Abstract. – AIM: The current study aims to explore the possible molecular mechanism of hyperhomocysteinemia (HHcy) mediated atherosclerosis (AS) and to find an effective intervention method for AS.

MATERIALS AND METHODS: A total of 40 Wistar rats were equalized into four groups: blank control, HHcy, folacin intervention, and lutein intervention groups. HHcy rat models were established. The intervention groups were respectively lavaged with folacin and lutein. Oxidative stress states, the levels of nitric oxide (NO) and endothelin-1 (ET-1), as well as the expression of nuclear factor (NF)-κB p65 and intercellular adhesion molecule (ICAM)-1 were compared.

RESULTS: In the HHcy rats, the activity of serum superoxide dismutase (SOD) and glutathione peroxidase (GPx) significantly decreased, whereas the malondialdehyde content and hydroxyl radical level noticeably increased, indicating that the rats stayed in aggravated oxidative stress states. Lutein intervention inhibited HHcy-induced oxidative stress excitement. In the HHcy rats, the NO level significantly decreased, whereas the ET-1 level significantly increased, indicating that HHcy mediated vascular endothelial dysfunction. Lutein reversed such dysfunction. In the HHcy rats, the mRNA and protein expression of SOD2 and GPX1 in the aortic wall tissue decreased, whereas that of NF-κB p65 and ICAM-1 increased. Lutein significantly upregulated the mRNA and protein expression of SOD2 and GPX1 and downregulated the expression of NF-κB p65 and ICAM-1.

CONCLUSIONS: Oxidative stress and inflammation are the important mechanisms of HHcy-mediated AS. In particular, HHcy-induced aggravated oxidative stress may function as the initial AS-mediating mechanism, upregulating the expression of NF-κB p65 and ICAM-1 and thereby becoming associated with AS. Lutein noticeably intervenes in and inhibits Hcy-mediated oxidative stress excitement and downregulates the expression of inflammation-associated informational molecules.

Key Words: Homocysteine (Hcy), Atherosclerosis (AS), Oxidative stress, Lutein.

Introduction

Atherosclerosis (AS) is the pathophysiological basis of cardiovascular diseases. However, an agreement over its etiology and pathogenesis has not been reached. Homocysteine (Hcy) is the side product of methionine methyl transfer reactions. Hyperhomocysteinemia (HHcy) is a critical independent risk factor of AS. Yet, the biochemical and molecular mechanisms concerning how HHcy mediates AS remain uncertain. As a result, an effective intervention and prevention method for both AS and cardiovascular diseases has not been found. Therefore, exposing the AS-mediating mechanism of Hcy from biochemical and molecular angles and finding an effective method as well as clues for the intervention and prevention of AS are of great theoretical and practical significance.

HHcy can cause oxidative stress and endothelial function impairment, thereby associated with cardiovascular diseases. AS development is subject to a chronic inflammation process. During this process, endothelial function impairment may function as the initial link of the cardiovascular disease-mediating process of HHcy. Hcy inhibits nitric oxide (NO) production of endothelial cells. Being an endothelium derived relaxing factor, NO plays important roles in blood flow and pressure maintenance, as well as vasculogenesis. However, whether HHcy-mediated aggravated oxidation stress leads to endothelial dysfunction and increased expression of some proinflammatory cytokines remains to be explored.

Lutein is a natural plant chemical that belongs to an oxygenated carotenoid in structure. By virtue of its special chemical constitution, Lutein is endowed with anti-oxidative stress activity and multiple biological functions. Although lutein has a preventive effect on AS, its exact biochemical and molecular mechanisms remain unclear. Studies on the intervention effect of lutein on HHcy-mediated AS and the underlying molecular mechanism have not been found in literature.

The aims of this study were to explore the oxidative stress state of rats with HHcy as well as the intervention effect and associated mechanisms of lutein.
Materials and Methods

Hhc y Rat Model Establishment and Lutein Intervention

A total of 40 male Wistar rats (8 weeks; 200 ± 20 g) were supplied by the Laboratory Animal Center of Henan (animal certification number: Henan 2010-0132S). All rats were fed in bedded cages. They were allowed free autoclaved water and food. Room temperature was controlled at 22 ± 2 °C with natural light. After 1 week of adaptive feeding, HHcy rat models were established according to Luzeng Wang’s method\textsuperscript{14-16}. The animals were equally randomized into four groups: control, HHcy, folacin intervention, and lutein intervention groups. They were weighed every week. According to the weights, intragastric dosages were determined. The blank control group was lavaged with 2 ml of 1% carboxymethylcellulose once each day, the HHcy group with 3% methionine suspension according to 1.5 g · kg\textsuperscript{-1}, the folacin intervention group with 3% methionine suspension plus folacin suspension (60 mg · kg\textsuperscript{-1}), and the lutein group with 3% methionine suspension plus lutein suspension (20 mg · kg\textsuperscript{-1}). After lavaging, animals in all groups were fed normally. All treatments lasted 8 weeks. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Zhengzhou University.

Sample Collection and Handling

At 8 weeks, each animal was intraperitoneally injected with 10% chloral hydrate (0.36 g/kg) for anaesthesia. Blood was extracted from the heart and blood serum was centrifugally separated. The serum Hcy concentration of each group was determined by enzyme linked immunosorbent assay (ELISA; the kit was the product of Wuhan Boster Biotechnology Co., Ltd., China). Oxidative stress associated indices such as the activity of serum superoxide dismutase (SOD), glutathion peroxidase (GPx) and catalase (CAT), hydroxyl radical (OH\textsuperscript{•}) level, as well as the contents of malondialdehyde (MDA), NO and endothelin-1 (ET-1) were also determined (the kits were the products of Nanjing Jiancheng Bioengineering Institute, Nanjing City, China). Part of the aortic wall tissue was taken for determining the mRNA expression of SOD2, GPx1, nuclear factor-κB (NF-κB) p65, and intercellular adhesion molecule-1 (ICAM-1) using real-time polymerase chain reaction (RT-PCR). The upstream and downstream primers for rat SOD2 amplification were 5’TGACCTTGCTTACGACTATG3’ and 5’TGGAGAAATGCTGTGTATT3’, with an amplified fragment length of 224 bp. The upstream and downstream primers for rat GPx1 amplification were 5’GGAGATGGCAAGAGATGGAAGA3’ and 5’ATGTCGATGCTGCCAACG3’, with an amplified fragment length of 312 bp. The upstream and downstream primers for rat NF-κBp65 amplification were 5’AGGCTTCTGGGACATATG3’ and 5’TGGCTCTTAGTGTATCTGTGC3’, with an amplified fragment length of 130 bp. The upstream and downstream primers for ICAM-1 amplification were 5’CTCTCTCAAGCTGACACGACAT3’ and 5’CCAGCCCGGATGTCG3’, with an amplified fragment length of 292 bp. The upstream and downstream primers for β-actin (internal reference) were 5’CCCATCTAAGGGTGATCAC3’ and 5’GGGAGGTGGACAGTGAG3’, with an amplified fragment length of 568 bp. The protein expression of SOD2, GPx1, NF-κBp65, and ICAM-1 in the aortic wall tissue was detected by Western blot analysis. Total protein was extracted and quantitated using the Bradford method. The sample in an appropriate amount was electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and then electro-transferred to a polyvmeldene difluoride (PVDF) membrane. The membrane was treated with 5% skimmed milk at 4°C for 6 h of blocking. After Tris-Buffered Saline and Tween (TTBS) washing (5 min × 3 times), it was incubated with primary antibody at room temperature for 1 h. After TTBS washing (5 min × 3 times), it was incubated with HRP-conjugated corresponding antibodies at room temperature and oscillated for 1 h. After washed thrice (5 min per wash), the membrane was colorated by enhanced chemoluminescence.

Statistical Analysis

Data were presented by means ± standard error of means (x ± s). Statistical analysis was carried out by SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) using one-way ANOVA for comparisons among groups and Newman Keuls tests for comparisons between groups. p < 0.05 was considered statistically significant.
Results

Serum Hcy, NO, and ET-1 Levels

The serum Hcy level in the model group was noticeably higher than that in the control group. The serum Hcy level in the folacin intervention group significantly decreased compared with that in the model group. Although the serum Hcy level in the lutein intervention group showed a decrease compared with that in the model group, no significant difference was observed. Compared with the control group, the model group exhibited a noticeably-decreased NO level and a noticeably-increased ET-1 level. Both folacin and lutein significantly increased the serum NO level and decreased the ET-1 content. The results are summarized in Table I.

Serum OH• and MDA

Compared with the control group, the HHcy model group showed marked increases in the OH• level and MDA content, indicating that HHcy rats stayed in oxidative stress excitement. Compared with the model group, both the folacin and lutein intervention groups showed significantly-decreased OH• and MDA, indicating that lutein noticeably intervened in the oxidative stress state of HHcy rats. The results are summarized in Table II.

Serum SOD, GPx, and CAT

Compared with the control group, the HHcy model group showed significantly-decreased SOD and GPx activity. Compared with the HHcy model group, both the folacin and lutein intervention groups showed significantly-increased SOD and GPx activity. These findings indicated that the noticeably-decreased antioxidase activity in HHcy rats might be ascribed to consumption due to Hcy-caused increased oxidative stress. However, no significant differences in CAT activity were observed between the model group and the intervention groups. The results are summarized in Table III.

RT-PCR

Compared with the control group, the HHcy model group exhibited decreased mRNA expression of SOD2 and GPx1 in the aortic tissue. Lutein significantly upregulated the mRNA expression of SOD2 and GPx1 and enhanced the anti-oxidative stress ability of organisms. Compared with the control group, the HHcy model group exhibited increased mRNA expression of NF-κB p65 and ICAM-1. Lutein significantly downregulated the mRNA expression of NF-κB p65 and ICAM-1. The results are shown in Figure 1.

Western Blot Analysis

Compared with the control group, the HHcy model group exhibited decreased protein expression of SOD2 and GPx1 in the aortic tissue. Lutein significantly upregulated the protein ex-

Table I. Levels of serum Hcy, NO, and ET-1 in different groups (x ± s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Case number</th>
<th>Hcy (µmol/L)</th>
<th>NO (µmol/L)</th>
<th>ET-1 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>6.88 ± 0.59</td>
<td>56.26 ± 10.06</td>
<td>34.12 ± 6.05</td>
</tr>
<tr>
<td>HHcy</td>
<td>10</td>
<td>17.26 ± 1.13*</td>
<td>38.88 ± 8.23*</td>
<td>56.25 ± 9.86*</td>
</tr>
<tr>
<td>Folacin</td>
<td>10</td>
<td>11.25 ± 0.81*</td>
<td>53.36 ± 11.12*</td>
<td>35.30 ± 5.78*</td>
</tr>
<tr>
<td>Lutein</td>
<td>10</td>
<td>15.39 ± 0.96</td>
<td>54.46 ± 10.31*</td>
<td>36.22 ± 6.32*</td>
</tr>
</tbody>
</table>

Notes: *p < 0.05, compared with the control group; #p < 0.05, compared with the model group; §p > 0.05, compared with the model group.

Table II. OH• and MDA in different groups (x ± s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Case number</th>
<th>OH• (U/L)</th>
<th>MDA (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>0.53 ± 0.05</td>
<td>5.11 ± 0.68</td>
</tr>
<tr>
<td>HHcy</td>
<td>10</td>
<td>0.92 ± 0.09*</td>
<td>7.65 ± 0.87*</td>
</tr>
<tr>
<td>Folacin</td>
<td>10</td>
<td>0.74 ± 0.06*</td>
<td>6.44 ± 0.91*</td>
</tr>
<tr>
<td>Lutein</td>
<td>10</td>
<td>0.69 ± 0.06*</td>
<td>5.62 ± 0.78*</td>
</tr>
</tbody>
</table>

Notes: *p < 0.05, compared with the control group; #p < 0.05, compared with the model group.
expression of SOD2 and GPx1 and enhanced the anti-oxidative stress ability of organisms. Compared with the control group, the HHcy model group exhibited increased protein expression of NF-κB p65 and ICAM-1. In contrast, lutein significantly downregulated the protein expression of NF-κB p65 and ICAM-1. The results are shown in Figure 2.

### Discussion

AS involves a variety of biochemical and molecular mechanisms, among which endothelial injury and inflammation are the most influential ones. An increase in Hcy is one of the independent risk factors of AS. Although HHcy may get involved in both mechanisms of endothelial injury and inflammation, its exact association path remains to be clarified. Oxidative stress may be an important initial factor of AS-associated endothelial injury and inflammation. Hcy induces oxidative stress excitement and increases reactive oxygen species (ROS) production of endothelial cells, which leads to increased endothelial cell lipid peroxidation and, finally, results in endothelial dysfunction. However, the exact molecular mechanism underlying HHcy-induced endothelial cell anti-oxidative stress excitement remains to be established. Apart from the Hcy sulfhydryl antioxidative chemical mechanism, there may exist more important ROS-producing pathways such as the biochemical mechanism involving changes in endothelial NO synthase activity, and meanwhile these pathways can be enlarged by some intracellular antioxidases such as inhibitors of SOD and GPx.

Further, all mechanisms should support Hcy to increase the levels of superoxide anion free radical and OH•, with the two latter prominently interacting with endothelial NO to produce peroxynitrite and, thereby, reducing the biological function of NO and resulting in endothelial dysfunction.

In the pathophysiological mechanism of AS, NF-κB activation plays a key role. It induces AS development by regulating the expression of numerous types of inflammatory factors. NF-κB is an important cellular transcriptional factor. One of its principle characteristics is manifested by the activation of cell adhesion factors as well as of some inflammatory cytokines such as ICAM-1 and TNF-α. HHcy-caused oxidative stress excitement and increased ROS have been assumed to enable the activation of inflammatory
signal transduction pathways of endothelial cells such as NF-κB to further promote the expression of some endothelial cell chemoattractive factors and adherence factors (such as ICAM-1). These expression changes are then carried into vascular endothelium by white blood cells through the circulation of recruitment, adhesion and migration. In this study, the HHcy rats exhibited oxidative stress excitement, which was manifested by decreased activity of SOD and GPx, as well as an increased lipid peroxidation level. In the HHcy model group, NO decreased and ET-1 increased, indicating that HHcy mediated vascular endothelial dysfunction. Furthermore, RT-PCR and western blot analysis showed decreased mRNA and protein expression of SOD2 and GPX1 and increased expression of NF-κB and ICAM-1 in these rats. Moreover, although lutein did not significantly decrease serum Hcy, it greatly intervened in and changed the biochemical behaviours mediated by Hcy, which were manifested by the upregulated expression of SOD2 and GPX1, strengthened anti-oxidative stress ability of organisms, and downregulated expression of NF-κB p65 and ICAM-1.

ROS is an important angiocellular signaling molecule. Oxidative stress excitement increased ROS production in HHcy rats. This phenomenon activated and upregulated NF-κB p65 expression and then induced the upregulation of ICAM-1 expression, thereby mediating AS. Antioxidants can decrease NF-κB p65 synthesis in varied cells as well as ICAM-1 expression in vascular endothelial cells. As a confirmed antioxidant, lutein can significantly downregulate the expression of NF-κB p65 and ICAM-1 induced by oxidative stress due to increased Hcy in HHcy rats, thus playing an important regulatory role in the ROS-inflammatory cycle mechanism of AS. This may be an important molecular mechanism of the potential protective effect of lutein on cardiovascular diseases. Lutein inhibits endotoxin-induced uveitis by inhibiting NF-κB-dependent signal transduction pathways and reducing the production of proinflammatory mediators such as NO, TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) \textsuperscript{30}. HHcy is associated with NF-κB-mediated inflammatory reaction by activating the ERK(1/2)/p38MAPK (extracellular stress-related kinase-1/p38 mitogen-activated protein kinase) pathway \textsuperscript{31}. However, whether the downregulatory effects of lutein on the expression of NF-κB p65 and ICAM-1 are mediated by the approved ERK(1/2)/p38MAPK pathway remains to be explored.

Conclusions

HHcy functions as an independent risk factor of AS. HHcy-induced aggravated oxidative stress, as well as so-caused concomitant endothelial cell injury and inflammation, is an important initial mechanism for mediating AS. In particular, HHcy-induced aggravated oxidative stress can induce the expression of NF-κB and the downstream inflammatory factors such as ICAM-1, thereby connected with AS development. Lutein significantly inhibits Hcy-mediated oxidative stress excitement and downregulates inflammation-related informational molecular expression. Therefore, the intervention and protective effect of lutein on early AS may arise from its direct effect of being an antioxidant rather than the indirect effect of decreasing serum Hcy. This explanation provides a new idea for the application of lutein in the effective prevention and cure of AS from the molecular level.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References


