbial, immunological, or thermal damage. In summation, the wound is the deterioration in epithelial integrity accompanied by loss of function and/or structure.<sup>[1]</sup>

Multiple phases of wound healing are summarized as: inflammation, cell migration, cell proliferation, angiogenesis, and tissue remodeling. It is known that intercellular signal-

ing, some growth factors, and several hormones are effective for the correct functioning of these processes.<sup>[2]</sup>

Many studies worldwide have examined the wound healing processes and thus have established better healing procedures. Further, there is increasing research in the area of wound healing specifically examining the effectiveness of natural products.<sup>[3]</sup>

Resveratrol (RSV) is a polyphenol compound found naturally in fruits such as blueberries and grapes. Numerous

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published studies indicate Resveratrol's antioxidant, anti-aging, cardioprotective, anti-inflammatory, anti-diabetic, and anti-carcinogenic effects.<sup>[4]</sup>

RSV is a natural source of polyphenols, and has been seen to have beneficial antioxidant effects. Recent studies have shown that RSV rapidly neutralizes reactive oxygen and nitrogen species and upregulates genes based on oxidative stress damage. It is believed that RSV can contribute to and accelerate wound healing.<sup>[5]</sup>

This study aims to determine the effects of RSV on perfusion, neovascularization, inflammation, fibroblast activity, and granulation stages of wound healing. Glutathione peroxidase (GPX), malondialdehyde (MDA), and superoxide dismutase (SOD) levels were used as comparisons to evaluate the antioxidant effects of RSV; frequently mentioned in rat excisional wound models in the literature.

## Methods

### Experimental Animals:

Sixteen female young Wistar Albino rats, each weighing approximately 200-230 grams were randomly divided into two groups. One group was named as the treatment group (= Resveratrol (RSV) group), while the other group was named as the control group. All animals were kept in separate and sterile cages maintaining standard environmental conditions for temperature, light, and humidity. Standard rodent food and ad libitum water were provided.

### Excisional Wound Model:

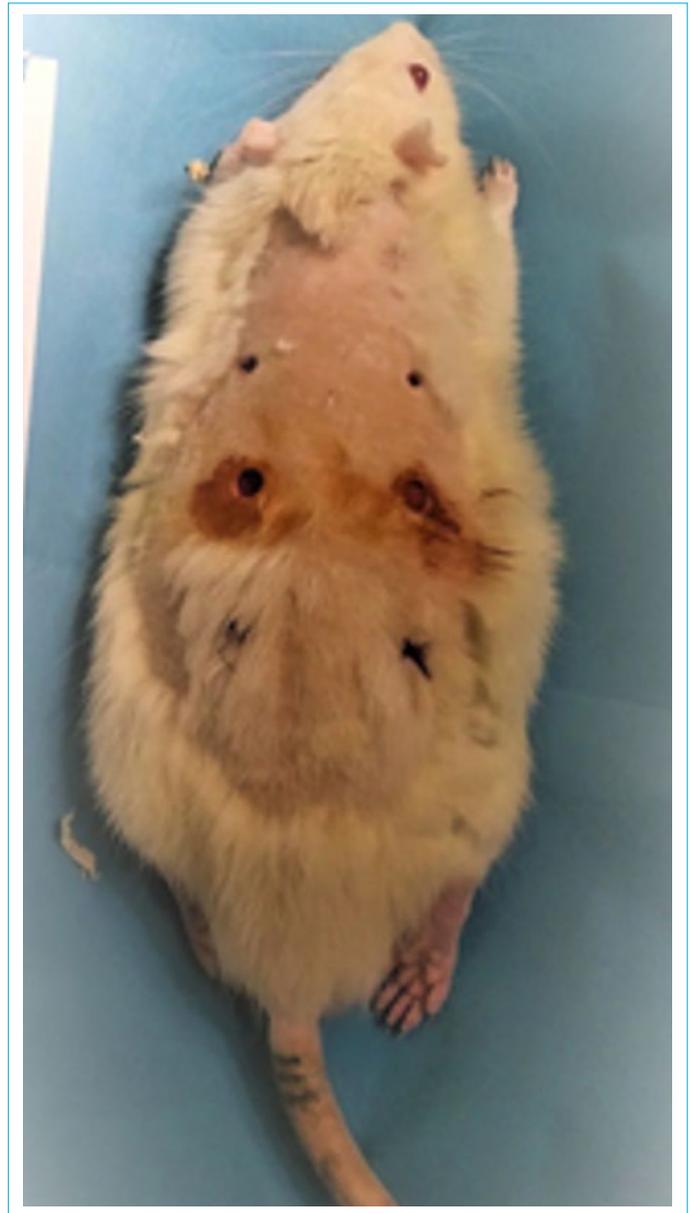
The dorsal skin of the rats in both the control and RSV groups was shaved and cleaned with 70% isopropyl alcohol. Before the surgical intervention, intraperitoneal anesthesia was applied with 35 mg/kg ketamine and 7 mg/kg xylazine. Six wounds were made in each rat with a 6-mm punch tool. Each of these wounds was 1 cm apart from the other and the midline. Three of the wounds were left open (open wound group) whereas the other three were sutured using 4/0 vicryl (closed wound group). The RSV group was administered one application daily of the RSV solution to both the open and closed wounds. No topical agent was applied to the rats in the control group (Fig. 1).

### Preparation of RSV

99% pure RSV was obtained from Sigma Aldrich and used topically in the study.

### Evaluation:

Punch biopsies were taken from the rats in both the open and closed wound groups on the 7<sup>th</sup>, 14<sup>th</sup>, and 21<sup>st</sup> days, and histopathological examinations were performed. An addi-



**Figure 1.** A rat wounded with 6-mm punch biopsy tool.

tional biopsy was taken on the 14th day, and SOD, GPX, and MDA standard Optical Density (OD) and concentration values were calculated on this material by the enzyme linked immunosorbent assay (ELISA) method. Blood perfusion of the open and closed wounds in both RSV and control groups were measured using the PeriScan PIM 3 System Laser Doppler Blood Perfusion Imager on days 7, 14, and 21.

### Statistical Analysis

SPSS 22.0 Statistical package program was used for statistical analysis. Certain comparisons were made to examine the effects of control and RSV groups on subjects. Before these comparisons were made, the conformity of the values of the subjects to the normal distribution was tested

by the Shapiro Wilk method. Parametric tests (Independent t-test) were applied on values that are suitable for normal distribution. Nonparametric tests (Mann-Whitney U test) were used for values that were not suitable for normal distribution. Chi-Square analysis was made in the comparison of categorical data. Comparison Results in 95%; evaluated at a significance level of  $p < 0.05$ .

## Results

Blood perfusion was assessed with PeriScan PIM 3 System Laser Doppler Blood Perfusion Imager. GPX, MDA, and SOD values were evaluated with ELISA and histopathological examination was performed to compare wound healing in control and RSV groups.

### PeriScan PIM 3 System Laser Doppler Blood Perfusion Imager-Evaluation of Blood Perfusion

#### -Open wound group

There was no statistically significant difference between the control and RSV groups on days 0, 7, and 14 ( $p > 0.05$ ). On day 21, a statistically significant difference was identified between the control ( $78.91 \pm 7.63$ ) and the RSV ( $59.40 \pm 3.42$ ) groups ( $p = 0.05$ ;  $p \leq 0.05$ ).

The perfusion rates of the control group were higher than the RSV group.

#### -Closed wound group

In this group, there was no statistical difference between control, and RSV groups on days 0, 7, 14, and 21 ( $p > 0.05$ ) (Table 1).

### Enzyme-linked Immunosorbent Assay (ELISA):

On day 14, after the initial excisions, punch biopsies were taken to evaluate GPX, SOD, and MDA levels. GPX, SOD, and MDA levels were evaluated according to the directives of the company (Sunred Biotechnology), which were provided by the sandwich ELISA method.

GPX Catalog number: 201-11- 1705

SOD Catalog number: 201-11-0169

MDA Catalog number: 201-11-0157

### Rat GPX

#### -Open wound group

There was no statistically significant difference between control ( $0.52 \pm 0.16$ ) and RSV ( $0.64 \pm 0.13$ ) groups in the standard OD values of rats with open wounds ( $p > 0.05$ ). In addition, regarding the concentration (ng/ml) values, there was no statistically significant difference between control ( $21.78 \pm 8.54$ ) and RSV ( $28.29 \pm 8.50$ ) group type ( $p > 0.05$ ).

#### -Closed wound group

Standard OD values of subjects with RSV group type were found to be significantly higher than those of subjects with control group ( $p = 0.000$ ;  $p < 0.05$ ). In addition, concentration (ng/ml) values were found to be higher in the RSV group compared to control group ( $p = 0.001$ ;  $p < 0.05$ ).

### Rat MDA

#### -Open wound group

There was no statistically significant difference between control and RSV groups in terms of both standard OD and

**Table 1.** Blood Perfusion Measured by PeriScan PIM 3 System Laser Doppler Blood Perfusion Imager

Open	Group type		t	p
	Control (n=3)	RSV (n=3)		
Day 0	61.83±4.60	59.14±1.78	0.942	0.400
Day 7	74.68±10.98	62.62±5.49	1.702	0.164
Day 14	80.95±20.11	70.39±9.78	0.817	0.460
Open	Group type		U	p
	Control (n=3)	RSV (n=3)		
Day 21	78.91±7.63	59.40±3.42	0.000	0.050*
Closed	Group type		t	p
	Control (n=3)	RSV (n=3)		
Day 0	75.99±14.80	57.80±6.82	1.934	0.125
Day 7	81.35±9.97	63.46±14.21	1.785	0.149
Day 14	75.17±13.61	66.62±11.32	0.836	0.450
Day 21	69.64±8.57	66.99±10.72	0.333	0.756

\* $p < 0.05$ .

concentration (ng/ml) values ( $p>0.05$ ).

#### -Closed wound group

There was no statistically significant difference between control and RSV groups in terms of both standard OD and concentration (ng/ml) values ( $p>0.05$ ).

#### Rat SOD

##### -Open wound group

There was no statistically significant difference between control and RSV groups between standard OD and concentration (ng/ml) values ( $p>0.05$ ).

##### -Closed wound group

There was no statistically significant difference between control and RSV groups in terms of either standard OD or concentration (ng/ml) values ( $p>0.05$ ) (Table 2).

#### Histopathological evaluation:

- All the obtained tissues were fixed in 10% formaldehyde solution and embedded into paraffin. Tissue pieces taken with thinness of 4  $\mu\text{m}$  were stained with hematox-

ilin-eosin (H&E). Histopathological examinations were performed under a light microscope by a blinded pathologist who was not included in the study.

- All histopathological slides were scanned using a digital pathology system (3D Histech company, P250 - Flash III Digital Scanner, 20x), and the images were photographed with special software (3D Histech company, CaseViewer program, tiff format, and 300 dpi).
- Microscopic areas evaluating neovascularization, inflammation, fibroblast generation, and granulation were evaluated with this software, and the ratio in the whole tissue for both control and RSV groups was calculated separately for each parameter examined. A special scale was used for histopathological evaluation.<sup>[6,7]</sup> According to this:

Level 1: 0-20%

Level 2: 21-40%

Level 3: 41-60%

Level 4: 61-80%

Level 5: 81-100%

**Table 2.** ELISA Measurement of GPX, MDA and SOD

Rat-GPX	Group Type		t	p	
	Open	Control (n=6)			RSV(n=6)
Standard OD		0.52±0.16	0.64±0.13	-1.369	0.201
Concentration(ng/ml)		21.78±8.54	28.29±8.50	-1.322	0.216
Closed	Group Type		t	p	
	Control (n=6)	RSV(n=6)			
Standard OD		0.40±0.097	0.70±0.098	-5.163	0.000*
Concentration(ng/ml)		15.66±4.69	31.52±6.18	-5.005	0.001*
Rat-MDA	Group Type		t	p	
	Open	Control (n=6)			RSV(n=6)
Standard OD		0.24±0.11	0.15±0.019	1.929	0.083
Concentration(ng/ml)		4.82±2.53	2.86±0.23	1.877	0.118
Closed	Group Type		U	p	
	Control (n=6)	RSV(n=6)			
Standard OD		0.15±0.029	0.14±0.013	16.500	0.809
Concentration(ng/ml)		2.84±0.35	2.73±0.14	16.500	0.809
Rat-SOD	Group Type		t	p	
	Open	Control (n=6)			RSV(n=6)
Standard OD		0.49±0.051	0.58±0.086	-2.130	0.059
Concentration(ng/ml)		8.54±0.879	10.10±1.55	-2.146	0.057
Closed	Group Type		t	p	
	Control (n=6)	RSV(n=6)			
Standard OD		0.64±0.21	0.49±0.074	1.581	0.145
Concentration(ng/ml)		11.41±4.10	8.54±1.27	1.639	0.153

\* $p<0.05$ .

**\*Granulation:** Presence or absence of histopathologically significant granulation tissue. Abnormal appearance of tissue after wound healing: presence or absence of components of general granulation tissue consisting of inflammatory cells, edema, angiogenesis, and fibroblasts

**\*Inflammation:** Presence or absence of histopathologically significant inflammatory cells in the wounded region and the whole tissue. Evaluation of only the lymphocytes and macrophages: cells of the acute inflammation

**\*Neovascularization:** Evaluation of the development of new vessels

**\*Fibroblast generation:** Histopathologically significant, increased level of fibroblasts in the wounded region and the whole tissue

#### **Day 7:**

##### **-Open wound group:**

There was no statistically significant relationship between neovascularization and granulation levels and group type ( $p > 0.05$ ).

##### **-Closed wound group**

Inflammation, granulation, neovascularization, and fibroblast levels were obtained as a single level within the control and RSV groups. Level comparisons could not be made on the 7th day, as no other level was found (Level 3 for all of the parameters in each group).

#### **Day 14:**

##### **-Open wound group:**

There was no statistically significant relationship between inflammation levels and group type ( $p > 0.05$ ). On the 14th day, a statistical difference was found between the neovascularization levels and the groups ( $p = 0.001$ ;  $p < 0.05$ ). Accordingly, 1st level neovascularization was found to be higher in the control group, and 2nd level neovascularization was found to be higher in the RSV group. In other words, neovascularization level was found to be higher in the RSV group.

There was no statistically significant difference between fibroblast levels between control and RSV groups ( $p > 0.05$ ).

##### **-Closed wound group**

There was no statistical relationship between neovascularization levels and group type ( $p > 0.05$ ). There was a statistically significant difference between the fibroblast levels and the groups ( $p = 0.001$ ;  $p < 0.05$ ). Accordingly, the ratio of the 2nd level fibroblast level in RSV group was higher than control group. However, the ratio of 3rd level fibroblasts in control group was higher than the RSV group. In other words, fibroblast level, which is one of the improvement indicators, was higher in control group, not in RSV group as expected.

#### **Day 21:**

##### **-Open wound group:**

There was no statistically significant relationship between inflammation, neovascularization, granulation, and fibroblast levels between the groups ( $p > 0.05$ ).

##### **-Closed wound group**

There was no statistically significant difference between neovascularization levels and group type ( $p > 0.05$ ). A statistically significant relationship was found between granulation levels and group type ( $p = 0.000$ ;  $p < 0.05$ ). Accordingly, 1st level granulation rate in control group was higher compared to RSV group. In RSV group, 2nd level granulation rate was found to be higher than the control group. A statistically significant relationship was found between fibroblast levels and group type ( $p = 0.001$ ). While 2nd level fibroblast levels were higher in RSV group, 3rd level fibroblast levels were higher in control group. While the granulation tissue was higher in RSV group, the fibroblast level was higher in control group (Table 3, 4).

## **Discussion**

Oxidative stress is characterized by an increase in endogenous reactive oxygen species such as hydrogen peroxide and superoxide. Low levels of hydrogen peroxide regulate physiological cellular events, while high levels of hydrogen peroxide observed in the inflammatory state generally cause loss of function and/or cytotoxicity in skin cells. In these conditions, hydrogen peroxide plays an indirect role by regulating metabolic pathways and intracellular signal cascades. Oxidative stress plays an important role in the pathogenesis of diseases such as: cardiovascular diseases, cancer, neurological diseases, diabetes, and aging. Defense mechanisms related to oxidative stress include: blocking and repair of these mechanisms, physical defense, and antioxidant defense. Enzymatic antioxidant defenses include: superoxide dismutase (SOD), glutathione peroxidase (GPXs), and catalase.<sup>[8-11]</sup>

Glutathione peroxidases (GPXs) are the enzyme groups that reduce hydroperoxides to water and alcohol. These enzymes provide an antioxidant protective shield by removing reactive oxygen and nitrogen species from the body. In addition, GPXs are not only critical for this detoxification process, but also play a key role in regulating apoptosis, inflammation, and other processes.<sup>[12]</sup> Superoxide dismutases (SODs), a group of metalloenzymes, release hydrogen peroxide and molecular oxygen by splitting superoxide anion free radicals and reducing O<sub>2</sub><sup>-</sup> levels.<sup>[13]</sup> Malondialdehyde (MDA) is a lipid peroxidation product and has been reported in several studies to be an indicator of oxidative stress. When free radicals are formed in the body, they at-

**Table 3.** Histopathological Parameters of the Open Wounds

Day	Open	Group type			Chi square	p
		Level	Control (n=8)	RSV (n=8)		
7	Neovascularization	2	0 (0%)	1 (12.5%)	1.067	1.000
		3	8 (100%)	7 (87.5%)		
	Granulation	2	0 (0%)	1 (12.5%)	1.067	0.302
		3	8 (100%)	7 (87.5%)		
14	Inflammation	1	8 (100%)	7 (0%)	1.067	1.000
		2	0(0%)	1 (100%)		
	Neovascularization	1	7	0 (0%)	12.444	0.001*
		2	1 (12.5%)	8 (100%)		
	Fibroblast	1	1 (12.5%)	1 (12.5%)	0.000	1.000
		2	7 (87.5%)	7 (87.5%)		
21	Inflammation	1	8 (100%)	7 (87.5%)	1.067	0.302
		2	0 (0%)	1 (12.5%)		
	Neovascularization	1	1 (12.5%)	0 (0%)	1.067	0.302
		2	7 (87.5%)	8 (100%)		
	Granulation	1	1 (12.5%)	2 (25%)	0.410	1.000
		2	7 (87.5%)	6 (75%)		
	Fibroblast	1	1 (12.5%)	0 (0%)	1.067	0.302
		2	7 (87.5%)	8 (100%)		

\*p&lt;0.05.

tack polyunsaturated fatty acids.<sup>[14]</sup>

GPX enzyme is a selenium-dependent enzyme and is known to have eight subtypes in the human body. In previously conducted studies, a strong mRNA expression of the GPX1 enzyme in the inflammatory phase of wound healing provides a positive contribution to healing.<sup>[15]</sup> In the study conducted by Luchi et al in 2010, it was reported that GPX1 enzyme levels have decreased in the early phase of the wound in immunosuppressive rats which negatively affected the wound healing.<sup>[16]</sup> A separate study, identified an increase of GPX1 within 3-7 days after skin damage.<sup>[17]</sup> It becomes clear that low levels of GPX1 enzyme, which

is known to play an important role in scavenging oxygen radicals, will adversely affect wound healing. This study found that GPX levels measured by ELISA in the RSV group were higher than in the control group. The excess amount of GPX indicates that the detoxification of reactive oxygen radicals is disproportionate, suggesting that it prevents cellular damage and delay in wound healing. We posit RSV may have a positive effect on wound healing due to its protective antioxidant effect.

Superoxide dismutase (SOD) is an important antioxidant that quickly cleanses reactive oxygen from the body and is known to have three subtypes. The more actively it can

**Table 4.** Histopathological Parameters of the Closed Wound

Day	Closed	Group type			Chi square	p
		Level	Control (n=8)	RSV (n=8)		
14	Neovascularization	1	0 (0%)	1 (12.5%)	1.067	1.000
		2	8 (100%)	7 (87.5%)		
	Fibroblast	Level	Control (n=8)	RSV (n=8)	Chi square	p
		1	0 (0%)	1 (12.5%)	12.500	0.001*
		2	1 (12.5%)	7 (87.5%)		
		3	7 (87.5%)	0 (0%)		
21	Neovascularization	Level	Control (n=8)	RSV (n=8)	Chi square	p
		1	1 (12.5%)	0 (0%)	1.067	0.302
		2	7 (87.5%)	8 (100%)		
		Level	Control (n=8)	RSV (n=8)		
	Granulation	1	8 (12.5%)	0 (25%)	16.000	0.000*
		2	0 (87.5%)	8 (75%)		
	Fibroblast	Level	Control (n=8)	RSV (n=8)	Chi square	p
		2	1 (12.5%)	8 (100%)	12.444	0.001*
		3	7 (87.5%)	0 (0%)		

remove reactive oxygen radicals, the stronger its potency is considered. In other words, more SOD activity corresponds with the removal of various oxygen types from the body.<sup>[18]</sup> In our study, no statistically significant difference was found in the amount of SOD between the RSV and control groups on the 14th day. The primary reason for this may be that only the total amount of SOD was measured, and no analysis was made for the subtypes of this enzyme in our study. For this reason, this study suggested that the activity of SOD should be evaluated by enzyme-isotyping in future studies. In addition, if this measurement had been made earlier, on the 7th day, maybe different results could have been obtained since the inflammation was more intense in this period. This situation should also be considered in future studies.

Polyphenols are a family of antioxidants that include flavonoids, anthocyanins, phenolic acids, lignans, and stilbens. All are derivatives of phenyl alanine and contain aromatic ring with reactive hydroxyl group. RSV (3,5,4-trihydroxystilbene), which is a subclass of stilbens, has trans and cis isomeric forms.<sup>[19]</sup> RSV functions as an antioxidant in vivo and can capture peroxy radicals in the skin; thus, it protects the skin from ischemia-reperfusion damage. Many studies have shown that RSV has the ability to capture both superoxide and hydroxyl radicals. RSV's in vivo antioxidant properties are strengthened by its ability to increase nitric oxide synthesis. Here, as an in vivo antioxidant, nitric oxide is capable of capturing superoxide. RSV

also maintains the intracellular concentrations of antioxidants found in biological systems. The measurement of oxidative stress in organisms can be made by evaluating MDA production. Cell and tissue damage caused by reactive oxygen radicals can be assessed with lipid peroxidation products such as MDA.<sup>[20]</sup> RSV, a polyphenol found in grape, red wine, mulberry, and peanuts and is well-known for antioxidant properties. Its antioxidant capacity is due to the OH-group yielding electrons and its aromatic ring.<sup>[21]</sup> In our study, biopsies were taken and evaluated on the 14th day and unexpected MDA levels may be related to timing. Possibly, lower MDA levels in the RSV group would have been detected if biopsies were taken during the period of higher inflammation. We encourage further studies to confirm this theory.

RSV induces anti-apoptotic signals to protect the skin. It was reported that RSV reduces protein tyrosine phosphorylation mediated by endothelin-1 and has anti-apoptotic effects.<sup>[22]</sup> Based on this information, it can be speculated that RSV may have an anti-apoptotic effect due to its partially positive effect on wound healing, but a parameter evaluating this effect was not used in this study. Further studies are needed to evaluate the possible anti-apoptotic effects of RSV with definitive parameters.

Blood flow to damaged skin is related to the extent of vascularization. Laser Doppler flowmetry may be useful for the evaluation of vasculature. In this study, blood flow was evaluated with the PeriScan PIM 3 System Laser Doppler

Blood Perfusion Imager. Microcirculation of the skin can be measured with the said device. While there was no difference between the groups on days 0, 7, and 14 in open wounds, the blood flow was found to be higher in the control group on day 21. The increase in perfusion in the open wound on the 21st day in control group was evaluated as a positive finding that RSV decreased the wound perfusion towards the last stage of healing. However, in histopathological studies, no statistical difference was found between the RSV and control groups on the 21st day. Examining the values of the 14th day, although neovascularization was higher in the RSV group, this could not be supported by Laser Doppler flowmetry. This can be attributed to the fact that the flow has not increased in immature vessels despite the increased neovascularization, but this must be proven by further studies. Besides, the higher incidence of neovascularization in the RSV group on day 14 indicates the efficacy of RSV in the mid-stage of wound healing. Although there was no difference in perfusion, from the current situation, the effectiveness of carrier molecules in RSV may be responsible. In closed wounds, a statistically significant difference was not detected in the control and RSV groups on days 0, 7, 14, and 21. In histopathological studies, again, no significant difference was found in the control and RSV groups in terms of neovascularization. Accordingly, RSV may not be effective in the neovascularization phase of wound healing, but future controlled trials are needed to prove this.

It is known that vascular endothelial growth factor (VEGF) plays a specific and powerful role in the growth and migration of endothelial cells; thus having a central role in angiogenesis. RSV plays a role in angiogenesis by providing a regulatory effect on VEGF expression with the help of polyphenols it contains. Previous studies have shown VEGF initiates the formation of granulation tissue.<sup>[23,24]</sup> In our study, the increased neovascularization (on day 14-open wounds) and granulation (on day 21-closed wounds) may have occurred due to RSV causing VEGF secretion. However, VEGF measurement to prove this was not included in the design of this study. In our study, RSV caused significant histopathological changes in higher level of neovascularization and granulation rate compared with the control group.

This study adds to the existing literature by identifying the partial effects of RSV on wound healing. It is recommended to evaluate the effects of RSV on wound healing in both closed and open wounds with the additional parameters specified in the article.

### Study Limitations:

It is recommended to evaluate the effects of RSV on wound healing in both closed and open wounds with the addi-

tional parameters specified in the article. More robust parameters are required for future research.

## Conclusion

Despite the lack of complete evidenciary support, this research indicates that RSV does have a positive effect on wound healing conditions in various ways. It was believed that the results of this study may lead to the production of new studies that may shed light on new therapeutic approaches for use in the reversal of oxidative stress in wound-causing diseases.

### Disclosures

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**Ethics Committee Approval:** The study protocol was approved by the Local Ethics Committee for Animal Experimentation, University of Health Sciences, Istanbul Mehmet Akif Ersoy Animal Experiments Production and Experimental Research Center (Approval number: 2019/12).

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**Conflict of Interest:** None declared.

**Authorship Contributions:** Concept – T.B.; Design – N.C.; Supervision – A.N.A., T.B.; Materials – T.B.; Data collection &/or processing – T.B.; Analysis and/or interpretation – N.C., T.B.; Literature search – N.C.; Writing – A.N.A., N.C., T.B.; Critical review – A.N.A., T.B.

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