Mediation of inflammation, obesity and fatty liver disease by advanced glycation endproducts

D.-D. XIONG¹, M. ZHANG¹, N. LI², J.-F. GAI³, L. MAO³, M. LI³

¹Department of Laboratory, Binzhou Central Hospital, Binzhou, Shandong, China
²Department of Pediatrics, Binzhou Central Hospital, Binzhou, Shandong, China
³Department of Gynaecology, Binzhou Central Hospital, Binzhou, Shandong, China

Abstract. – OBJECTIVE: Fatty liver may induce various complications including chronic hepatitis or liver cirrhosis, and is frequently occurred in obesity individuals. Advanced glycosylation end products (AGEs) were known to play a critical role in multiple liver diseases. This study, therefore, aimed to study the effect of AGEs on obesity, related liver cirrhosis and inflammation, on an obesity fatty liver rat model.

MATERIALS AND METHODS: A total of 60 Sprague Dawley (SD) rats were randomly divided into control, model and AGEs inhibitor groups (n=20 each). AGEs level, body weight and liver function were examined in each animal, followed by hematoxylin-eosin (HE) staining to detect the pathological change of liver. Further Real-time PCR and enzyme-linked immunosorbent assay (ELISA) were employed to detect inflammatory cytokine levels including tumor necrosis factor (TNF)-α and interleukin (IL)-6.

RESULTS: AGEs level was significantly elevated in obesity fatty liver model rats, which also had higher total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL-C) and high-density lipoprotein (HDL-C) levels, along with deteriorated liver function and higher TNF-α and IL-6 levels. The application of AGEs inhibitor aminoguanidine significantly improved liver functions and lower TNF-α or IL-6 levels when compared to the model group (p<0.05 in all cases).

CONCLUSIONS: Obesity fatty liver can promote AGEs level, further causing pathological changes and increased secretion of inflammatory cytokines. The inhibition of AGEs can improve the metabolism of fatty acids, decrease inflammatory cytokines and benefit the treatment of obesity fatty liver.

Key Words: Obesity fatty liver, Inflammatory cytokines, Advanced glycosylation end products.

Introduction

With the economic progress and change of diet structure worldwide, the incidence of obesity is increasing continuously. As shown by the WHO survey, the percentage of overweight (body mass index, BMI between 25 and 29.9) and obesity (BMI>30) in the whole population is 11.2%, with increasing obesity population in China¹, ². Obesity, as caused by the metabolic disorder, is manifested with increased number and volume of adipocytes, causing abnormally elevated fatty acid percentage. Obesity has been known to be related to various systemic diseases such as diabetes and cardiovascular disease³,⁴. Fatty liver is one of the common chronic liver diseases and is caused by the imbalance of total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL-C) and high density lipoprotein (HDL-C), thus leading to over-deposition of fatty acids in hepatocytes, causing multiple complications such as chronic inflammation or liver cirrhosis⁵,⁶. Fatty liver can occur in all age groups and most often in young adults, thus causing a major health concern. Fatty liver is the second most commonly occurred liver disease next to viral hepatitis⁷,⁸. Fatty liver is often developed in obesity people, due to their elevated free fatty acids, which can be converted into triglyceride⁹. Currently no drugs have been available for obesity fatty liver with confirmed efficacy¹⁰.

Advanced glycosylation end products (AGEs) are a kind of fluorescent compounds formed by irreversible non-enzymatic glycosylation reaction between aldehyde/ketone groups of reducing sugar took (including fructose, glucose, xylose and pentose) and free amino group of lipids, proteins and nucleotides¹¹. AGEs exert their functions with binding onto specific receptor RAGE or modifying structure and function of lipids, proteins and nucleotides¹². The over-deposition of AGEs may cause metabolic disorders and lead to multiple pathological damages¹³. Elevation of both blood glucose and reactive oxygen during the progres-
tion of various diseases including diabetes, ischemia-reperfusion damage, hypoxemia and atherosclerosis can facilitate AGEs production, further aggravating the disease progression\textsuperscript{14,15}. The effect of AGEs on obesity fatty liver and inflammation, however, remained unclear. This study, therefore, aimed to investigate the effect of AGEs on obesity, fatty liver and inflammation on an obesity fatty liver rat model.

**Materials and Methods**

**Animals**

A total of sixty healthy male specific pathogen free (SPF)-grade Sprague Dawley (SD) rats (age: 2 month old, body weight: 250 ± 20 g) were purchased from the Laboratory Animal Center of Shandong University and were kept in an SPF-grade facility. This work has been pre-approved by the Ethical Committee of Binzhou Central Hospital.

**Animal Model and Sample Collection**

All rats were housed under normal conditions for 2 weeks, and then were randomly divided into 3 groups (n=20 each) including control, obesity fatty liver and AGEs inhibitor groups. Obesity fatty liver model was prepared by the application of high-fat diet (including 30% fatty acid and 40% carbohydrate) and subcutaneous injection of CCl\textsubscript{4} (20% in castor oil) at 0.2 mg/100 g for six weeks as previously reported\textsuperscript{16, 17}. The specific inhibitor for AGEs, aminoguanidine (100 mg/kg·d, Invitrogen/Life Technologies, Carlsbad, CA, USA), was given by subcutaneous injection simultaneously.

After 6 weeks, all rats were anesthetized by 10% chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA). Blood samples were collected from abdominal aorta followed by centrifugation (1000×g for 10 min) and serum separation. Liver tissues were also collected for fixation in 4% neutral buffered formaldehyde or for storage at -80°C in Real-time PCR.

**Histopathological Examination of Liver Tissues**

After 48-hour fixation, liver tissues were dehydrated in gradient ethanol (50%, 60%, 70%, 80%, 90% and 95%), followed by xylene treatment, paraffin incubation and embedding. Tissue blocks were sectioned into slices, which were de-waxed in xylene followed by hematoxylin-eosin (HE) staining and microscopic observation.

**Liver Function Assay and AGEs Quantification**

Both body weights and liver (wet) weights were measured in all groups. Total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bile acids (TBIL). AGEs level in serum was measured by fluorescent spectrometry (370 nm excitation, 440 nm emission).

**Real-time PCR**

Liver tissue sample (20 mg) was homogenized and centrifuged in 1.5 mL TRIlol (Invitrogen/Life Technologies, Carlsbad, CA, USA) for total mRNA extraction. cDNA was then synthesized using specific primers (Table I). Real-time PCR was used to quantify the expression levels of target genes. The PCR consisted of 35 cycles each containing: 90°C denature for 30 s, 58°C annealing for 50 s, and 72°C elongation for 35 s. Fluorescent PCR cyclor collected data for calculating CT value with reference to GAPDH gene. Further quantitative analysis was performed by 2\textsuperscript{−ΔΔCt} method.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Serum TNF-α and IL-6 levels were measured by ELISA kits (Abcam Biotech., Cambridge, MA, USA) following manual instructions. In brief, serially diluted standard samples were seeded into 96-well plate, along with test samples at gradient concentrations. After gentle washing, 50 μl enzyme-linked reagents were added into each well except the blank control. After 37°C incubation for 30 min, chromogenic substrate A and B were sequentially added, followed by further 10 min-incubation at 37°C. The reaction was stopped by adding 50 μl stopping buffer. Optical density (OD) values in each well at 450 nm was measured by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). A standard curve was firstly plotted based on the standard sample, followed by calculating sample concentration.

**Statistical Analysis**

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) package was used to process all collected data, of which measurement data were presented as mean ± standard deviation (SD). Tukey’s post hoc test was used for comparing measurement data between groups. A statistical significance was identified when \(p<0.05\).
Results

Body Weight and Liver Weight
Both body weight and (wet) liver weight of obesity fatty liver model rats were significantly higher than those in control rats (Table II, *p*<0.05 in both cases). The application of AGEs inhibitor significantly decreased both body weight and liver weight when compared to model rats (Table II, #*p*<0.05 in both cases).

Body Weight and Liver Weight
In histological examination, no fatty degeneration or morphological change of liver tissue/hepatic lobule was observed, neither did the infiltration of inflammatory hepatocytes. In model rats, there was certain hepatic fatty degeneration including both macrovesicular and microvesicular steatosis, in addition to large amounts of collagens in the portal area, separating and de-structuring hepatic lobular structures. Furthermore, inflammatory cells occurred in the hepatic tissues, which had structural alternations. The treatment using aminoguanidine inhibitor decreased the fatty degeneration and alleviated inflammatory infiltration (Figure 1). All these results suggest the participation of AGEs in the formation and progression of obesity fatty liver. The inhibition of AGEs formation can impede the occurrence of fatty liver to certain extents.

Serum AGEs Levels
We further tested the serum level of AGEs, which was significantly elevated in model rats compared to controlled ones (Figure 2,* p*<0.05). The application of AGEs inhibitor, aminoguanidine, significantly depressed AGEs level (Figure 2,* #p*<0.05). These results suggest the deposition of AGEs in obesity rats, while aminoguanidine effectively inhibits AGEs formation.

Table I. Primer sequence.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TCTCA TGCCA CTGGG CAGGA T</td>
<td>ACTAT TGACG TGGGT GTGCC G</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CTCTC AGATG CGAAA CGTGA A</td>
<td>ATTCT ACTCC GCCAC GCCGT</td>
</tr>
<tr>
<td>IL-6</td>
<td>ACTTC AGAAG CTGGG TCAGA</td>
<td>GCCTC AGGAT CTATA AGC</td>
</tr>
</tbody>
</table>

TNF-α: tumor necrosis factor α, IL-6: interleukin 6.

Table II. Body weight and liver weight.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Liver wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>472.15±28.24</td>
<td>10.01±0.75</td>
</tr>
<tr>
<td>Model</td>
<td>20</td>
<td>675.32±39.73*</td>
<td>14.89±0.82*</td>
</tr>
<tr>
<td>AGEs inhibitor</td>
<td>20</td>
<td>569.71±31.25**</td>
<td>11.46±0.66#</td>
</tr>
</tbody>
</table>

Note: *, *p*<0.05 compared to control group; **, #*p*<0.05 compared to model group.

Figure 1. Rat liver tissues by HE staining (200 ×). A, control; B, model; C, AGEs inhibitor. AGEs: advanced glycosylation end products, HE: hematoxylin-eosin.
Blood Fat Level

We further tested various indexes reflecting fat level in abdominal aorta blood, including TC, TG, LDL-C and HDL-C. Results (Table III) showed significantly elevated TC, TG, LDL-C levels in model rats, whose HDL-C level; however, it was significantly depressed ($p<0.05$ in all cases). The application of AGEs inhibitor, effectively depressed TC, TG and LDL-C level, while increased HDL-C level ($p<0.05$ in all cases when compared to those in model group). Those results showed increased blood fatty acid secretion in obesity rats accompanied with higher AGEs. The inhibition of AGEs, can improve the blood fat secretion.

Rat Liver Function

Serum separated from abdominal aorta blood samples were further tested for liver function indexes. ALT, AST and TBIL in model rats were significantly elevated in model rats when compared to control ones (Table IV, $p<0.05$). AGEs inhibitor, however, effectively depressed all those indexes ($p<0.05$ compared to model group). Therefore, obesity fatty liver rats had higher AGEs level, accompanying with aggravated liver functions. The inhibition of AGEs production effectively improved liver functions.

![Figure 2](image)

**Figure 2.** Serum AGEs level. *, $p<0.05$ compared to control group; #, $p<0.05$ compared to model group. AGEs: advanced glycosylation end products.

TNF-α and IL-6 mRNA Expression

Using Real-time PCR, we quantified mRNA levels of inflammatory cytokines including TNF-α and IL-6 in rat liver tissues. Results showed significantly elevated TNF-α and IL-6 mRNA levels in obesity fatty liver rats (Figure 3, $p<0.05$ compared to control ones). The application of AGEs inhibitor, however, effectively inhibited expression of both cytokines ($p<0.05$ compared to model group), which returned to similar levels as those in control group.

Serum TNF-α and IL-6 Levels

ELISA method was used to describe serum levels of TNF-α and IL-6. Consistent results have been obtained as those for mRNA levels: obesity fatty liver rats had significantly elevated serum TNF-α and IL-6 (Figure 4, $p<0.05$ compared to control group). The application of AGEs inhibitor, however, effectively inhibited expression of both cytokines ($p<0.05$ compared to model group), which returned to similar levels as those in control group. Those results suggest the induction of both serum and liver expressions of TNF-α and IL-6, further facilitates inflammation. The inhibition of AGEs can depress TNF-α and IL-6 expression, indicating the correlation between obesity fatty liver-related inflammation and AGEs deposition. The depression of AGEs, therefore, can improve related inflammation.

Discussion

Obesity, with its increasing incidence worldwide, has become one research focus in clinics. Due to larger volume of food intake, obesity patients frequently had over-load livers, which are required for fat metabolism. The deposition of fatty acid in liver tissues lead to higher incidence of fatty liver in obesity patients, who may develop into liver fibrosis or cirrhosis, severely compromising patients’ life quality.$^5,6$
AGEs, including pyrraline, pentosidine, hydroxymethyl lysine, were a group of non-degradable complex compounds with auto-fluorescence formed by various non-enzymatic glycosylation reaction (including rearrangement, dehydration, oxidation and dehydrogenation) of amino acid residues or amino groups of lipid peroxidation products such as malonaldehyde and ascorbic acid under pathological or stress condition including metabolic disorders, inflammation, atherosclerosis, ischemia-reperfusion injury and aging. AGEs were known to play certain role in pathology of diseases including diabetes and osteoporosis. It has been known that obesity fatty liver disease further disrupts patient’s metabolic functions, causing inflammation. Therefore, it was speculated that AGEs may participate in the formation and inflammation of obesity fatty liver.

In this study, obesity fatty liver rat model was prepared by high-fat diet combined with subcutaneous injection of CCl₄, which can suppress the lipolysis of liver by altering mitochondria function, further aggravating fatty liver disease. Both increased liver weight and hepatic fatty degeneration occurred in model rats, which also had elevated AGEs level, suggesting the over-deposition of AGEs in obesity fatty liver.

Aminoguanidine, as specific inhibitor of AGEs, blocks its synthesis by binding onto 3-deoxy-glucosone, which is an intermediate in non-enzymatic glycosylation reaction. In this paper, the application of aminoguanidine effectively decreased body weight and liver weight, along with alleviating hepatic fatty degeneration, suggesting the inhibition of AGEs may slow down or even reverse the fatty liver disease progress.

It has been known that blood fatty acid level was positively correlated to BMI. Previous studies have shown elevated TG, TC and LDL-C, but lower HDL-C levels in obesity people compared to age-matched control group. This work revealed increased AGEs contents in obesity rats, suggesting the participation of AGEs in facilitating blood fat secretion. Further liver function assays revealed that the inhibition of AGEs significantly improved liver function. Due to the frequent occurrence of inflammation in obesity fatty liver disease, this study further analyzed the correlation between AGEs and inflammatory cytokines. Our results showed the induction of hepatic and serum expressions of TNF-α and IL-6 by obesity fatty liver, proving the occurrence of inflammation. The suppression of AGEs formation, significantly inhibited expression of those two

**Table IV. Liver function indexes.**

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>TBIL (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37.35±4.16</td>
<td>225.89±41.62</td>
<td>1.26±0.21</td>
</tr>
<tr>
<td>Model</td>
<td>438.43±52.16*</td>
<td>667.37±78.68*</td>
<td>4.27±0.56*</td>
</tr>
<tr>
<td>AGEs inhibitor</td>
<td>236.41±67.18*#</td>
<td>395.13±92.68*#</td>
<td>2.98±0.67*#</td>
</tr>
</tbody>
</table>

Note: *, p<0.05 compared to control group; †, p<0.05 compared to model group. ALT: alanine aminotransferase, AST: aspartate aminotransferase, TBIL: total bile acids, AGEs: advanced glycosylation end products.
Metabolites and fatty liver disease

Cytokines, providing a novel way for alleviating inflammation.

Conclusions

AGEs were abundantly expressed in obesity fatty liver disease, further causing pathological changes and increased secretion of inflammatory cytokines. The inhibition of AGEs may improve lipid metabolism, inhibit production of inflammatory cytokines, thus benefiting the treatment of obesity fatty liver disease.

References


8) Luckhoff HK, Krueger FC, Kotze MJ. Composite prognostic models across the non-alcoholic fatty liver disease spectrum: clinical application in developing countries. World J Hepatol 2015; 7: 1192-1208.


18) Kandel-Kifer M, Almog T, Shashi A, Shlomai G, Anafi L, Aviv C, Barshack I, Grosskopf I, Harats D, Kama- ri Y. Interleukin-1alpha deficiency attenuates en-


21) Ganji S, Kashyap ML, Kamanna VS. Niacin inhibits fat accumulation, oxidative stress, and inflammatory cytokine IL-8 in cultured hepatocytes: impact on non-alcoholic fatty liver disease. Metabolism 2015; 64: 982-990.