Original Article

Enalapril protects endothelial cells against induced apoptosis in Alzheimer's disease

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Background: Alzheimer's disease (AD) is a progressive neurodegenerative disease in which endothelial cell (EC) can be affected. In brain, functional changes in ECs contribute to reductions in resting blood flow. Furthermore, angiotensin-converting enzyme inhibitors (ACE-I) have beneficial effects on endothelial dysfunction. This is the first study that presents direct experimental evidence associating endothelial apoptosis as a basis of AD pathogenesis and response to an ACE-I therapy. **Materials and Methods:** Human umbilical vein ECs (HUVECs) were treated with sera from AD patients and sera from healthy volunteers (each group, $n = 10$). Apoptosis was determined by annexin V–propidium iodide staining and cell death detection kit. The effect of 50 μM enalapril on endothelial apoptosis was assessed. Nitrite (NO_2^-) levels were determined in the culture supernatants. **Results:** Enalapril suppressed the induction of apoptosis by the serum of patients only when used before treating HUVECs with the sera of AD. Mean ± SD of apoptosis induction in the control group was 6.7 ± 3.69; in the group treated with sera of AD for 24 h was 47.78 ± 0.65; in the group wherein sera from AD was added (pretreatment) after exposure of HUVECs by 50 μM enalapril for 24 h was 26.6 ± 2.63; and in the group wherein HUVECs were exposed in the sera of AD for 24 h and then 50 μM enalapril was added to these cells for another 24 h (post-treatment) was 56.87 ± 5.51. Also, the mean ± SD of NO_2^- concentration showed significantly greater levels of dissolved NO_2/NO_3 metabolite in the culture media of untreated HUVECs by enalapril (1.03 ± 0.06) as compared with control (0.26 ± 0.13; $P < 0.05$), while the rate of nitric oxide (NO) significantly decreased when enalapril was presented in culture both in the pretreatment (0.07 ± 0.003) and in the post-treatment group (0.06 ± 0.005; $P < 0.05$). **Conclusion:** It could be concluded that EC treated with sera from AD patients activates apoptosis in HUVECs; this effect was reversed by enalapril pretreatment. This can be proposed as a therapeutic approach for Alzheimer's patients.

Key words: Apoptosis, enalapril, endothelial cell

INTRODUCTION

Alzheimer's disease (AD) is an irretrievable neurodegenerative disease that causes dementia in the elderly.[1] The etiopathogenesis of AD is still unclear. Recently, new concepts have emerged regarding the role of endothelial cells (ECs), vascular disease, and oxidative stress in the pathogenesis of AD and mechanisms that contribute to these events.[2,3] Overproduction of reactive oxygen and NO were observed in many neurodegenerative disorders.[4,5]

Endothelial dysfunction has been linked with many acute and chronic neuroinflammatory diseases.[6] Many stimuli can induce apoptosis in ECs *in vitro*, proposing that endothelial apoptosis is the main mechanism in CNS vascular injury, leading to diminished barrier, immune cell penetration of the CNS, and proceeding inflammation.[7]

In circulation, ECs affect blood vessels, especially in the brain. It is newly transpired that endothelial dysfunction and vascular disease is related on the role of this cell type.[8]

In brain, functional changes in ECs contribute to reductions in resting blood flow (hypoperfusion), impairment of vasodilator responses, and subsequent cellular injury.[9-12] Identification of endogenous molecules and pathways that protect the vasculature may result in targeted approaches to prevent or slow the progression of vascular disease that causes or contributes to the vascular component of dementia and AD.[11]

Activation of the rennin–angiotensin system plays a prominent role in vascular dysfunction. Some clinical studies shows that angiotensin-converting enzyme inhibitors (ACE-I), which has clinically been widely used as an anti-hypertensive agent, reduces the incidence of dementia or slows down the rate of cognitive decline in patients with hypertension.[13,14] Ohrui *et al*.[15] showed that active ACE-I, but not non-centrally active ACE-I, could

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slow down the rate of cognitive decline in mild-to-moderate AD patients.[16]

As regards to beneficial effects of ACE-I on endothelial dysfunction[16-18] and endothelial apoptosis, the same can be observed in many inflammatory and vascular injury disorders. This is the first study that presents direct experimental evidence associating endothelial apoptosis as a basis of AD pathogenesis and response to an ACE-I therapy.

MATERIALS AND METHODS

The study was conducted with the collaboration of the Departments of Physiology, Applied Physiology Research Center, and Neurology Outpatient Department of Al-Zahra hospital, Isfahan University of Medical Sciences between July 2010 and June 2011. A complete explanation of the study was given to each patient and written informed consent was received from all patients. The study protocol was reviewed and approved by the ethics in Research Committee, Isfahan University of Medical Science.

Patients

In this study, 10 patients with AD and 10 healthy controls (age- and sex-matched healthy subjects) were recruited. Diagnosis of AD was based on the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and AD and Related Disorders Association.[19] Patients with a history of drug abuse, chronic systemic diseases such as diabetes mellitus, hypertension, coronary heart disease, cigarette smoking, alcohol abuse, or acute illness, severe head injury, or seizure disorders, and who were treated with electroconvulsive therapy, major depression, cerebrovascular disease, intoxication, metabolic abnormalities, and dementia caused by diseases other than AD were not included in the study.

Sample collection and preparation

Peripheral venous blood from ADs was sampled into serum tubes. Then serum was centrifuged within 30 min to reduce platelets and stored at –80°C for further analysis; all measurements were performed at the same time.

Cell culture

Human umbilical vein ECs (HUVECs) (National Cell Bank of Iran affiliated with the Pasteur Institute, Tehran, Iran) were cultured in endothelial basal medium (EBM) supplemented with gentamicin, amphotericin B, and 10% fetal calf serum (FCS) until the 3rd passage before the experiments was performed.

For evaluation effects of enalapril on HUVECs treated with sera of AD, we arranged different groups; in the first group, HUVECs were only treated by sera from AD for 24 hours, in the second group, HUVECs were treated by 50 μM enalapril (dissolved in 0.9% NaCl[20,21]) for 24 h, and then sera from AD was added to these cells for another 24 h. In the third group, HUVECs were exposed in the sera of AD for 24 h and then 50 μM enalapril was added to these cells for another 24 h. In the fourth group, HUVECs were treated by sera from healthy individuals for 24 h.

Apoptosis analysis

The rate of apoptosis in HUVECs was evaluated by flowcytometry and Cell-Death Detection kit. For each treatment, a total number of 10^5 cells were washed with ice-cold PBS once and were stained with annexin-propidium iodide (PI) as follows: Cells (10^5/ml) were incubated with 1 μl annexin V-fluorescein isothiocyanate and 0.5 μl PI (10 mg/ml) in binding buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; 1.8 mM $CaCl_2$). Subsequently, the cells were analyzed by fluorescence-activated cell sorting (FACScan, Becton-Dickinson). Apoptotic cells were designated as annexin-V^+/PI^- cells. Data were analyzed by Cell Quest software. As an additional measure of apoptotic cell death, we assessed the formation of histone-associated DNA fragments by the Cell-Death Detection ELISA kit from Roche (Basel, Switzerland)[21] as previously mentioned or according to the manufacturer instruction.[22]

NO metabolite (NO_2) measurement

NO_2, an important NO metabolite in culture supernatants, was determined using the Griess reaction (Parameter TM, total NO Assay kit, R and D Systems, USA) according to the manufacturer's instructions. Briefly, in this assay, NO_2 is detected colorimetrically as an Azo dye product of the Griess Reaction. The Griess Reaction is based on the two-step diazotization reaction in which acidified NO_2 produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophore azoderivative that absorbs light at 560 nm wavelength. Values were calculated using a standard curve using a standard curve produced with sodium NO_2.[22]

Statistical analysis

The data were reported as mean ± SE (standard error of mean). One way analysis of variance (ANOVA), followed by the Tukey's *post hoc* test was used for data analysis. All experiments were repeated in three independent replicates. P value of less than 0.05 was considered significant. Statistical analyses were performed using SPSS version 16.

RESULTS

A 24-h treatment of HUVECs with the sera of untreated AD resulted in significantly greater apoptosis than in healthy

controls as measured by flow cytometry and Cell-Death Detection Kit ($P < 0.05$) [Figure 1]. There were no significant differences in the apoptosis rates of HUVECs between patients with AD.

We examined the effects of enalapril on cultured EC apoptosis in two groups: 24 h before (pretreatment) and 24 h after (post-treatment) adding patients' serum. The addition of enalapril suppressed markedly the induction of apoptosis by the serum of patients only when used before treating HUVECs with the sera of AD ($P \leq 0.05$) [Figure 1a] [(mean ± SD) in (groups) control; 6.7 ± 3.69, in patients; 47.78 ± 0.65, in pretreatment; 26.6 ± 2.63; and in post-treatment; 56.87 ± 5.51. Also, the rate of apoptosis in different groups was assessed by Cell-Death Detection kit that detects internucleosomal degradation of genomic DNA during apoptosis. In this experiment, we again observed increasing apoptosis rate in AD group in comparison with control group. Data showed enalapril pretreatment and post-treatment prevented the induction of apoptosis by the serum of AD in HUVECs [Figure 1b].

Also, the mean ± SD of NO_2 concentration showed significantly greater levels of dissolved NO_2/NO_3 metabolite in the culture media of untreated HUVECs by enalapril (1.03 ± 0.06) as compared with the control 0.26 ± 0.13 ($P < 0.05$), while the rate of NO significantly decreased when enalapril was presented in culture both in the pretreatment (0.07 ± 0.003) and in the post-treatment groups (0.06 ± 0.005; $P < 0.05$) [Figure 1c].

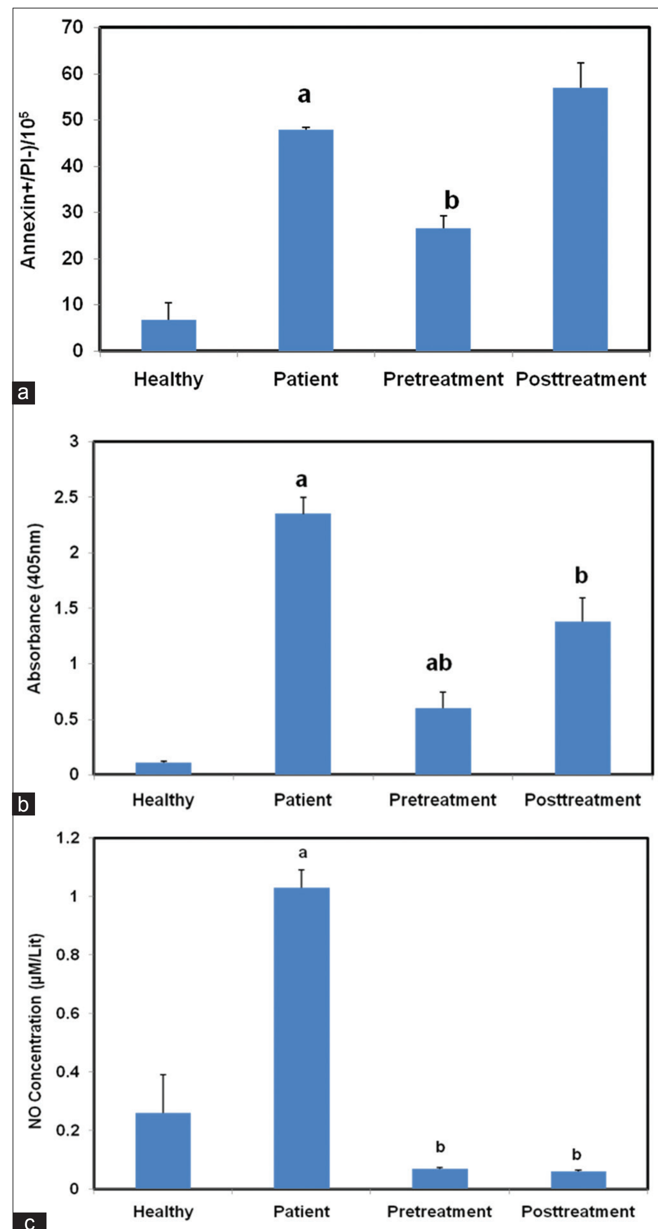

Figure 1: Determination of apoptosis induction in human umbilical vein endothelial cells after exposure with sera from Alzheimer's disease patients vs. the control group. Apoptosis rates of HUVECs were measured after exposure to media containing 10% serum of patients with AD or the control group for 24 h. Assessment of suppression of AD serum-induced apoptosis by pre and post-treatment of enalapril (50 μM) for 24 h with flow cytometry (the number of apoptotic cells were designated as annexin-$V^+/PI^-/10^5$ cells) (a) a; $P < 0.001$ and Cell-Death Detection Kit (b), a; $P < 0.05$ vs. healthy, b, ab; $P < 0.05$ vs. patients. Enalapril (50 μM) was added to HUVECs 24 h before and after treating with sera from AD patients, the concentration (μm/L) of nitrite in the culture medium was determined. a; $P < 0.05$ vs. healthy individuals and b; $P < 0.05$ vs. patients (c)

DISCUSSION

In this study, we discovered that NO_2 concentration and apoptotic measurements were significantly higher in the HUVEC media treated by AD serum as compared with the control, and elevation levels of dissolved NO_2/NO_3 metabolite was significantly reduced by co-incubation of an ACE-I both in the pretreatment and post-treatment groups of serum. However, for apoptotic markers, this reduction occurred only when we added ACE-I before treatment of HUVECs with AD's serum.

Etiopathogenesis of AD, which leads to demientia, is still unclear. Several studies suggested a possible role of oxidative stress in the pathogenesis of AD.[23,24] In our experimental model, apoptotic measurements in AD were significantly high in comparison with several studies that indicated that apoptosis might contribute to onset and progression of AD. P53 protein plays a part in neuronal apoptosis in the brain of these patients.[25]

Furthermore, in cultures of neurons and astrocytes of human and rat as well as in peripheral blood lymphocytes and brain of patients with AD, increasing in the level of p53 protein has been observed.[26,27]

It is also believed that inflammatory cytokines in AD, such as TNF-α, are stimulated by activated microglia, which cause increase of oxidative stress and markers of inflammation in AD patients as well as nitrate generation.[27] Elevated NO_2 concentration in our study could explain that

overproduction of reactive oxygen and nitrogen species occurs in neurodegenerative disorders including AD. In the nervous system, NO seems to have both neurotoxic and neuroprotective properties.[27]

This NO can derive from overactivation of constitutive isoform of the enzyme NOS (neuronal NOS) or from the expression of inducible isoform of NOS.[27] We believe that evidence of this process could be detectable by using plasma measures and increased level of NO_2 concentration in treated HUVECs by AD's serum. In a previously reported study, this hypothesis has been confirmed that there is an increase in serum NO levels in AD patients as compared to that in controls.[28]

We used cultured ECs from HUVEC. In these cells, expression of ACE has been shown.[29] The activation of renin–angiotensin system in the brain of patients with AD has been shown in previous reports.[30-32] Certain drugs of ACE-I may decline the rate of cognitive deterioration in patients with AD.[31] Binding of captopril, a kind of ACE-I to endothelial ACE may results in a "site-specific" potentiate in antioxidant defenses. This property of ACE-I has been demonstrated *in vitro*[33] and *in vivo*.[34,35] ACE-I attenuates oxidative stress-induced EC apoptosis via p38 MAP kinase inhibition.[35]

In our study, pretreatment with enalapril could decline both apoptotic measurement and NO_2 concentration. It seems that enalapril scavenged free radicals and peroxynitrate that are induced in AD serum. The prophylactic treatment of ACE-I in some disease, like in migraine, has been proven.[22]

CONCLUSION

Our study revealed that enalapril can possibly decline elevated levels of NO in serum and apoptotic measurement that are presented in probable AD patients. There are evidences confirming beneficial effects of ACE-I for AD prophylaxis.[36] However, the role of the elevation of NO level of serum on AD pathogenesis is unclear, and it requires further investigation. Also, in this study, we demonstrated increased apoptosis of HUVECs following incubation of these cells with sera from AD patients and provided evidence supporting a direct stabilizing effects of enalapril on the endothelium.

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REFERENCES

1. Faraci FM. Protecting against vascular disease in brain. Am J Physiol Heart Circ Physiol 2011;300:566-82.
2. Casado A, Encarnacion Lopez-Fernandez M, Concepcion Casado M, de La Torre R. Lipid peroxidation and antioxidant enzyme activities in vascular and Alzheimer dementias. Neurochem Res 2008;33:450-8.
3. Delibas N, Ozcankaya R, Altuntas I. Clinical importance of erythrocyte malondialdehyde levels as a marker for cognitive deterioration in patients with dementia of Alzheimer type: A repeated study in 5-year interval. Clin Biochem 2002;35:137-41.
4. Yi J, Horky LL, Friedlich AL, Shi Y, Rogers JT, Huang X. L-arginine and Alzheimer's disease. Int J Clin Exp Pathol 2009;2:211-38.
5. Law A, Gauthier S, Quirion R. Say NO to Alzheimer's disease: The putative links between nitric oxide and dementia of the Alzheimer's type. Brain Res Brain Res Rev 2001;35:73-96.
6. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: A marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 2003;23:168-75.
7. Alexander JS, Zivadinov R, Maghzi AH, Ganta VC, Harris MK, Minagar A. Multiple sclerosis and cerebral endothelial dysfunction: Mechanisms. Pathophysiology 2011;18:3-12.
8. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation 2004;109:27-32.
9. Hossmann KA. Variability thresholds and the penumbra of focal ischemia. Ann Neurol 1994;36:557-65.
10. Iadecola C, Davisson RL. Hypertension and cerebrovascular dysfunction. Cell Metab 2008;7:476-84.
11. Joutel A, Monet-Lepretre M, Gosele C, Baron-Menguy C, Hammes A, Schmidt S, *et al*. Cerebrovascular dysfunction and microcirculation rarefaction precede white matter lesions in a mouse genetic model of cerebral ischemic small vessel disease. J Clin Invest 2010;120:433-45.
12. Marshall RS, Lazar RM. Pumps, aqueducts, and drought management: Vascular physiology in vascular cognitive impairment. Stroke 2011;42:221-6.
13. Croog SH, Levine S, Testa MA, Brown B, Bulpitt CJ, Jenkins CD, *et al*. The effects of antihypertensive therapy on the quality of life. N Engl J Med 1986;314:1657-64.
14. Sink KM, Leng X, Williamson J, Kritchevsky SB, Yaffe K, Kuller L, *et al*. Angiotensin-converting enzyme inhibitors and cognitive decline in older adults with hypertension: Results from the Cardiovascular Health Study. Arch Intern Med 2009;169:1195-202.
15. Ohrui T, Tomita N, Sato-Nakagawa T, Matsui T, Maruyama M, Niwa K, *et al*. Effects of centrally active ACE inhibitors on Alzheimer disease progression. Neurology 2004;63:1324-5.
16. Meier P, Maillard M, Burnier M. The future of angiotensin II inhibition in cardiovascular medicine. Curr Drug Targets Cardiovasc Haematol Disord 2005;5:15-30.
17. Hornig B, Arakawa N, Drexler H. Effect of ACE inhibition on endothelial dysfunction in patients with chronic heart failure. Eur Heart J 1998;19:48-53.
18. Cesari M, Kritchevsky SB, Atkinson HH, Penninx BW, Di Bari M, Tracy RP, *et al*. Angiotensin-converting enzyme inhibition and novel cardiovascular risk biomarkers: Results from the Trial of Angiotensin Converting Enzyme Inhibition and Novel Cardiovascular Risk Factors (TRAIN) study. Am Heart J 2009;157:334.
19. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. Neurology 1984;34:939-44.
20. Wu J, Lei MX, Xie XY, Liu L, She YM, Mo J, *et al*. Rosiglitazone

inhibits high glucose-induced apoptosis in human umbilical vein endothelial cells through the PI3K/Akt/eNOS pathway. Can J Physiol Pharmacol 2009;87:549-55.
21. Hagiwara S, Iwasaka H, Matumoto S, Hidaka S, Noguchi T. Effects of an angiotensin-converting enzyme inhibitor on the inflammatory response in *in vivo* and *in vitro* models. Crit Care Med 2009;37:626-33.
22. Javanmard SH, Sonbolestan SA, Heshmat-Ghahdarijani K, Saadatnia M, Sonbolestan SA. Enalapril improves endothelial function in patients with migraine: A randomized, double-blind, placebo-controlled trial. J Res Med Sci 2011;16:26-32.
23. Calissano P, Matrone C, Amadoro G. Apoptosis and *in vitro* Alzheimer disease neuronal models. Commun Integr Biol 2009;2:163-9.
24. Rohn TT, Head E, Nesse WH, Cotman CW, Cribbs DH. Activation of caspase-8 in the Alzheimer's disease brain. Neurobiol Dis 2001;8:1006-16.
25. De la Monte SM, Wands JR. Molecular indices of oxidative stress and mitochondrial dysfunction occur early and often progress with severity of Alzheimer's disease. J Alzheimers Dis 2006;9:167-81.
26. Dorszewska J, Florczak J, Rozycka A, Jaroszewska-Kolecka J, Trzeciak WH, Kozubski W. Polymorphisms of the CHRNA4 gene encoding the alpha4 subunit of nicotinic acetylcholine receptor are related to the oxidative DNA damage and the level of apoptotic proteins in lymphocytes of the patients with Alzheimer's disease. DNA Cell Biol 2005;24:786-94.
27. Dezor M, Dorszewska J, Florczak J, Kempisty B, Jaroszewska-Kolecka J, Rozycka A, *et al*. Expression of 8-oxoguanine DNA glycosylase 1 (OGG1) and the level of p53 and TNF-alpha proteins in peripheral lymphocytes in patients with Alzheimer's disease. Folia Neuropathol 2011;49:123-31.
28. Vural H, Sirin B, Yilmaz N, Eren I, Delibas N. The role of arginine-nitric oxide pathway in patients with Alzheimer disease. Biol Trace Elem Res 2009;129:58-64.
29. Persson K, Whiss PA, Nyhlén K, Jacobsson-Strier M, Glindell M, Andersson RG. Nitric oxide donors and angiotensin-converting enzyme inhibitors act in concert to inhibit human angiotensin-converting enzyme activity and platelet aggregation *in vitro*. Eur J Pharmacol 2000;406:15-23.
30. Yamada K, Horita T, Takayama M, Takahashi S, Takaba K, Nagata Y, *et al*. Effect of a centrally active angiotensin converting enzyme inhibitor, perindopril, on cognitive performance in chronic cerebral hypo-perfusion rats. Brain Res 2011;1421:110-20.
31. De Gennaro Colonna V, Rossoni G, Rigamonti A, Bonomo S, Manfredi B, Berti F, *et al*. Enalapril and quinapril improve endothelial vasodilator function and aortic eNOS gene expression in L-NAME-treated rats. Eur J Pharmacol 2002;450:61-6.
32. Dong YF, Kataoka K, Tokutomi Y, Nako H, Nakamura T, Toyama K, *et al*. Perindopril, a centrally active angiotensin-converting enzyme inhibitor, prevents cognitive impairment in mouse models of Alzheimer's disease. FASEB J 2011;25:2911-20.
33. Benzie IF, Tomlinson B. Antioxidant power of angiotensin-converting enzyme inhibitors *in vitro*. Br J Clin Pharmacol 1998;45:168-9.
34. de Cavanagh EM, Inserra F, Ferder L, Fraga CG. Enalapril and captopril enhance glutathione-dependent antioxidant defenses in mouse tissues. Am J Physiol Regul Integr Comp Physiol 2000;278:572-7.
35. Ceconi C, Francolini G, Bastianon D, Gitti GL, Comini L, Ferrari R. Differences in the effect of angiotensin-converting enzyme inhibitors on the rate of endothelial cell apoptosis: *In vitro* and *in vivo* studies. Cardiovasc Drugs Ther 2007;21:423-9.
36. Higashi Y, Chayama K, Yoshizumi M. Angiotensin II type I receptor blocker and endothelial function in humans: Role of nitric oxide and oxidative stress. Curr Med Chem Cardiovasc Hematol Agents 2005;3:133-48.

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