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# The effect of resveratrol on expression of matrix metalloproteinase 9 and its tissue inhibitors in vascular smooth muscle cells

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Original Article

## Abstract

**BACKGROUND:** Matrix metalloproteinase 9 (MMP-9) is involved in extracellular matrix degradation and remodeling. An increase in MMP-9 expression by vascular component cells plays an important role in atherosclerotic plaque formation and rupture. Resveratrol, a polyphenolic substance, was suggested to play a role in preventing the progress of atherosclerotic disease. The aim of this study was to investigate the effect of resveratrol on MMP-9 and tissue inhibitors of metalloproteinases (TIMPs) in vascular smooth muscle cells (VSMCs) after treatment with $H_2O_2$.

**METHODS:** Cultured VSMCs were pre-treated with 0.2 mM of $H_2O_2$ before stimulation with different concentration of resveratrol. Expression of MMP-9, TIMP-1, and TIMP-3 genes were measured using real-time polymerase chain reaction (PCR) method, and MMP-9 protein level was detected using western blot analysis.

**RESULTS:** Resveratrol at 120 µmol/l concentration reduced the elevated level of MMP-9 induced by $H_2O_2$ in VSMCs as 1.85 ± 0.35 folds ($P < 0.05$) and 8.70 ± 1.20 folds ($P < 0.05$) after 24 and 48 hours, respectively. Resveratrol increased the diminished level of TIMP-1 induced by $H_2O_2$ as 2.5 ± 0.48 folds following the treatment with 120 µmol/l after 48 hours ($P < 0.05$).

**CONCLUSION:** Resveratrol as an antioxidant can decrease MMP-9 production, not only by suppressing MMP-9 expression, but also by augmenting TIMP-1 production. Altogether, resveratrol as an antioxidant can regulate the MMP-9/TIMP-1 balance, and may be considered as a preservative agent in the treatment and prevention of atherosclerosis.

**Keywords:** Matrix Metalloproteinase 9, TIMPS, Resveratrol, Vascular Smooth Muscle

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## Introduction

Atherosclerosis is the most common cause of death in the world, and oxidative stress has a critical role in the pathogenesis and development of atherosclerosis. Matrix metalloproteinase 9 (MMP-9) is a member of the family of endopeptidases, that is involved in extracellular matrix degradation and remodeling. Increased expression of MMP-9 by vascular component cells during vascular injury and inflammation plays an important role in atherosclerotic plaque formation and rupture.[1]

MMPs activity is regulated by tissue inhibitors of metalloproteinases (TIMPs).[2] Changes in TIMP levels are considered to be important due to their direct effect on MMP activity. Resveratrol is a natural polyphenolic antioxidant, and several studies have confirmed its protective effects on the cardiovascular system in patients with coronary artery disease (CAD).[3,4]

Resveratrol has a cardioprotective activity via various mechanisms such as anti-inflammatory and antioxidant activity.[5,6]

Resveratrol reduces neural damage after cerebral ischemia. The elevated levels of MMP-9 were notably diminished in the resveratrol-treated mice as compared to the vehicle MCAO mice; suggesting that resveratrol can protect neural tissue against acute ischemic stroke, which could be attributed to its activity against MMP-9 by inhibiting JNK and PKC signal transduction.[7,8]

It seems that inhibition of MMPs activation or prevention of their upregulation could inhibit the

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effects of oxidative stress on atherosclerotic plaque formation. The aim of this study was to investigate the effect of resveratrol on MMP-9 and TIMPs in vascular smooth muscle cells (VSMCs) after treatment with $H_2O_2$.

## Materials and Methods

In this experimental study, human aorta-vascular smooth muscle cells (HA-VSMC) were purchased from cell bank of Pasteur Institute of Iran in Tehran, Iran. The cells were cultured in F12K growth medium. F12K media contained 10% fetal bovine serum (FBS), 0.05 mg/ml ascorbic acid, 0.01 mg/ml insulin, 0.05 mg/ml transferrin, 10 ng/ml sodium selenite, 0.03 mg/ml human epidermal growth factor (EGFs), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to a final concentration of 10 mM, penicillin (100 U/ml), and streptomycin (100 U/ml).

Cells were cultured in a humidified incubator containing 5% $CO_2$ at 37 °C. Daily control of cell growth and cell division in culture condition were done. Cells used for the experiments were 3 to 7 passages.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine resveratrol concentration causing 50% cell death (the half maximal inhibitory concentration or IC50). Briefly, the cells were seeded at a density of $15 \times 10^3$ in 96-well plates. After achieving approximately 80% confluence, cells were treated with increasing concentration of resveratrol (40 to 200 µmol/l) for 48 hours. Thereafter, 20 µl MTT (5 mg/ml, Sigma-Aldrich, Germany) solution was added to each well. The formazan crystal was dissolved by adding 150 µl dimethyl sulfoxide (DMSO) to each well after 4 hours, and the optical density of each well was measured at 490 nm on an enzyme-linked immunosorbent assay (ELISA) plate reader (Awareness, USA) following 4 hours of incubation at 37 °C.

The cells were seeded in a 12-well plate at a density of $1 \times 10^4$ cells per well. When the cells achieved approximately 80% confluence, they were pretreated with physiologic concentration (0.2 mM) of $H_2O_2$ for inducing oxidative stress.[9] Then, the cells co-cultured with 80, 100, and 120 µmol/l resveratrol (Sigma, USA) for 24 and 48 hours.

***RNA isolation and cDNA synthesis:*** Total RNA was extracted from the cells using Trizol (Invitrogen, USA) according to the manufacturer instructions. RNA was quantified using a Nanodrop 2000C spectrophotometer (Thermo Scientific, USA) and treated with DNase. Then, cDNA was synthesized from 0.5 mg total RNA using random primer and the cDNA Synthesis kit (Thermo Fisher Scientific, USA).

***Real-time polymerase chain reaction (Real-time PCR):*** The expression of MMP-9, TIMP-1, and TIMP-3 genes was measured using quantitative real-time PCR. The experiments were performed using Rotor-Gene 3000 real-time DNA amplification system (Corbett Research, Australia) and SYBR green method. Primers used for real-time PCR are listed in table 1. Experiments were performed in triplicate,[6] using 5 µl SYBR green PCR Master Mix, 0.2 µl primer sets, 2 µl cDNA (40 ng), and 3.6 µl nuclease-free $H_2O$ to yield a 10 µl reaction. The amplification conditions were as follow: initial denaturing at 94 °C for 5 minutes, then 40 cycles of 95 °C for 15 seconds, 59 °C for 20 seconds, and 72 °C for 30 seconds. The comparative cycle threshold (CT) (ΔΔCT) method was used for data quantitation, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an endogenous reference.

**Table 1.** Primer sequences and product length

| Genes | Genes Primer sequences (5'-3') | Amplicon length (bp) | Gene bank reference |
|---|---|---|---|
| MMP-9 | Forward: GCTCACCTTCACTCGCGTGTA<br>Reverse: TCCGTGCTCCGCGACA | 70 | NM_004994.2 |
| TIMP-1 | Forward: CCTGGCTTCTGGCATCCTG<br>Reverse: CCACGAACTTGGCCCTGATG | 125 | NM_003254.2 |
| TIMP-3 | Forward: GTCGCGTCTATGATGGCAAG<br>Reverse: AAGCAAGGCAGGTAGTAGCA | 151 | NM_000362.4 |
| GAPDH | Forward: ACACCCACTCCTCCACCTTTG<br>Reverse: TCCACCACCCTGTTGCTGTAG | 112 | NM-002046.5 |

MMP-9: Matrix metalloproteinase 9;TIMP: tissue inhibitors of metalloproteinases; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

***Western blot analysis:*** Western blot analysis was used for detecting MMP-9 protein expression. VSMCs were lysed in ice-cold radioimmune precipitation assay buffer (6 ×) containing protease inhibitor cocktail. Samples were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to nitrocellulose membranes, and incubated overnight at 4 °C with blocking solution [5% nonfat dried milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20]. Membranes were incubated with MMP-9 antibody (1:3000) at room temperature for 2 hours. After washing, the membrane was incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (1:10,000) at room temperature for 90 minutes. Finally, the color was developed with the addition of 3,3',5,5'-tetramethyl-benzidine membrane peroxidase substrate. Beta-actin was detected as a sample loading control.

All experiments were done in triplicate. Results are expressed as the mean ± standard error of mean (SEM). Statistical analysis was done using nonparametric Kruskal-Wallis test. The nonparametric Mann-Whitney U test was used to compare differences between control and test groups. P value of less than 0.050 was considered as the level of significance.

## Results

The IC50 of resveratrol estimated by MTT assay was about 120 µmol/l. This concentration of resveratrol was chosen for further experiments.

In this study, the resveratrol effects on expression of MMP-9 and its inhibitors (TIMP-1 and -3) were investigated in VSMCs after inducing with $H_2O_2$. $H_2O_2$ used at the non-toxic concentration of 0.2 mM. MMP-9 expression increased 1.43 ± 0.29 and 1.98 ± 0.54 folds after 24 and 48 hours, respectively ($P < 0.050$ for both) after treatment of the cells with $H_2O_2$ without resveratrol. In contrast, resveratrol at different concentrations decreased MMP-9 expression, when given simultaneously with $H_2O_2$.

After 24 hours, MMP-9 expression was decreased 1.60 ± 0.21, 1.57 ± 0.30, and 1.85 ± 0.35 folds following the treatment with 80, 100, and 120 µmol/l resveratrol when compared with the $H_2O_2$-treated group ($P < 0.050$ for all) (Figure 1).

After 48 hours, resveratrol at 80, 100, and 120 µmol/l concentrations reduced the elevated level of MMP-9 induced by $H_2O_2$ as 6.20 ± 1.28, 5.50 ± 1.96, and 8.70 ± 1.20 folds, respectively, when compared with the $H_2O_2$-treated group ($P < 0.050$ for all) (Figure 1).

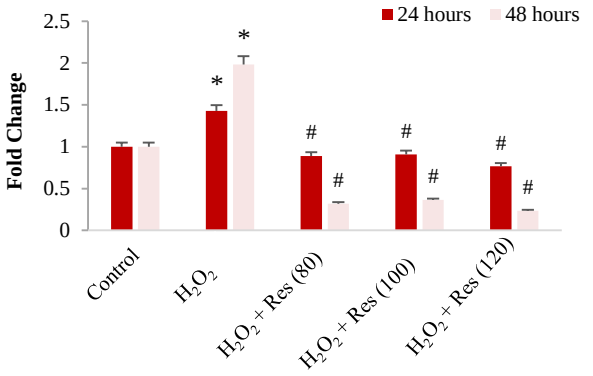


**Figure 1.** $H_2O_2$ upregulated the expression of matrix metalloproteinase 9 (MMP-9) in vascular smooth muscle cells (VSMCs). Resveratrol at 80, 100, and 120 µmol/l concentrations reduced the elevated level of MMP-9 induced by $H_2O_2$.
* $P < 0.050$ compared with control group
# $P < 0.050$ compared with $H_2O_2$-treated group

Western blot analysis confirmed the changes observed at MMP-9 mRNA level (Figure 2). In western blot analysis, beta-actin (42 kDa) was used as internal control.

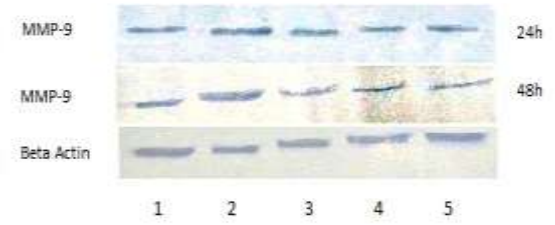


**Figure 2.** The expression of matrix metalloproteinase protein 9 (MMP-9) determined by western blot analysis after treatment with 80, 100, 120 µmol/l resveratrol in vascular smooth muscle cells (VSMCs). Beta-actin (42 kDa) was used as an internal control to standardize the protein loading in western blotting.
Lane 1: control; Lane 2: treated with $H_2O_2$; Lane 3-5: treated with $H_2O_2$ and various concentration of resveratrol (80, 100, and 120 µmol/l, respectively).

After treating the cells with $H_2O_2$ without resveratrol, TIMP-1 expression decreased 1.73 ± 0.26 folds after 48 hours ($P < 0.050$). In contrast, resveratrol at different concentrations increased TIMP-1 expression when given simultaneously with $H_2O_2$.

After 48 hours, resveratrol at 80, 100, and 120 µmol/l concentrations increased the diminished level of TIMP-1 induced by $H_2O_2$ about 1.74 ± 0.51,

1.93 ± 0.37, and 2.50 ± 0.48 folds, respectively, compared with the $H_2O_2$-treated group ($P < 0.050$ for all) (Figure 3). There was no significant change in the expression of TIMP-1 after 24 hours.

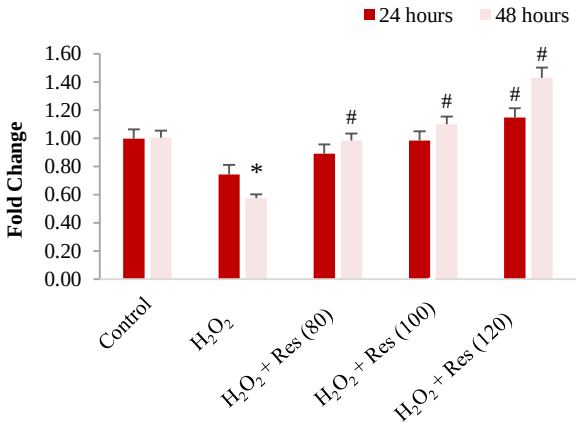


**Figure 3.** $H_2O_2$ downregulated the expression of tissue inhibitors of metalloproteinase 1 (TIMP-1) in vascular smooth muscle cells (VSMCs). Resveratrol in 80, 100, and 120 µmol/l concentrations increased the diminished level of TIMP-1 induced by $H_2O_2$ after 48 hours.
* $P < 0.050$ compared with control group
# $P < 0.050$ compared with $H_2O_2$-treated group

We found no significant effect by resveratrol on TIMP-3 expression by real time PCR (not shown).

## Discussion

In this study, we investigated the resveratrol effect on MMP-9 expression in $H_2O_2$-induced VSMCs. Resveratrol decreased MMP-9 expression in $H_2O_2$-induced VSMCs.

Matrix metalloproteinases play important roles in cardiovascular diseases, and oxidative stress has destructive effect on vascular biology through the activation of MMPs.[10]

MMP-9 is involved in the breakdown of extracellular matrix proteins in various physiological and pathological conditions, such as angiogenesis, bone development, and atherosclerosis plaque rupture.[1]

Enhanced expression and activities of MMP-9 have been observed in vascular injury. TIMP-1 is endogenous inhibitor of MMP-9, and it is suggested that TIMP-1 plays an important role in cardiovascular disease.

Regarding the effects of oxidative stress in vascular remodeling, antioxidant approaches are used to reduce the upregulation of MMPs, and attenuate the tissue dysfunction and remodeling during vascular diseases.[11-13]

In this study, $H_2O_2$ increased MMP-9 expression in VSMCs. Upregulation of MMP-9 is not unexpected in conditions that free radicals were increased by $H_2O_2$ or other oxidants. Elevated levels of MMP-9 due to oxidant stress in VSMCs has been demonstrated in many studies.[10,14]

Resveratrol decreased MMP-9 expression in $H_2O_2$-induced VSMCs. Resveratrol displays potent antioxidant activity, thereby can scavenge free radicals produced by $H_2O_2$, and so reduce the oxidative effects of $H_2O_2$ in the environment.

Previous studies have demonstrated that polyphenols interfere with MMP-9 expression, and reduce inflammatory angiogenesis through MMP-9 inhibition.[15,16] The results of this study and other studies[17,18] support a potential protective role for dietary polyphenols in cardiovascular diseases.

Resveratrol inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signal transduction pathway that mediates the expression of MMPs and many other genes involved in inflammatory and pro-oxidant processes associated with vascular disease development.[19] The capability of resveratrol in reducing of NF-κB activity may be considered indicative to change MMP-9 expression in this study.

The MMPs are tightly regulated, not only at the transcriptional level, but also by their specific inhibitors.[20,21] The effect of antioxidant on MMP or TIMP expression in different cells has been previously reported.[22-24]

Epigallocatechin-3-gallate, as an antioxidant agent, reduces the activity and expression of MMP-9, and enhances the expression of TIMP-1 in cancer cell line MDA-MB-231.[22]

In a recent study, resveratrol attenuated blood-brain barrier dysfunction via regulation of MMP-9 and TIMP-1.[23]

According to a study, use of polyphenol extract from olive pomace oil down-regulated the levels of matrix MMP-2 and MMP-9, and increased TIMP-1 expression in human endothelial cells,[24] which could represent a powerful tool for the prevention and treatment of endothelial dysfunction-associated vascular disease.

Matrix metalloproteinases contribute to plaque rupture, atherothrombosis, and myocardial infarction.[25] For this reason, using MMP inhibitors can be considered as a preservative agent for the prevention and treatment of cardiovascular diseases.[26]

The results of this study showed that resveratrol, as an antioxidant, can decrease MMP-9 expression,

not only by suppressing MMP-9 production, but also by augmenting TIMP-1 production.

It seems that due to the dual role of resveratrol with regard to regulation of the MMP-9/TIMP-1 balance, it could be a good choice for preventing and treating cardiovascular diseases.

## Conclusion

Altogether, the results of this study showed that resveratrol, as an antioxidant, can regulate the MMP-9/TIMP-1 balance, and may be considered as a preservative agent in the prevention and treatment of atherosclerosis. To better understand the atherosclerosis prevention and treatment with resveratrol, further studies with animal models are necessary.

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## Conflict of Interests

Authors have no conflict of interests.

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