

# THE EFFECT OF ASPIRIN ON SERUM VASCULAR ENDOTHELIAL GROWTH FACTOR AND NITRIC OXIDE CONCENTRATION IN HIGH-CHOLESTEROL FED RABBITS

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## Abstract

**INTRODUCTION:** Hypercholesterolemia is one of the major risk factors for atherosclerosis which is characterized by endothelial dysfunction. This study was designed to investigate the effect of aspirin on serum vascular endothelial growth factor (VEGF) and nitric oxide (NO) concentrations in hypercholesterolemic animals.

**METHODS:** Sixteen male rabbits were randomly divided into two groups, aspirin-treated and control. Aspirin (10 mg/kg/day) was administered orally using feeding tube. All animals were fed with high-cholesterol diet (1%) during the experiment. After five weeks, blood pressure, serum lipid and lipoprotein profiles, serum VEGF and NO concentrations were measured.

**RESULTS:** Aspirin did not change blood pressure. Aspirin significantly decreased serum LDL (1276±72.1 vs. 1505±68.03 mg/dl) and triglyceride (477.5±8.3 vs. 649.1±15.2 mg/dl) (P<0.05). High-cholesterol diet significantly decreased serum VEGF level in both groups (control: 24.59±0.42 vs. 38.09±2.49 pg/ml; aspirin: 24.72±0.84 vs. 42.29±2.03 pg/ml) (P<0.05) and aspirin did not change serum VEGF level in hypercholesterolemic animals (P>0.05). Serum NO concentration was also significantly decreased after five weeks of high-cholesterol diet (control: 5.87±0.33 vs. 8.67±0.68 µmol/lit; aspirin: 5.66±0.33 vs. 8.58±0.60 µmol/lit) (P<0.05). Aspirin did not change serum NO level (P>0.05).

**CONCLUSIONS:** We conclude that under the conditions of this study, aspirin cannot change serum VEGF and NO concentrations in high-cholesterol fed animals.

**Keywords:** Hypercholesterolemia, nitric oxide, vascular endothelial growth factor, aspirin.

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## Introduction

Hypercholesterolemia is an established risk factor for atherosclerosis and cardiovascular disease.<sup>1</sup> One of the critical early events in atherosclerosis is endothelial dysfunction,<sup>2</sup> which is characterized by decreased synthesis and/or activity of endothelium-derived Nitric Oxide (NO).<sup>3</sup> A deficiency in NO bioavailability causes vasoconstriction, proliferation of smooth muscle cells, increased platelet activation and aggregation, and leukocyte adhesion.<sup>4-6</sup>

Growth factors play an important role in the pathogenesis of cardiovascular disease and atherosclerosis.<sup>7</sup> Vascular Endothelial Growth Factor (VEGF), a potent angiogenic growth factor, stimulates endothelial cell proliferation and migration,

and angiogenesis.<sup>8,9</sup> These actions of VEGF are thought to prevent the atherosclerosis processes.<sup>10,11</sup> Non-steroidal anti-inflammatory drugs (NSAIDs), which block the enzyme cyclooxygenase, have been widely used for analgesic and anti-inflammatory purposes in cardiovascular disease. Clinical and experimental studies have reported that aspirin reduces cardiovascular death and slows the development of atherosclerotic lesions.<sup>12-15</sup> Several mechanisms have been proposed for the anti-atherosclerotic effects of aspirin.<sup>12,14</sup> However, the exact mechanism is not clear. The aim of this study was to evaluate the effect of aspirin on serum VEGF and NO concentrations in experimentally induced atherosclerosis in rabbits.

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### Materials and methods

Sixteen male rabbits were purchased from the Pasteur Institute of Iran. The animals were housed two per cage in animal room at room temperature with 12h light/dark cycle. The Ethics Committee of Isfahan University of Medical Sciences approved all of the experimental procedures.

All of the animals were fed with high-cholesterol diet (1%) during the experiment. Cholesterol-rich diet was prepared by adding 1 g cholesterol (Merck, Germany) in 4 ml olive oil to 0.1 kg of commercial rabbit chow. It has been demonstrated by previous studies that this diet induces atheromatous lesions in the arteries after 4-6 weeks.<sup>16-18</sup>

After one week of habituation in the laboratory, overnight fasting blood samples were taken to measure serum lipid and lipoprotein profiles (cholesterol: CHO, triglyceride: TG, high-density lipoprotein: HDL, and low-density lipoprotein: LDL), NO and VEGF concentrations. The blood samples were centrifuged and kept in separate Eppendorf tubes at -70 °C until analysis.

The animals were randomly divided into two groups. All animals had free access to high-cholesterol diet and water ad libitum. Group 1 (n=8) received 10 mg/kg/day of aspirin (Sigma) dissolved in carboxy methyl cellulose.<sup>19-21</sup> Group 2 (n=8) received carboxy methyl cellulose as control. Aspirin was administered orally using feeding tube. After five weeks, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) (Sigma). A polyethylene catheter was inserted into the right femoral artery and direct blood pressure was measured by physiograph (Bioscience, England). Then, blood samples were taken, centrifuged and kept

in separate Eppendorf tubes at -70°C for determination of serum NO and VEGF concentrations.

Serum VEGF concentration was measured using enzyme-linked immunosorbent assay using available reagents and recombinant standards (R & D systems, USA). Briefly, 50 µl of standard or serum was added to the wells of microplate precoated with monoclonal antibody for VEGF and was incubated for 2 hours at room temperature. After any unbound substances had been washed away, an enzyme-linked polyclonal antibody against VEGF was added to the wells and incubated for 2 hours. After a wash, 100 µl substrate solution was added to the wells and incubated for 30 minutes. 100 µl of stop solution was then added for color development. The optical density was determined at 450 nm using microplate reader. The VEGF assay has a minimum sensitivity of 3.0 pg/ml. Serum NO concentration was determined by Griess reagent method (Promega Corp, U.S.A, Cat#G2930) using available reagents.<sup>22</sup> Briefly, serum samples were added to the wells (96-well enzymatic assay plate). Sulfanilamide solution was added to all experimental samples, and after incubation, N-1-naphthylethylenediamine dihydrochloride solution was added. Absorbance was then measured by microreader at the wavelength of 520 nm. The NO concentration in samples was determined in comparison to nitrite standard reference curve. The limit detection was 2.5 µM nitrite.

Data are reported as means ± SEM. T-test was used to compare data between the two groups. Paired t-test was used to compare data before and after the experiment. Statistical values of less than 0.05 were considered as significant.

**TABLE 1.** Systolic, diastolic and mean arterial pressure (mmHg) and body weight (Kg) in two experimental groups

Group	n	Systolic pressure	Diastolic pressure	Mean arterial pressure	Body weight (before)	Body weight (after)
1 (Aspirin)	8	94.3±9.4	69.3±7.3	77.6±7.8	1.68±0.35	1.94±0.39
2 (Control)	8	95.6±10.5	66.8±15.1	76.4±12.2	1.63±0.35	2.32±0.97
P (t-test)		ns*	ns	ns	ns	ns

\*ns: no significant difference

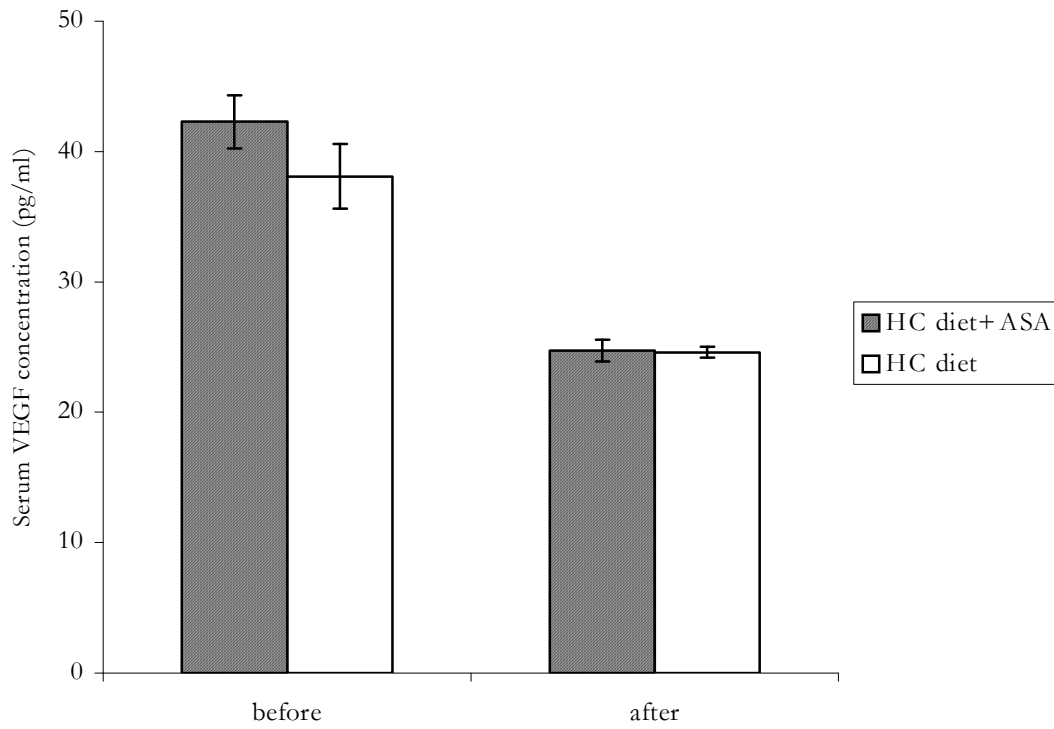
Data are expressed as Mean ± SEM

**TABLE 2.** Serum cholesterol (CHO), triglyceride (TG), HDL and LDL concentrations (mg/dl) before and after the experiment

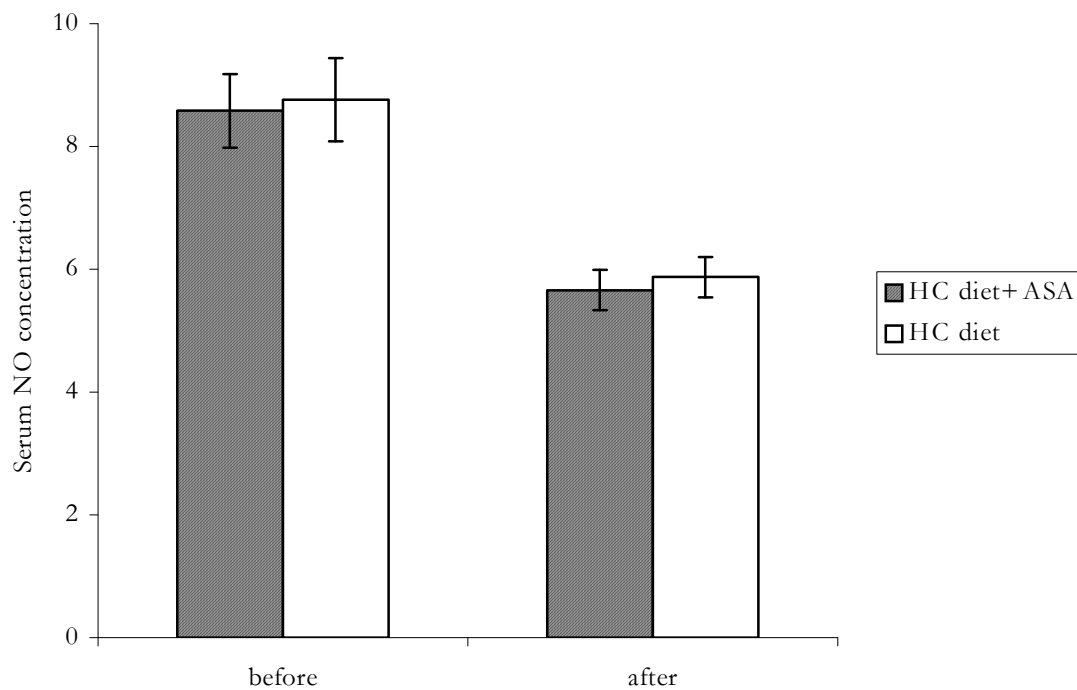
Group	CHO		TG		HDL		LDL	
	before	after	before	after	before	after	before	after
1 (Aspirin)	146.7±4.8	1552.8±81.1	96.1±11.3	477.5±8.3	28.5±2.4	143.2±9.8	98.9±3.1	1276±72.1
2 (Control)	140.5±2.7	1745.4±77.8	73.0±3.7	649.1±15.2	28.9±2.3	144.1±15.3	96.1±2.5	1505±68.3
P (t-test)	ns*	P=0.09	ns	P<0.05	ns	ns	ns	P<0.05

\*ns: no significant difference

Data are expressed as Mean ± SEM



**FIGURE 1.** Serum VEGF concentration (pg/ml) in two experimental groups. Aspirin could not change serum VEGF level in hypercholesterolemic animals ( $P>0.05$ ). (HC: High- Cholesterol diet, ASA: Aspirin)



**FIGURE 2.** Serum NO concentration ( $\mu\text{mol/lit}$ ) in two experimental groups. Aspirin could not change serum NO concentration ( $p>0.05$ ). (HC: High-Cholesterol diet, ASA: Aspirin)

## Results

Table 1 shows mean body weight, systolic pressure, diastolic pressure, and mean arterial pressure. There was no significant difference in the weight of animals between aspirin-treated and control groups ( $P>0.05$ ). Aspirin did not change systolic, diastolic and mean arterial pressure ( $P>0.05$ ).

There were no significant differences in the baseline values of serum CHO, HDL, LDL and TG. Aspirin significantly decreased serum LDL ( $1276\pm 72.1$  vs.  $1505\pm 68.03$  mg/dl) and TG ( $477.5\pm 8.3$  vs.  $649.1\pm 15.2$  mg/dl) levels ( $P<0.05$ ).

Figure 1 illustrates the serum VEGF concentration in aspirin-treated and control groups. At the end of the experiment, serum VEGF concentration was significantly lower than before the experiment (control:  $24.59\pm 0.42$  vs.  $38.09\pm 2.49$  pg/ml; aspirin:  $24.72\pm 0.84$  vs.  $42.29\pm 2.03$  pg/ml) ( $P<0.05$ ). Aspirin did not change serum VEGF level in hypercholesterolemic animals ( $24.72\pm 0.84$  vs.  $24.59\pm 0.42$  pg/ml) ( $P>0.05$ ).

Figure 2 shows serum NO concentration in two experimental groups. Results showed that serum NO concentration had significantly decreased after five weeks on high-cholesterol diet (control:  $5.87\pm 0.33$  vs.  $8.67\pm 0.68$   $\mu\text{mol/lit}$ ; aspirin:  $5.66\pm 0.33$  vs.  $8.58\pm 0.60$   $\mu\text{mol/lit}$ ) ( $P<0.05$ ). Aspirin did not change serum NO level in hypercholesterolemic rabbits ( $5.66\pm 0.33$  vs.  $5.87\pm 0.33$   $\mu\text{mol/lit}$ ) ( $P>0.05$ ).

## Discussion

The effect of aspirin on serum VEGF and NO concentrations in hypercholesterolemic rabbits was the objective of this study. We found that aspirin did not change serum NO and VEGF concentrations in high-cholesterol fed animals. Our results showed that aspirin did not change blood pressure.

In a previous study, aspirin did not change blood pressure in hypertensive animals.<sup>23</sup> Low-dose aspirin does not interfere with the blood pressure-lowering effect of antihypertensive drugs.<sup>24</sup> It is suggested that the effect of aspirin on blood pressure may be related to dose and time of administration time.<sup>25</sup> Under the conditions of this study, aspirin did alter the blood pressure.

The administration of a high-cholesterol diet was accompanied by an increase in serum CHO, TG and LDL in all animals. The administration of aspirin was followed by a significant decrease in serum LDL and TG. Similar results have been previously reported<sup>26,27</sup> and can be explained by the direct antilipolytic effect

of aspirin or its effect on insulin metabolism which results in a reduced rate of lipolysis and finally reduced serum LDL and TG.<sup>28,29</sup>

Hypercholesterolemia is one of the major risk factors of atherosclerosis which is characterized by endothelial dysfunction.<sup>1</sup> One of the most important endothelium-derived mediators is nitric oxide (NO), which is formed from L-arginine by the action of NO synthetase enzyme (NOS).<sup>6</sup> NO has several antiatherogenic actions including inhibition of platelet aggregation, monocyte migration and lipid oxidation.<sup>4-6</sup> Although it has been suggested that endothelial NOS is a site of action for aspirin and protects endothelial cells via the NO/cGMP pathway,<sup>30</sup> our results showed that serum NO level did not change after aspirin administration, which may be related to hypercholesterolemia.

Hypercholesterolemia is characterized by impaired NO release.<sup>31</sup> Elevated plasma level of asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthetase inhibitor, and oxidative stress reduce NO bioavailability in hypercholesterolemic conditions.<sup>31,32</sup>

Our results also showed that serum VEGF concentration decreased following a high-cholesterol diet and aspirin did not change serum VEGF concentration. The impairment of angiogenesis in hypercholesterolemic conditions has been previously reported.<sup>32,33</sup> Hypercholesterolemia impairs angiogenesis by suppressing endothelial and tumoral bFGF and VEGF expression.<sup>34</sup> Aspirin was found to decrease VEGF release and VEGF mRNA;<sup>35</sup> this could be related to antiplatelet effect of aspirin which decreases VEGF production. VEGF, a potent angiogenic growth factor, stimulates endothelial cell proliferation and migration, and angiogenesis<sup>36,37</sup> and may prevent the atherosclerosis processes.<sup>11,38</sup>

In our study, we did not find any change in serum VEGF level after aspirin treatment. Apparently, at least this dose of aspirin (10 mg/kg/day) cannot change serum VEGF in high-cholesterol fed animals. The ineffectiveness of aspirin, particularly at low doses in the atherosclerosis process has been reported.<sup>39-41</sup> Serum NO and VEGF concentration may change at other doses of aspirin. Antioxidative and antiplatelet actions of aspirin have been proposed as the mechanisms underlying the antiatherosclerotic effects of aspirin.<sup>42,43</sup> Aspirin also reduces ICAM-1 expression, VCAM-1 and E-selectin induction and subsequent monocyte adhesion.<sup>41,44</sup>

We conclude that under the conditions of this study, aspirin cannot change serum VEGF or NO concentrations in high-cholesterol fed animals.

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