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Effects of resveratrol on the alterations of cavernosal eNOS and LOX-1 expression in the hypercholesterolemic condition: a preliminary study

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Background/aim: The aim of this study was to determine the effects of resveratrol on the alterations of cavernosal eNOS and LOX-1 mRNA expression in the hypercholesterolemic condition.

Materials and methods: Twenty-one New Zealand white male rabbits were separated into three groups. Rabbits were fed with a normal dietary intake for the control group and a 2% cholesterol diet for the hypercholesterolemia and resveratrol groups for 6 weeks. Resveratrol 4 mg/kg daily was administered for the resveratrol group. Cavernosal LOX-1 and eNOS mRNA expressions were determined with real-time RT-PCR in all groups. The statistical analysis was performed with the Kruskal–Wallis and Mann–Whitney U tests.

Results: We found no difference between mean LOX-1 mRNA expression levels in the three groups. Lower mean eNOS mRNA expression level was determined in the hypercholesterolemia group when compared with the control group ($P = 0.011$). Mean eNOS mRNA expression level in the resveratrol group was similar to that in the control group but significantly higher than that in the hypercholesterolemia group ($P < 0.001$).

Conclusion: This preliminary study demonstrates the beneficial effects of resveratrol on cavernosal eNOS expression. The presence of cavernosal LOX-1 expression was also shown for the first time. Resveratrol may be an alternative option in hypercholesterolemic erectile dysfunction with further studies supporting its beneficial effects on the corpus cavernosum.

Key words: Hypercholesterolemia, erectile dysfunction, resveratrol, eNOS, LOX-1

1. Introduction

Erectile dysfunction (ED) and atherosclerosis share common risk factors such as obesity, diabetes mellitus, hypercholesterolemia, sedentary lifestyle, and metabolic syndrome (1). Hypercholesterolemia both impairs endothelium-dependent cavernosal smooth muscle relaxation and inhibits vascular smooth muscle relaxation (2–4). However, the underlying molecular mechanisms are still unclear. Oxidized low-density lipoprotein (ox-LDL) has many harmful effects on NO bioactivity in endothelial dysfunction in the early stages of atherosclerosis, whereas controversial data exist for its effects on corpus cavernosum (5–7). Lectin-like oxidized LDL receptor-1 (LOX-1) is the main receptor type for the proatherogenic effects of ox-LDL and responsible for the binding, internalization, and degradation of ox-LDL in endothelial cells (8). LOX-1 expression is upregulated in atherosclerosis of large arteries and in pathologic conditions such as hypertension, diabetes mellitus, and hyperlipidemia (9). Its expression in the corpus cavernosum has not been demonstrated before.

The most accepted pathophysiologic mechanism for both atherosclerosis and ED is endothelial dysfunction (10). Nitric oxide (NO) is the key molecule for the regulation of endothelial function in the vascular walls as well as for the induction and maintenance of erections (11). The decreased bioactivity of NO in the penis regardless of the reason causes vasculogenic ED (5). Endothelial nitric oxide synthase (eNOS) is the main molecule responsible for NO production in vascular and cavernosal structures, so it is not surprising that decreased cavernosal eNOS expression causes diminished NO bioactivity and subsequently ED.

Resveratrol is a polyphenol that has various rehabilitative biochemical and physiologic effects on endothelial functions and is found in large amounts in *Polygonum cuspidatum*, grape skin, and red wine. Resveratrol augments NO bioavailability by some mechanisms, one of which is the increased expression of endothelial nitric oxide synthase (eNOS) in myocardium and vascular endothelial cells (12–14). Resveratrol significantly improved erectile functions and demonstrated relaxant

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effects on cavernosal tissues in hypercholesterolemic and diabetic animal models (15–17). This preliminary study explores the effects of resveratrol on cavernosal eNOS and LOX-1 expression in hypercholesterolemic rabbits.

2. Materials and methods

2.1. Experimental animals and hypercholesterolemia conditioning

The study protocol was approved by the institutional review board (Protocol No. 99/2007). Twenty-one 3-month-old New Zealand white male rabbits weighing 2600–3200 g were obtained from the Department of Experimental Animals in our university. They were randomly divided into three groups with 7 rabbits in each group: control, hypercholesterolemia (HC), and resveratrol groups. Rabbits were housed individually in different cages in an air-conditioned room under a 12-h light/dark cycle. All rabbits were fed with a standard laboratory diet for at least 7 days before the initiation of the study. The rabbits in the control group were fed with a regular diet and drinking water for 6 weeks. The rabbits in the hypercholesterolemia and resveratrol groups were fed with a 2% (w/w) hypercholesterolemic diet and drinking water for 6 weeks. Resveratrol (Sigma Aldrich) at a daily dosage of 4 mg/kg was also administered via drinking water for the resveratrol group (15,18). All rabbits were weighed at the beginning and the end of the study. After 6 weeks, the rabbits were sacrificed with high-dose thiopental. Blood samples were obtained for the measurement of plasma total, LDL, and HDL cholesterol levels (Roche Diagnostic, Tokyo, Japan). The penis was excised and the corpus cavernosum was dissected free from surrounding structures such as the tunica albuginea and corpus spongiosum.

2.2. Real time reverse transcription-polymerase chain reaction (RT-PCR)

TRIzol reagent (RNA-tidy-G, AppliChem) was used for total RNA isolation. One milliliter of TRIzol solution was added for 100 mg of tissue sample. The suspension was treated with DNase, and after a waiting period of 15 min at room temperature, the suspension was homogenized with an autohomogenizator (Heidolph Silent Crusher S). After

centrifugation RNA was separated from DNA and protein with 0.2 mL of chloroform (AppliChem). Isopropanol (0.5 mL; AppliChem) was used for the precipitation of RNA. The RNA pellet was washed with 1 mL of 75% ethanol (Merck) twice and the ethanol was removed. After 60 min of air-drying, 50 μ L of RNase-free water (QIAGEN) was used for the solubilization of the RNA pellet and total RNA was isolated. The amount of RNA was determined by measuring the absorbance at 260 nm on a spectrophotometer (OPTIMA SP-3000 PLUS). Then 1 μ g of total RNA was reverse-transcribed into cDNA with the Roche Transcriptor First Strand cDNA Synthesis Kit according to the manufacturer's protocol. The obtained cDNA was preserved at -20°C until RT-PCR application. Quantitative RT-PCR was performed with the Roche Light Cycler 1.5 using 2 μ L of cDNA for each sample. Proper temperature adjustments were set for denaturation, cooling, elongation, and melting phases. Amplification was carried out with the FastStart DNA Master HybProbe (Roche) by specific mRNA primer pairs and hybridization probes (TIB MOLBIOL) for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), eNOS, and LOX-1 (Table 1). Amplified products were visualized on 1.5% agarose gels with the use of ethidium bromide.

Standard curves were made up with the dilution method using Light Cycler 3.0 software and absolute mRNA expression levels for the internal control GAPDH and the target genes LOX-1 and eNOS were determined with the comparative threshold cycle (Ct) method. GAPDH was used as an internal control for RT-PCR analysis because it is one of the most universally used internal controls and its expression is consistent at different time points and various experimental manipulations (19). The relative amount of the expression levels of target genes' LOX-1 and eNOS mRNA to the housekeeping GAPDH mRNA expression levels were calculated for the three groups and mean expression levels were compared.

2.3. Statistical analysis

All data were expressed as mean \pm standard error. Statistical analysis was performed with the Kruskal–Wallis test for the comparisons between all experimental groups and the

Table 1. Rabbit GAPDH, LOX-1, and eNOS specific primer sequences.

	FW	RW
GAPDH	5'- TgTgAAgCTCATTTCCTggTATg	5'- gTTTCATgACAAggTAgggCTC
LOX-1	5'- gAATATACTggAgggACAggTCTTAg	5'- CTggAATgggAAgTCgCT
eNOS	5'- CTggAggATgTggCCgT	5'- CTCTggCCTTCTgCTCATTCT

FW: Forward primer, RW: reverse primer, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, LOX-1: lectin-like oxidized LDL receptor-1, eNOS: endothelial nitric oxide synthase.

Mann–Whitney U test for two groups at a significance level of $P < 0.05$ with SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Body weight and plasma cholesterol levels

There was not any difference ($P > 0.05$) in mean body weight of the rabbits between the control, hypercholesterolemia, and resveratrol groups both at baseline and at the end of the trial (Table 2). Plasma total, LDL, and HDL cholesterol levels were significantly higher in the hypercholesterolemia and resveratrol groups compared with the control group ($P < 0.001$). Lower total and LDL cholesterol levels were observed in the resveratrol group when compared with the hypercholesterolemia group ($P < 0.01$). HDL cholesterol levels did not differ significantly between these two groups ($P > 0.05$, Table 3).

3.2. LOX-1 expression

LOX-1 and GAPDH expressions for the three groups are shown by ethidium bromide staining in Figure 1. Mean expression levels of the amount of the target LOX-1 mRNA relative to the housekeeping GAPDH mRNA in the control, hypercholesterolemia, and resveratrol groups were 0.67 ± 0.32 , 0.39 ± 0.22 , and 0.28 ± 0.13 , respectively (Figure 1) ($P > 0.05$).

3.3. eNOS expression

Mean expression levels of the amount of the target eNOS mRNA relative to the housekeeping GAPDH mRNA in the control, hypercholesterolemia, and resveratrol groups were 4.81 ± 2.46 , 0.28 ± 0.22 , and 5.21 ± 1.10 , respectively (Figure 2). Statistical analysis showed that mean cavernosal eNOS mRNA expression level for the resveratrol group was similar to that of the control group ($P > 0.05$), which was significantly higher than that of the hypercholesterolemia group ($P < 0.001$).

4. Discussion

Risk factors for the development of ED show close similarity with vascular risk factors for the development of atherosclerosis. The most accepted pathophysiologic mechanism for both conditions is endothelial dysfunction (10). The fact that hypercholesterolemia impairs vascular and cavernosal endothelium-dependent smooth muscle relaxation and may cause vasculogenic ED has been demonstrated in previous isometric tension studies (2–4). In this study, the hypercholesterolemic rabbit model formed by a 2% (w/w) high-cholesterol diet reached approximately 72-fold higher total and LDL cholesterol levels when compared with the control group at the end of the sixth week, which was high enough for the development of impaired cavernosal functions according to these studies. In a study conducted by Seo et al. in rabbits fed with a 2% cholesterol diet for 4 or 8

Table 2. Mean body weights of the rabbits in the three groups at baseline and the end of the trial. Mean body weights did not differ significantly in the three groups ($P > 0.05$).

	Body weight (g)	
	Baseline	Study end
Control (n = 7)	2752.8 ± 59.0	2862.8 ± 78.5
Hypercholesterolemia (n = 7)	2708.6 ± 65.3	2878.6 ± 86.2
Resveratrol (n = 7)	2861.4 ± 57.3	2993.6 ± 75.4

weeks, acetylcholine-induced relaxation (endothelium-dependent) of the corpus cavernosum was significantly reduced in the hypercholesterolemia group compared to the control group, whereas no difference was observed with direct smooth muscle relaxant papaverine (endothelium-independent) between the experimental and control groups (20). They also determined decreased eNOS activity in the hypercholesterolemia group and concluded that impaired cavernosal relaxation in hypercholesterolemia may be related to the functional impairment of eNOS. The hypercholesterolemic rabbits in the present study also had a lower eNOS expression level compared to the control group, which is postulated as one of the reasons for decreased eNOS activity. We also demonstrated impaired cavernosal functions with the same experimental method in our previous study; this is why we did not perform functional studies again in the present study (15).

There is debate over the effects of ox-LDL on corpus cavernosum smooth muscle. While some authors suggested that ox-LDL decreased endothelium-dependent relaxation responses, others reported that ox-LDL did not change cavernosal relaxation responses (5–7). Direct contractile effects of ox-LDL on cavernosal smooth muscle have also been demonstrated (21). Ox-LDL has a significant role in proatherogenic endothelial dysfunction. The main receptor for the endothelial dysfunction in proatherogenic processes with ox-LDL is LOX-1. Sawamura et al. showed that LOX-1 is essential for the binding, internalization, and degradation of ox-LDL in endothelial cells and it is highly expressed in vascular-rich organs such as the placenta, lungs, and brain as well as in atherosclerotic lesions (8). In spite of its vascular-rich structure, the presence of LOX-1 in the corpus cavernosum and its possible role in hypercholesterolemia-associated impotence have not been explored previously. To our knowledge, cavernosal LOX-1 expression and possible alterations in the hypercholesterolemic condition were evaluated for the first time in the present study. We found no significant difference between mean cavernosal LOX-1 expression level in the control, hypercholesterolemia, and resveratrol

Table 3. Average plasma cholesterol levels at the end of the sixth week. Total and LDL cholesterol levels were significantly lower in the resveratrol-treated group compared to those in the hypercholesterolemia group.

	Control (n = 7)	Hypercholesterolemia (n = 7)	Resveratrol (n = 7)
Total cholesterol (mg/dL)	35.0 ± 5.7	2508.0 ± 252.0 ^a	1357.8 ± 342.4 ^{a,b}
LDL (mg/dL)	6.4 ± 1.3	859.1 ± 73.0 ^a	495.6 ± 107.0 ^{a,b}
HDL (mg/dL)	6.4 ± 0.9	27.1 ± 1.7 ^a	23.7 ± 3.8 ^a

^aControl group vs. hypercholesterolemia and resveratrol groups; $P < 0.001$.

^bResveratrol group vs. hypercholesterolemia group; $P < 0.01$.

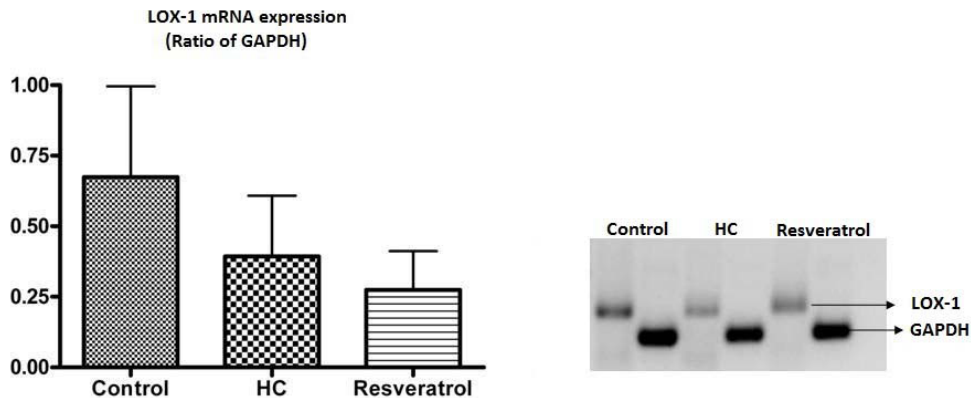


Figure 1. Cavernosal mean LOX-1 mRNA expression level in the three groups and the demonstration of cavernosal LOX-1 expression with ethidium bromide staining using one representative sample from each group. Quantitative analysis showed no significant difference between groups ($P > 0.05$). HC: Hypercholesterolemia.

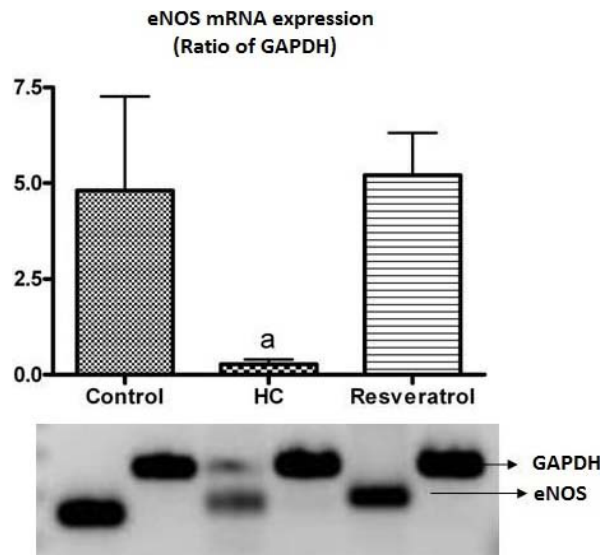


Figure 2. Cavernosal mean eNOS mRNA expression level in the three groups and visualization of PCR products on agarose gel with ethidium bromide staining with one representative sample from each group. Lower eNOS signal intensity in the sample from the hypercholesterolemia group is observed in comparison to the control and resveratrol groups. HC: Hypercholesterolemia, a; $P < 0.001$ for HC vs. control and HC vs. resveratrol groups.

groups in this preliminary study. It is known that LOX-1 expression is upregulated in the atherosclerosis of large arteries in pathologic conditions such as hypertension, diabetes mellitus, and hyperlipidemia, all of which are also important risk factors for vasculogenic erectile dysfunction (9). One might thus expect the impairment of cavernosal LOX-1 expression in the hypercholesterolemic condition. Considering the results of the present study, it may be hypothesized that cavernosal structures are composed of small vessels and LOX-1 may not have a pioneering role in small vessels as it has in large arteries. Nevertheless, in order to clarify whether LOX-1 has any role in hypercholesterolemia-induced ED or not, further trials are mandatory before making any clear-cut recommendations.

Resveratrol was proposed as a promising agent in cardioprotection in a recent report by Penumathsa et al. with its various biochemical and physiologic effects (22). The most prominent effect of resveratrol seems to come from the upregulation of NO bioavailability in pathologic conditions such as ischemia/reperfusion injury, hypercholesterolemia, or diabetes. Wallerath et al. studied the effects of resveratrol on eNOS activity and expression in human umbilical vein endothelial cell cultures. They found that resveratrol upregulated eNOS mRNA expression, eNOS protein expression, eNOS activity, and eNOS promoter activity (23). In a previous study conducted by our group, we evaluated the effects of resveratrol on different vascular structures such as the thoracic aorta, mesenteric artery, renal artery, and corpus cavernosum by isolated tissue bath experiments in the hypercholesterolemic status. We demonstrated that a 2%

high cholesterol diet for 6 weeks significantly impaired cavernosal relaxation responses. Endothelium-dependent relaxation responses induced by acetylcholine were significantly higher in the control and resveratrol groups when compared with the hypercholesterolemia group in that study (15). In this preliminary study, we evaluated cavernosal eNOS expression in three groups with the same experimental model and determined that mean eNOS expression levels in the resveratrol and control groups were significantly higher than that in the hypercholesterolemia group. With these findings we propose that protected cavernosal eNOS expression by resveratrol may be one of the precluding effects of resveratrol on the corpus cavernosum in the hypercholesterolemic condition. One limitation of the present study is that we solely evaluated eNOS mRNA expression, not the protein expression or eNOS phosphorylation, which are also important aspects of NO bioactivity.

In conclusion, we demonstrated for the first time that resveratrol has beneficial effects on cavernosal eNOS expression in the hypercholesterolemic condition. This might be considered as one of the mechanisms for the restorative effects of resveratrol on cavernosal structures in vasculogenic impotence. The presence of cavernosal LOX-1 expression was also shown for the first time. However, more extensive research on this topic is necessary in order to better identify and support these findings.

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