The intervention effect of zinc supplementation on irbesartan treatment for atherosclerosis of ApoE$^{-/-}$ mice

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Abstract: To explore the influence of zinc supplementation on irbesartan treatment for atherosclerosis of ApoE gene-deleted mice. Atherosclerosis model mice induced by normal feed were randomly divided into ApoE$^{-/-}$ control group, irbesartan group and zinc sulfate+ irbesartan group, 6 mice each group; C57BL/6J mice with normal feed were regarded as blank control group (n=6). Blank control group and ApoE$^{-/-}$ control group were not given any medical treatment, and irbesartan group were treated with irbesartan (50mg/kg/d), and zinc sulfate+irbesartan group were given zinc sulfate (25mmol/L) treatment besides the administration of irbesartan group. The blood pressure of each mouse was recorded and the blood lipid level was detected after 15 weeks' treatment. The internal inflammatory reaction of the mice was evaluated according to IL-6 and TNF-α level. The oxidative stress was evaluated according to MDA and SOD levels. And the atherosclerosis plaque was analyzed with immunohistochemistry. After 15 weeks of drug administration, it showed that the total cholesterol level, LDL-C, HDL-C and blood pressure level ($P<0.05$) of the mice were improved significantly with the administration of zinc sulfate+irbesartan compared with irbesartan group, and the oxidative stress response and inflammatory reaction of mice in zinc sulfate+irbesartan group decreased more significantly than those of irbesartan group ($P<0.05$). In the detection of atherosclerosis plaque, the ratio of plaque area and tube area as well as the improvement degree of mean aortic IMT in zinc sulfate+irbesartan group were superior to those of irbesartan group ($P<0.05$). Zinc supplementation has certain therapeutic effect on the advanced atherosclerosis of ApoE gene-deleted mice, which can significantly improve the efficacy of irbesartan.

Keywords: Atherosclerosis, ApoE$^{-/-}$ mice, zinc, irbesartan.

INTRODUCTION

Atherosclerosis (AS) has been established as a complex inflammatory disease, which is the accumulation of lipids and fibrous materials cell debris in the arteries (Caralgol and Kartal, 2010). And it is mediated by complicated molecular interactions, in which chemokines play a key role (Tuttolomondo *et al*., 2012). Rein-angiotensin system (RAS) plays an important part in the process of AS, and angiotensin II (ang II) is the intermediate product of RAS, which participates in the pathological process of AS through affecting vascular endothelial cell, monocyte /macrophage and smooth muscle cell, promoting angiogenesis and affecting lipid metabolism etc. (Wang *et al*., 2013). Angiontensin II type 1 receptor (AT1) mediates almost all the pathological and physiological functions of Ang II. Angiotensin receptor bloker (ARB) could inhibit the formation of atherosclerosis plaque of mice through blocking a series of biological effects generated in the combination of Ang II and AT1 (Poduri *et al.,* 2012). Irbesartan is one type of ARB, which, confirmed by research mitigates the occurrence and development of AS through the antagonism of AT1 receptor to decrease the activity of cell transcription factor NF-κB (Tian *et al*., 2012). AS damages in progress contains metal ions (such as Fe and Cu). Zn has the antioxidative effect; zinc deficiency could affect the activity of organism antioxidant enzymes, decrease the free radical scavenging rate and increase lipid per oxidation.

In this research, ApoE gene-deleted C57BL/6J mice were used in the modeling of atherosclerosis. On the basis of irbesartan treatment, the zinc supplementation was added. The effect of zinc supplementation on irbesartan treatment of AS was analyzed and detected, in which the function and possible mechanism of zinc were analyzed, which provided a new reference method for the clinical treatment of AS.

MATERIALS AND METHODS

Experimental animals

Eight-week-old male ApoE gene-deleted (ApoE$^{-/-}$) mice with the genetic background of C57BL/6J (n=18) and eight-week-oldmale wild-type C57BL/6J mice (n=6) were provided by Henan Experimental Animal Center. The mice were fed in the animal room with constant temperature (22.5±0.5) and constant humidity (50%±5%) in separate cages with 1mouse apiece, and the light-dark cycle was 12h. The mice were randomly divided into the cages, free to eat and drink, with diet pellet fed for 1 week of adaptive cultivation. All experiments were approved by the Medical Ethics Committee of People's

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Hospital of Zhengzhou and followed national guidelines for the care and use of animals.

Modeling of mice atherosclerosis

The mice were divided into groups after 1 week of adaptive cultivation for the 15-week modeling of mice atherosclerosis. 6 wild-type mice were used as blank control group and 18 ApoE$^{-/-}$ mice were randomly divided into three groups: ApoE$^{-/-}$ control group, irbesartan group and zinc sulfate + irbesartan group, 6 mice in each group. All the mice in four groups were fed normal feed, besides, the irbesartan group were fed irbesratan thoroughly mixed in the feed with a dosage of 50mg/kg/d. Zinc sulfate + irbesartan group were provided with free access to water containing 25mmol/L zinc sulfate for consecutive 15 weeks on the basis of irbesartan group. The drinking water of mice in the rest three groups was general deionized water.

After 15 weeks of drug administration, mice in each group were given intraperitoneal injection of pentobarbital sodium (50mg/kg) for anesthesia after an overnight fast. Then right common carotid artery was separated with the mice fixed in supine position and the arterial blood pressure was recorded with physiology signal acquisition device. After the recording of the blood pressure, the mice arterial blood was collected and centrifuged. And the supernatant serum was collected for lipid detection. Then the thoracic cavity was opened rapidly and perfusion of the whole body with 0.9% normal saline under the 100mmHg physiological pressure was conducted, then the whole aorta and heart were separated rapidly; brachiocephalic trunk vessel was regarded as anatomic landmark and aorta under brachiocephalic trunk was frozen at -80 refrigerator for protein detection; after the brachiocephalic trunk was fixed in 4% paraformaldehyde for 24h, it was made paraffin section for immunohistochemistry assay.

Serum lipid, inflammatory factor analysis and the detection of oxidative stress level in mice

After serum was diluted with normal saline, the total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were detected with micro plate reader according to specification standard process. Serium IL-6 and TNF-α levels were detected with ELISA. The activities of SOD in serum were measured by xanthine oxidase method, and thiobarbituric acid reactive substance was adopted to determine serum MDA level.

Analysis of atherosclerosis plaque with immunohistochemistry

Atherosclerosis area lesions of mice in each group were detected with immunohistochemistry. Paraffin sections of each group were selected and stained whit HE and mice sections of each group were obtained for Movat staining. The atherosclerotic lesions area, maximum degree of lumen stenosis and intra-aortic intima-media thickness (IMT) were analyzed by the Image-Pro Plus 5.1 software.

STATISTICAL ANALYSIS

The experiment results were indicated in mean ±SD. The SPSS 16.0 software package was used for professional statistical analysis. Normality and homoscedasticity were tested, and either single factor ANOVA variance analysis or Mann-Whitney U test was applied to be statistics, and $P<0.05$ difference was considered of statistical significance.

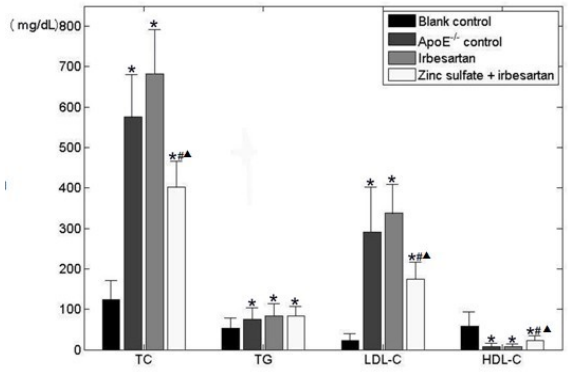

Fig. 1: The blood lipid levels of mice in each group after fed for 15 weeks

Note: α: compared with blank control group, $P<0.05$;#: compared with ApoE$^{-/-}$ control group, $P<0.05$;▲: compared with Irbesarban group.

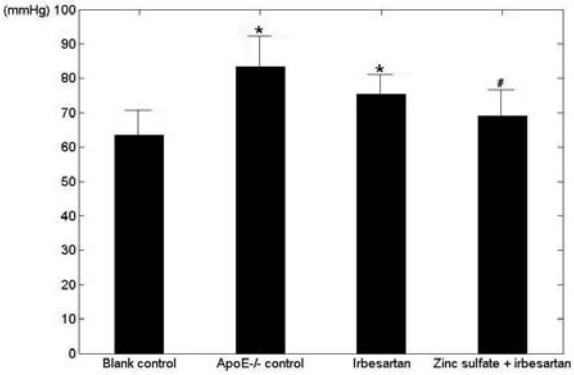

Fig. 2: the comparison of mean arterial pressure levels of mice in each group

Note: α: compared with blank control group, $P<0.05$; #: compared with ApoE$^{-/-}$ control group, $P<0.05$

RESULTS

Distribution of blood lipid and blood pressure of the mice

Due to the lack of apolipoprotein E, ApoE$^{-/-}$ mice spontaneously formed hypercholesterolemia induced by normal feed. After 15-week consecutive culture, the

Table 1: Analysis and comparison of atherosclerotic area of mice sections in each group (mean±SD, n=6)

Items	Blank control group	$ApoE^{-/-}$control group	Irbesartan group	Zinc sulfate + irbesartan group
Atherosclerosis area ($\times10^4$ μm^2)	6.0±0.5	17.6±4.5*	12.8±3.2*#	12.0±2.7*#
Maximum Degree of lumen stenosis (%)	18.5±1.8	59.1±5.8*	46.5±5.0*#	42.3±4.6*#
Plaque area/lumen area (%)	4.2±0.7	33.7±4.1*	23.5±3.2*#	19.3±2.9*#▼
Mean aortic IMT (μm)	152.6±15.2	293.4±62.7*	258.7±45.6*#	211.9±29.4*#▼

Note: α: compared with blank control group, P<0.05; #: compared with $ApoE^{-/-}$control group, P<0.05 ▼: compared with irbesartan group, P<0.05

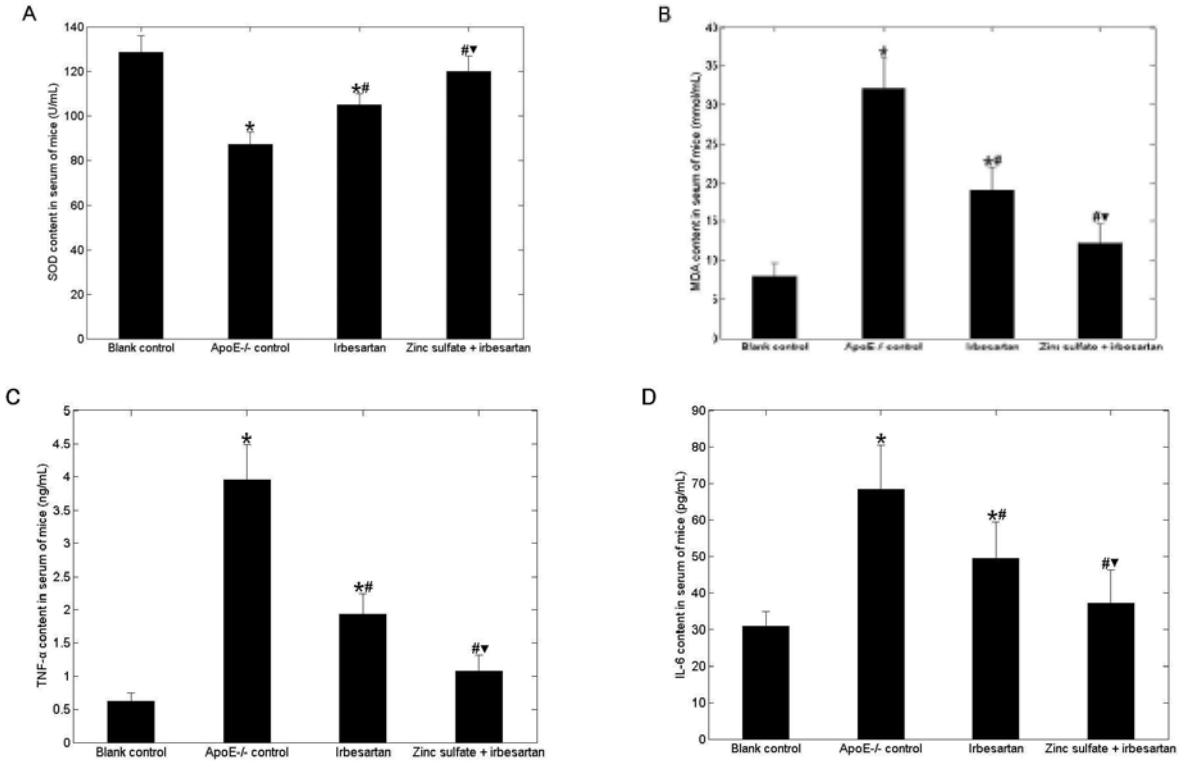

Fig. 3: Serum SOD, MDA and inflammatory factor level of mice in each group

A: Serum SOD level; B: Serum MDA level; C: Serum TNF-α level; D: IL-6 level Note: α: compared with the blank control group, P<0.05; #: compared with $ApoE^{-/-}$ control group, P<0.05; ▼: compared with irbesartan group, P<0.05.

serum lipid level in $ApoE^{-/-}$ control group, irbesartan group and zinc sulfate +irbesartan group all were higher than that of the blank control group mice. Fig. 1 indicated that through the comparison of total cholesterol level, LDL-C and HDL-C levels, zinc sulfate + irbesartan group and control group had obvious difference (P<0.05), while as to the triglyceride level, the difference between zinc sulfate + irbesartan group and $ApoE^{-/-}$ control group was not significant. There was no significant difference between the blood type of irbesartan group and that of $ApoE^{-/-}$ control group. The distribution of zinc among its major plasma proteins was altered. And it was observed in atherosclerosis. Zinc was transported into endothelial cells in an easy way. This suggested that endothelium could be particularly influenced by perturbations in zinc homeostasis and metabolism. Zinc supplementation was generally adopted in clinical trials, which showed a decrease of plasma HDL-C concentrations. And this leaded to an increase of heart disease risk. The results of the study showed that zinc sulfate could decrease the blood lipid level in mice and improve the efficacy of irbesartan.

At the same time, the recorded mean arterial pressure result of mice in four groups (fig. 2) indicated that the blood pressure of $ApoE^{-/-}$ control group and irbesartan group was significantly higher than that of the blank control group, while the blood pressure of zinc sulfate + irbesartan group was significantly lower than that of $ApoE^{-/-}$ group (P<0.05). The blood pressure of irbesartan group was lower than that of $ApoE^{-/-}$ group, but the difference was not obvious (P>0.05).

The detection of serum inflammatory factor and oxidative stress level of mice

It was found during the detection of serum inflammatory factor and SOD, MDA levels that there existed significant difference between ApoE$^{-/-}$ control group and the blank control group. But after drug treatment, there was a significant difference between the levels or content of SOD, MDA, TNF- α and IL-6 of irbesartan group and zinc sulfate + irbesartan group and the ApoE$^{-/-}$ control group ($P<0.05$). Fig. 3 also indicated that after zinc sulfate + irbesartan treatment, oxidative stress and inflammatory response in mice decreased significantly compared with those of irbesartan group ($P<0.05$). The results indicated that irbesartan could reduce the atherosclerosis condition in animals and keep the disease from developing, while it could significantly improve the efficacy of irbesartan with adequate intake of zinc.

The detection and analysis of arteriosclerosis area

We found that atherosclerotic lesion areas of ApoE$^{-/-}$ control group, irbesartan group, and zinc sulfate + irbesartan group were increased respectively compared with those of the blank control group (table 1). In the detection of the maximum degree of lumen stenosis, the ratio of plaque area and lumen area as well as mean aortic IMT, the blank control group was lower than that of the rest three groups ($P<0.05$). Compared with irbesartan group and zinc sulfate + irbesartan group, ApoE$^{-/-}$ control group was lower in respect of the maximum degree of lumen stenosis, the ratio of plaque area and lumen area as well as mean aortic IMT ($P<0.05$). While zinc sulfate + irbesartan group was superior to irbesartan group in respect of the improvement of the ration of plaque area and lumen area ($P<0.05$), as to atherosclerotic lesion area and the maximum degree of lumen stenosis, zinc sulfate+irbesartan group was improved compared with irbesartan group, but not significantly ($P>0.05$).

Zinc has the function of maintaining the structural integrity of 3,000 transcription factors in human genome, and it is of great importance for the biological activity of over 300zinc metalloenzymes (Vallee and Falchuk, 1993). The result of the study indicated that the zinc intake and supplement could improve the efficacy of irbesartan treatment for atherosclerosis. It could improve zinc homeostasis in the body or metabolism with the zinc supplement in the treatment of atherosclerosis mice, which achieved the effect of the improvement of vascular endothelial cell function and alleviation of pathological damage of atherosclerosis on vascular wall.

DISCUSSION

The study established AS animal model with ApoE$^{-/-}$ mice and based on the administration of antihypertensive drug irbesartan, irbesartan and zinc sulfate + irbesartan were applied in the treatment of atherosclerosis in mice. The intervention effect of zinc supplement on the treatment of AS with irbesartan was observed. The result presented that the efficiency of the improvement of blood lipid level with the single irbesartan was not ideal; while when adequate zinc supplement was added, TC and LDL-C levels in ApoE$^{-/-}$ mice were significantly decreased and the HDL-C level increased significantly. The addition of zinc significantly improved the lipid metabolism and balance in the animal body. As to the blood pressure, although the irbesartan addition could decrease the blood pressure of mice, it was not significant compared with ApoE$^{-/-}$ control mice ($P>0.05$), while zinc sulfate + irbesartan treatment could decrease arterial blood pressure of mice significantly ($P<0.05$). Yao R *et al* found the similar result in the study of irbesartan inhibiting molecular mechanism of AS pathogenesis. They found irbesartan could reduce the atherosclerosis degree of experimental animals and oxidative stress and decrease angiotensin II level, but it had no significant effect on decreasing systolic pressure and plasma lipid level (Yao *et al*., 2010).

Derosa G *et al* pointed out that irbesartan has peroxisome proliferator-activated receptor agonistic effects in *in vitro* studies, and they also demonstrated beneficial effects on inflammatory markers of atherosclerosis and endothelial function (Derosa *et al*., 2009). During the detection of serum inflammatory factors and SOD, MDA levels of mice in each group, we found that, after drug treatment, there was an obvious difference between the levels or content of SOD, MDA, TNF-α and IL-6 in irbesartan group and zinc sulfate + irbesartan group compared with that of ApoE$^{-/-}$ control group ($P<0.05$). The results indicated that irbesartan can attenuate atherosclerosis. This effect was partly related to the inhibition of oxidative stress and inflammatory signal transduction pathways, which eventually lead to the decrease in the expression of inflammatory cytokines. Ma C *et al* wrote that irbesartan can regulate vascular inflammatory gene expression profiles related to atherosclerosis in EA. hy926 cells. They found that, compared with the control group, 56 genes showed notable changes after irbesartan treatment, and that eight genes were involved in atherosclerosis and AT1R was down-regulated while angiotensin type 2 receptor (AT2R) was up-regulated in irbesartan-treated cells. Based on the results, they held the point that these inflammatory factors may promote the destabilization of atherosclerotic plaque possibly in relation to AT2R over expression (Ma *et al*., 2011).

On the other hand, zinc has also been related to several cardiovascular risk factors including lipoprotein concentrations and antioxidant status (Hughes and Samman, 2006). Impaired zinc homeostasis has been associated with increased levels of oxidative stress as well as the induction of widespread genomic and proteomic changes which relate to cardiovascular diseases (Meika and Samir, 2012). When stimulated with linoleic acid and TNF-α, endothelial cells made zinc

deficiency demonstrate considerably higher levels of apoptotic cells death and caspase-3 activity than control cells. And this effect was blocked by simultaneous administration of zinc to the culture medium (Meerarani *et al*., 2000). There were considerable clinical trials about increasing zinc intake, and all the experiment results confirmed that the decrease of HDL-C level could increase the risk of heart disease (Foster *et al*., 2010). The mechanism of zinc influencing the formation of AS may be formed through the interaction of zinc with a series of oxidation-reduction reaction or inflammatory reaction Zinc could affect the signal transduction of the signal pathway such as NF-κB, NO, PPAR and PKC, for example, the interaction of zinc and NO, which may promote the expression of Rrf2 in vascular cell and the formation of atherosclerosis. Zinc supplementation also could reduce the Cu/Zn ratio of the body, oxidative stress and inflammation. CD4 and CD19 leukocyte levels of patients who were given the new supplementation increased significantly as well as the CD4/CD8 ratio (Guo and Wang, 2013). In the study, after the treatment of zinc sulfate + irbesartan, the oxidative stress level and inflammation in mice decreased significantly compared with those of irbesartan group ($P<0.05$). And the zinc supplement could enhance the efficacy of irbesartan. During the detection of pathological damages of atherosclerosis area with immunohistochemistry we found that zinc sulfate + irbesartan group had better efficacy of improving the mean aortic IMT and the ratio of plaque area and lumen area than that of the irbesartan group. The researchers speculated that the reason probably was some enzymes synthesized by metal zinc involved the collaborative process. Metalloprotease chelating with zinc ion-adipocyte-derived leucine amino peptidase (ALAP) was the member of metalloprotease M1. The study found ALAP could hydrolyze many bioactive peptides such as Ang II to make it inactivate (Hattori *et al*., 2000; Hallberg *et al*., 2003). Irbesartan is a kind of ARB with the Ang II antagonism. Therefore, it can enhance the efficacy of irbesartan when the zinc and irbesartan are combined.

CONCLUSION

Although Irbesartan can alleviate the atherosclerosis degree and oxidative stress as well as decreasing the angiotensim II level, its effect on decreasing the systolic blood pressure and plasma lipid level is not significant. Supplement of zinc has significantly improved the blood metabolism and balance within animals' bodies. The employment of zinc supplement dosing method has a certain effect of treatment on the lesion of advanced atherosclerosis in rats with gene ApoE deletion, which can significantly better the therapeutic effect of irbesartan.

REFERENCES

Caralgol B and Kartal Ozer N (2010). Lipid rafts and redox regulation of cellular signaling in cholesterol induced atherosclerosis. *Curr Cardiol Rev.,* **6**: 309-324.

Derosa G, Ferrari I and Cicero AF (2009). Irbesartan and hydrochlorothiazide association in the treatment of hypertension. *Curr Vasc Pharmacol.,* **7**: 120-36.

Foster M, Petoca P and Samman S (2010). Effects of zinc on plasma lipoprotein cholesterol concentrations in humans: A meta-analysis of randomized controlled trials. *Atherosclerosis,* **210**: 344-352.

Guo CH and Wang CL (2013). Effects of zinc supplementation on plasma copper/zinc ratios, oxidative stress, and immunological status in hemodialysis patients. *Int. J. Med. Sci.,* **10**: 79-89.

Hattori A, Kitatani K, Matsumoto H, Miyazawa S, Rogi T, Tsuruoka N, Mizutani S, Natori Y, Tsujimoto M (2000). Characterization of recombinant human adipocyte-dereved leucine aminopeptidase expressed in Chinese hamster ovary cells. *J. Biochem.,* **128**: 755-762.

Hallberg P, Lind L, Michaëlsson K, Kurland L, Kahan T, Malmgvist K, Ohman KP, Nyström F, Lijedahl U, Syvänen AC and Melhus H (2003). Adipocyte-derived leucine aminopeptidase genotype and response to antihypertensive therapy. *BMC Cardiovasc. Disord.,* **3**: 11.

Hughes S and Samman S (2006). The effect of zinc supplementation in humans on plasma lipids, antioxidant status, and thrombogenesis. *J. Am. Coll. Nutr.,* **25**: 285-291.

Meerarani P, Ramadass P, Toborek M, Bauer HC, Bauer H and Henning B (2000). Zinc protects against apoptosis of endothelial cells induced by linoleic acid and tumor necrosis factor alpha. *Am. J. Clin. Nutr.,* **71**: 81-87.

Ma C, Lu XC, Fan L, Luo Y, Yang B, Gao Y, Liu XF (2011). Irbesartan regulates inflammatory gene expressions related to atherosclerosis in EA. hy926 cells. *Nan Fang Yi Ke Da Xue Xue Bao.,* **31**: 1835-1839.

Meika F and Samir S (2012). Zinc and regulation of inflammatory cytokines: Implications for cardiometabolic disease. *Nutrients,* **4**: 676-694.

Poduri A, Owens AP 3rd, Howatt DA, Moorleghen JJ, Balakrishnan A, Cassis LA and Daugherty A (2012). Regional variation in aortic AT1b receptor mRNA abundance is associated with contractility but unrelated to atherosclerosis and aortic aneurysms. *PLoS One*, **7**(10): e48462.

Tuttolomondo A, Di Raimondo D, Pecoraro R, Arnao V, Licata G (2012). Atherosclerosis as an inflammatory disease. *Curr. Pharm. Des.,* **18**: 4266-4288.

Tian HB, Wang XL, Lin LJ, Dong B, Li WH, Wang XL (2012). Effects of irbesartan on activity and expression

of nuclear factor-κB in cultured THP-1 cells. *Chinese Gene. Pract.,* **15**: 2295-2297.

Vallee BL and Falchuk KH (1993). The biochemical basis of zinc physiology. *Physiol Rev.,* **73**: 79-118.

Wang Y, Tikellis C, Thomas MC and Golledge J (2013). Angiotensin converting enzyme 2 and atherosclerosis. *Atherosclerosis,* **226**: 3-8.

Yao R, Cheng X, Chen Y, Xie JJ, Yu X, Liao MY, Ding YJ, Tang TT and Liao YH (2010). Molecular mechanisms of irbesartan suppressing atherosclerosis in high cholesterol-diet apolipoprotein E knock-out mice. *Int. J. Cardiol.,* **139**:113-22.