Original Article
Anti-oxidative stress role of valsartan and fluvastatin in rat mesangial cells cultured with high glucose

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Abstract: This study aims to investigate the roles of valsartan and fluvastatin in fighting against oxidative stress injury in high glucose cultured rat mesangial cells (HBZY-1). The rat mesangial cells were cultured in vitro, then divided into the control group, high glucose group, mannitol group, high glucose plus valsartan group, high glucose plus fluvastatin group, and high glucose plus valsartan and fluvastatin group. Indicators such as the cell morphology, cell viability, superoxide dismutase (SOD), nitric oxide (NO) and malondialdehyde (MDA) were compared. Compared with the control group, the high glucose culture could significantly reduce the SOD activity (61.54±0.30 vs 30.99±0.47, P<0.05) and the NO level (9.56±0.43 vs 17.11±0.24, P<0.05), while significantly increase the MDA concentration (36.2±0.13 vs 10.21±0.14, P<0.05) in the supernatant of rat mesangial cells. While the culture of high glucose with different concentrations of valsartan and fluvastatin could increase the SOD activity and NO level, and reduce the MDA concentration, among which the high glucose + valsartan + fluvastatin culture group exhibited the most significant effects. Valsartan and fluvastatin exhibited the dose-dependent effects of anti-oxidative stress injury in high glucose cultured rat mesangial cells, and the combination of these two drugs had better effects than single drug.

Keywords: Valsartan, fluvastatin, rat mesangial cells, oxidative stress

Introduction
Diabetic nephropathy (DN) is a common major complication of diabetes, and the leading cause of mortality and disability in diabetes. Epidemiological study showed that by 2030, the world’s diabetics would reach 366 million, and the patients with varying degrees of DN would be over 100 million [1]. The treatment of DN is difficult, with more costs and more time-consumption, thus bringing heavy burdens to societies [2]. The pathogeneses of DN are complex, and have not been entirely clear yet; presently, it’s considered to be the synergetic result of various factors [3-6], among which high blood sugar is an important initial factor, and oxidative stress (OS) plays an important role in the occurrence and development of DN [7, 8]. In diabetes, the proliferation of glomerular mesangial cells would be significantly enhanced under the stimulations of various factors. Angiotensin receptor blocker (ARB) [9] and Statins [10] have been confirmed to have renal protective effects in DN, but the combined effects and related mechanisms of these two have not been reported. Therefore, the present study observed the changes of OS indicators in the high glucose cultured rat mesangial cells when treated with valsartan and fluvastatin, aiming to explore the relationships between their protective effects and anti-OS, as well as to compare the effects of the individual and combined application of the above two drugs.

Materials and methods

Cell and regents
Cell: rat mesangial cells (Institute of Medical Science, First Affiliated Hospital of Wenzhou Medical College, cell number: HBZY-1). Reagents: raw material of valsartan and fluvastatin (Novartis, Beijing), low glucose dulbecco minimum essential medium (DMEM) (GIBCO, USA), fetal bovine serum (Evergreen, Hangzhou), methyl thiazolyl tetrazoliun (MTT) (Sigma, USA), and the detection kits for the superoxide dismutase (SOD) activity, nitric oxide (NO) content and malondialdehyde (MDA) content (Nanjing Jiancheng).
Cell culture and grouping

HBZY-1 was cultured in the 10% FBS-containing low glucose DMEM culture medium for the subculture; when the cells reached 80% fusion, serum-free medium was then given for 24 h synchronous static culture, followed by the experiments. The cells were divided into the following six groups: (1) the control group (group N): cultured in low glucose DMEM culture medium, with the concentration of glucose as 5.5 mmol/L; (2) the high glucose group (group H): with the concentration of glucose as 30 mmol/L; (3) the osmotic pressure control group (group M): cultured in 5 mmol/L glucose and 25 mmol/L mannitol-containing DMEM medium for 24 h; (4) high glucose + valsartan group (group V): intervened the cells with low (LV, 1 μmol/L), middle (MV, 10 μmol/L), and high (HV, 100 μmol/L) concentrations of valsartan for 24 h, and the concentration was finally decided as 10 μmol/L; (5) high glucose + fluvastatin group (group F): intervened the cells with low (LF, 1 μmol/L), middle (MF, 10 μmol/L), and high (HF, 100 μmol/L) concentrations of fluvastatin for 24 h, and the concentration was finally decided as 10 μmol/L; (6) high glucose + valsartan + fluvastatin group (group VF): the final drug concentrations were decided as 10 μmol/L.

Cytologic morphology observation

After treated the cells for 24 h and 48 h, the morphologies were observed and photographed under inverted microscope (When the experiment proceeded to 48 h, most cells in group H were already separated from the bottle wall and suspended in the culture supernatant. Therefore, the following experiments were carried out for only 24 h).

Detection of cell viability

The cell proliferation was detected by MTT assay, and the survival rate of HBZY-1 under high glucose and inhibition rate under valsartan and fluvastatin were calculated. Survival rate (%) = (OD value of group H/OD value of group N) × 100%; inhibition rate (%) = (OD value of group H-OD value of group V or F/OD value of group H) × 100%.

Figure 1. The morphology of various HBZY-1 groups at 24 h (× 40). A: Normal glucose group (N); B: High glucose group (H); C: Mannitol osmotic control group (M); D: High glucose plus valsartan group (V); E: High glucose plus fluvastatin group (F); F: High glucose plus valsartan and fluvastatin group (VF).
Table 1. OD value of various HBZY-1 groups (A, ±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>OD value at 0 h</th>
<th>OD value at 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.136±0.011</td>
<td>0.138±0.005</td>
</tr>
<tr>
<td>H</td>
<td>0.135±0.023</td>
<td>1.663±0.017</td>
</tr>
<tr>
<td>M</td>
<td>0.132±0.015</td>
<td>0.143±0.026</td>
</tr>
<tr>
<td>LV</td>
<td>0.143±0.015</td>
<td>0.604±0.012</td>
</tr>
<tr>
<td>V</td>
<td>0.137±0.024</td>
<td>0.389±0.009</td>
</tr>
<tr>
<td>HV</td>
<td>0.136±0.019</td>
<td>0.264±0.011</td>
</tr>
<tr>
<td>LF</td>
<td>0.135±0.017</td>
<td>0.648±0.013</td>
</tr>
<tr>
<td>F</td>
<td>0.134±0.025</td>
<td>0.317±0.015</td>
</tr>
<tr>
<td>HF</td>
<td>0.138±0.031</td>
<td>0.253±0.022</td>
</tr>
<tr>
<td>VF</td>
<td>0.131±0.014</td>
<td>0.139±0.017</td>
</tr>
</tbody>
</table>

Note: N: Normal group; H: High glucose group; M: Mannitol osmotic control group; LV: High glucose plus 1 μmol/L valsartan group; V: High glucose plus 10 μmol/L valsartan group; HV: High glucose plus 100 μmol/L valsartan group; LF: High glucose plus 10 μmol/L fluvastatin group; F: High glucose plus 10 μmol/L fluvastatin group; HF: High glucose plus 10 μmol/L valsartan and 10 μmol/L fluvastatin group. Compared with group N, *P<0.05; compared with group H, **P<0.01; compared with group V, ***P<0.05; compared with group F, ****P<0.05.

Detection of SOD, NO, and MDA in the cell supernatant

Collected the cell supernatants for the detection of the SOD, NO, and MDA levels in accordance with the instructions of detection kits, respectively.

Statistical analysis

Each experiment was repeated three times independently. SPSS10.0 software was used for data analysis; the data were expressed as mean ± standard deviation (±s); the intergroup comparison used t test, and the comparison among groups used the analysis of variance, with P<0.05 considered as statistically significant.

Results

Morphological observation

After 24 h intervention, the cells in group N exhibited normal morphologies, and the cellular structures were clear and identifiable. The cells in the rest groups exhibited significant morphological changes. The cells in group M exhibited similar morphologies to group N, while the cell number was slightly increased, and the cell body exhibited slight hypertrophy; the morphological changes of the cells in group V and F were similar: the cell gaps became enlarged, and the cellular structures were not clear, and the cells became flat; the cells in group H were disorganized, with more significant flat degree (Figure 1). When the experiment proceeded to 48 h, most cells in group H were already separated from the bottle wall and suspended in the culture supernatant.

Proliferation detection of the mesangial cells by MTT

0 h: the difference among the OD values of difference groups were not statistically significant (P>0.05). 24 h: the OD value of group H was increased significantly than group N, and the difference was statistically significant (P<0.01), and the survival rate was 172.8±6.5%. Compared with group H, the OD values of the rest groups were reduced, and the differences were statistically significant (P<0.05); the differences among the groups with 3 different concentrations of valsartan and fluvastatin were statistically significant (P<0.05), and were in a concentration-dependent manner. The OD value of group VF was statistically significantly reduced than group V and F (P<0.05, Table 1). The inhibition rate of group VF was 37.1±1.83%, indicating the combination of valsartan and fluvastatin exhibited significantly higher inhibitory effects towards cell proliferation than a single drug.

Detection of the SOD activity, NO and MDA content in the supernatant of mesangial cells of each group

0 h: the differences in the SOD activity, NO and MDA content among different groups were not statistically significant (P>0.05); 24 h: compared with group N, the SOD activity and NO content in group H were decreased, while the MDA content was increased significantly (P<0.05). Compared with group H, the SOD activities and NO contents in the cell supernatants of group V, H, and VF were increased, while the MDA contents were decreased (P<0.05), compared with group V and F, the SOD activity and NO content in group VF was increased (P<0.05), while the MDA content was decreased (P<0.05, Table 2), indicating that the anti-OS effects of the combination of valsartan and fluvastatin were better than a single drug.
Table 2. The activities of SOD and the level of NO and MDA of various HBZY-1 groups

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/ml)</th>
<th>NO</th>
<th>MDA (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
<td>0 h</td>
</tr>
<tr>
<td>N</td>
<td>67.17±0.37</td>
<td>66.39±0.28</td>
<td>17.23±0.12</td>
</tr>
<tr>
<td>H</td>
<td>61.54±0.30</td>
<td>30.99±0.47</td>
<td>14.15±0.27</td>
</tr>
<tr>
<td>M</td>
<td>56.35±0.31</td>
<td>48.49±0.23</td>
<td>16.54±0.14</td>
</tr>
<tr>
<td>V</td>
<td>59.27±0.38</td>
<td>45.46±0.13</td>
<td>14.68±0.24</td>
</tr>
<tr>
<td>F</td>
<td>58.34±0.12</td>
<td>47.55±0.24</td>
<td>15.07±0.15</td>
</tr>
<tr>
<td>VF</td>
<td>62.18±0.27</td>
<td>60.09±0.16</td>
<td>17.19±0.24</td>
</tr>
</tbody>
</table>

Note: N: Normal group; H: High glucose group; M: Mannitol osmotic control group; V: High glucose plus 10 µmol/L valsartan group; F: High glucose plus 10 µmol/L fluvastatin group; VF: High glucose plus 10 µmol/L valsartan and 10 µmol/L fluvastatin group. Compared with group N, a P<0.05; compared with group H, b P<0.05; compared with group V, c P<0.05; compared with group F, d P<0.05.

Discussion

DN is a common and serious chronic complication of diabetes, and its pathogenesis involves many aspects; OS is one of the common mechanisms in various DN pathogeneses [7, 8]. The proliferation of mesangial cells and abnormal expression of the related factors play an important role in the occurrence and development of glomerular sclerosis. Recent studies had shown that ARB and Statins had renal protective effects, while its mechanism is not fully elucidated.

Mesangium has such functions as contraction, phagocytosis, proliferation, and synthesizing mesangial matrix and collagen, etc., and could secrete a variety of bioactive mediators and cytokines. The pathological features of early DN are glomerular hypertrophy, glomerular basal membrane thickening and extracellular matrix (ECM) accumulation, which all could lead to glomerular ultrafiltration and microalbuminuria [9]. The mesangial cells could secrete ECM, and ECM also has feedback regulation towards the mesangial cells. Under physiological conditions, ECM would be in the equilibrium of production and degradation. While under pathological conditions, the proliferation of mesangial cells would lead to the accumulation of ECM. MTT mainly reflects the cellular energy metabolism, and is a simple and accurate method in detecting cell proliferation; it could help to reflect the integrity of the cell membranes and distinguish the necrotic cells. This study used high glucose emdium to culture the mesangial cells HEZY-1, and found by this method that after cultured in high glucose medium for a certain period of time, the proliferation of the mesangial cells was significantly increased, and the survival rate of the cells was significantly increased, further confirming the pro-proliferation effects of high glucose on the mesangial cells.

High glucose-resulted OS plays extremely important roles in the pathogenesis of DN, which could directly generate superoxide anion; therefore, the generation of the reactive oxygen species (ROS) inside the mitochondriae would be increased, or such antioxidant enzyme system as superoxide dismutase (SOD) might exhibit defects or functional deficiencies, followed by the imbalance of the generation, neutralization, and elimination of ROS in local renal tissues [7, 8]. Detecting SOD might reflect the degree of OS, as well as indirectly reflect the degree of cell damage. NO is a vascular protective factor produced by NO synthase (NOS)-catalyzed L-arginine inside the endothelium. Human body mainly have three types of NOS: eNOS is secreted by the endothelial cells, nNOS is secreted by the macula densa, and iNOS is largely secreted by the mesangial cells or immune cells in the pathological state. NO could inhibit the aggregation of platelets and white blood cells, thus reducing the generation of the collagen and fibrin, reducing the synthesis of ECM, and helping to prevent the renal fibrosis. Under normal circumstances, the production of NO is very sophisticated and coordinated in time and space. While in the most early stage of DN, nNOS inside the dense plaques would be downregulated, so that NO of efferent glomerular arteriole would be decreased, and the efferent glomerular arteriole would contract and GFR would be increased; in early DN, eNOS and iNOS in renal microvessels...
Antioxidation role of valsartan and fluvastatin

would be increased, resulting in the strong dilatation of afferent glomerular arteriole and weak relaxation of efferent glomerular arteriole, and GFR would be increased; in middle and advanced stage, renal NOS would be decreased, so that the synthesis of ECM inside the mesangial cells would be increased while the degradation would be reduced, and the glomerular functions would be decreased and GFR would be decreased [11]. High glucose could also mediate the generation of ROS indirectly through such end products of the advanced glycation as AGEs [5], TGF-β1 [12], and Ang II [13], etc. High glucose could also stimulate the mesangial cells to produce ROS through PKC-dependent NAD(P)H oxidase (the important enzymes to produce ROS) [14, 15]. ROS could attack the unsaturated fatty acids in vivo, resulting in the lipid peroxidation product MDA, and the latter was positively correlated with the renal impairment [16]. This experiment confirmed that high glucose could significantly decrease the SOD and NO activity and increase the MDA content in the supernatant of the mesangial cells, and further confirmed that high glucose could induce the generation of ROS in cells, which would lead to the enhanced lipid peroxidation reactions and decreased antioxidant capacities.

Numerous studies had shown that ARB had renal protective effects independent from its antihypertensive effects, could reduce the microalbuminuria in the patients with DN, and slowed the development of DN [9]. Basic and clinical studies had shown that on the one hand, ARB could impact the renal hemodynamics, thus reducing the high perfusion, high filtration and high intracapillary pressure of DN; on the other hand, it could also impact the non-kinetic effects of cytokines and growth factors to play the roles of treating DN and delaying the renal failure [17]. Ogawa et al [18] clinically applied candesartan and valsartan and found that ARB could reduce the OS levels in the diabetics and relieve the kidney damages, suggesting that the renal protective effects of ARB might be associated with its antioxidant effects.

Recent studies also showed that in addition to the lipid-regulation effects, Statins also had multiple effects, including anti-inflammations, anti-thrombosis and anti-oxidation [19]. Through upregulating eNOS, downregulating iNOS, and reducing the activity of NAD(P)H oxidase, Stains could exert their protective effects, Heusinger-Ribeiro et al [20] reported that 48 h after the administration of simvastatin (10 mol/L), the proliferation of the mesangial cells in SD rats was significantly inhibited, and the number of mesangial cells was also reduced. Casey et al [21] found that pravastatin could increase the expression of NOS, protect the functions of the microvascular endothelial cells in the early DN animal model under high glucose, and suppress the early diabetic microangiopathies. Wassmann et al [22] found from in vitro experiments and in vivo animal trails that Statins could not only reduce the plasma level of cholesterol, but also reduce the expression of AngII type 1 receptor (AT-1) gene, inhibit Rac-1, thus reducing the generation of ROS, suggesting that Statins had certain antioxidant effects.

This study found that valsartan and fluvastatin both had the roles of inhibiting the proliferation of mesangial cells induced by high glucose, and this kind of effects was positively correlated with the concentration of the drug, suggesting there presented a concentration-dependent manner. At the same time, both drugs could reverse the decreased activities of SOD and NO inside the high glucose cultured mesangial cells, and reduce the production of MDA; the combined effects of these two drugs were stronger, suggesting that valsartan and fluvastatin might reduce the production of ROS in the high glucose-induced cells, thus increasing the activities of the antioxidant enzymes, regulating the oxidation equilibrium inside the mesangial cells, inhibiting the high glucose-stimulated abnormal proliferation of the mesangial cells, and playing their renal protective roles.

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Disclosure of conflict of interest

None.
Antioxidation role of valsartan and fluvastatin

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