European Review for Medical and Pharmacological Sciences 2017; 21: 5172-5178

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

D.-D. XIONG[1], M. ZHANG[1], N. LI[2], J.-F. GAI[3], L. MAO[3], M. LI[3]

[1]Department of Laboratory, Binzhou Central Hospital, Binzhou, Shandong, China
[2]Department of Pediatrics, Binzhou Central Hospital, Binzhou, Shandong, China
[3]Department 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 China[1, 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 disease[3,4]. 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 cirrhosis[5,6]. 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 hepatitis[7,8]. Fatty liver is often developed in obesity people, due to their elevated free fatty acids, which can be converted into triglyceride[9]. Currently no drugs have been available for obesity fatty liver with confirmed efficacy[10].

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, protein and nucleotides[11]. AGEs exert their functions with binding onto specific receptor RAGE or modifying structure and function of lipids, proteins and nucleotides[12]. The over-deposition of AGEs may cause metabolic disorders and lead to multiple pathological damages[13]. Elevation of both blood glucose and reactive oxygen during the progres-

Corresponding Author: Min Li, MD; e-mail: minlikl@163.com

sion of various diseases including diabetes, ischemia-reperfusion damage, hypoxemia and atherosclerosis can facilitate AGEs production, further aggravating the disease progression[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_4 (20% in castor oil) at 0.2 mg/100 g for six weeks as previously reported[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 TRIzol (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 cycler collected data for calculating CT value with reference to GAPDH gene. Further quantitative analysis was performed by $2^{-\Delta Ct}$ 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 $p<0.05$.

Table I. Primer sequence.

Gene	Forward primer 5′-3′	Reverse primer 5′-3′
GAPDH	TCTCA TGCCA CTCGG CAGGA T	ACTAT TGACG TGGGT GTGCC G
TNF-α	CTCTC AGATG CGAAA CGTGA A	ATTCT ACTCC GGCAC GCGT
IL-6	ACTTC AGAAG CTCGG TCAGA	GCCTC AGGAT CTTAA AGC

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

Table II. Body weight and liver weight.

Group	n	Body weight (g)	Liver wet weight
Control	20	472.15±28.24	10.01±0.75
Model	20	675.32±39.73*	14.89±0.82*
AGEs inhibitor	20	569.71±31.25*#	11.46±0.66#

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

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 destructing 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.

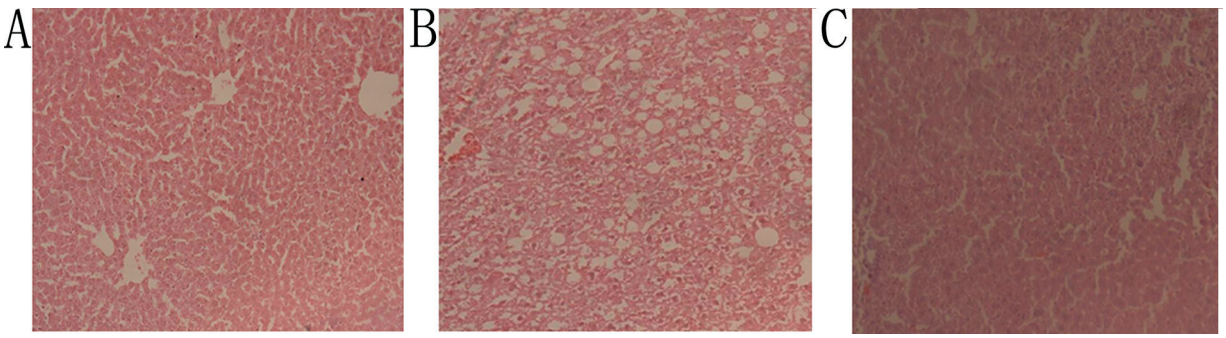

Figure 1. Rat liver tissues by HE staining (200 ×). ***A,*** control; ***B,*** model; ***C,*** AGEs inhibitor. AGEs: advanced glycosylation end products, HE: hematoxylin-eosin.

Table III. Blood fatty acid indexes.

Group	TC	TG	LDL-C	HDL-C
Control	1.21±0.09	1.23±0.18	0.71±0.09	0.86±0.11
Model	1.83±0.66*	2.65±0.75*	1.17±0.36*	0.51±0.08*
AGEs inhibitor	1.51±0.31*#	1.77±0.39*#	0.92±3.23#	0.78±0.09#

Note: *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group. TC: total cholesterol, TG: triglyceride, LDL-C: low-density lipoprotein, HDL-C: high-density lipoprotein, AGEs: advanced glycosylation end products.

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.

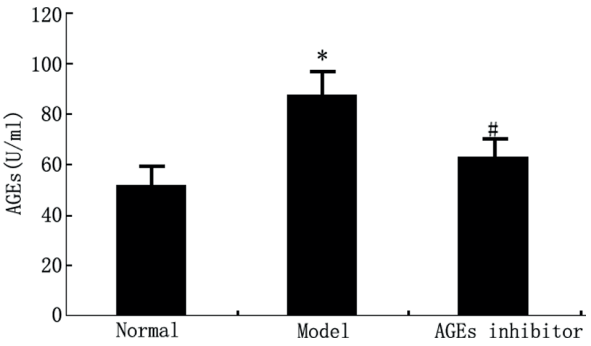

Figure 2. Serum AGEs level. *, $p<0.05$ compared to control group; #, $p<0.05$ compared to model group. AGEs: advanced glycosylation end products.

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].

Table IV. Liver function indexes.

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

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.

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[18,19]. 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 CCl_4, which can suppress the lipolysis of liver by altering mitochondria function, further aggravating fatty liver disease[16]. 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[17]. 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[20]. 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[21,22], 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

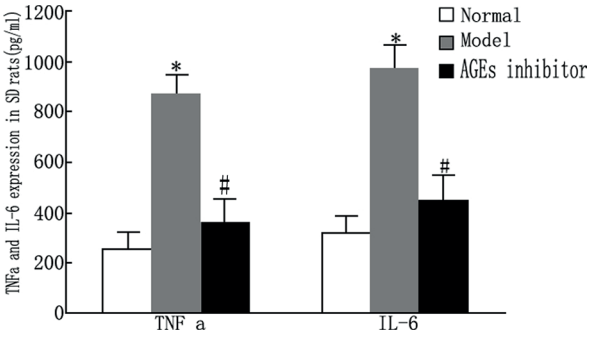

Figure 3. TNF-α and IL-6 mRNA levels in liver tissue. *, $p<0.05$ compared to control group; #, $p<0.05$ compared to model group. TNF-α: tumor necrosis factor α, IL-6: interleukin 6.

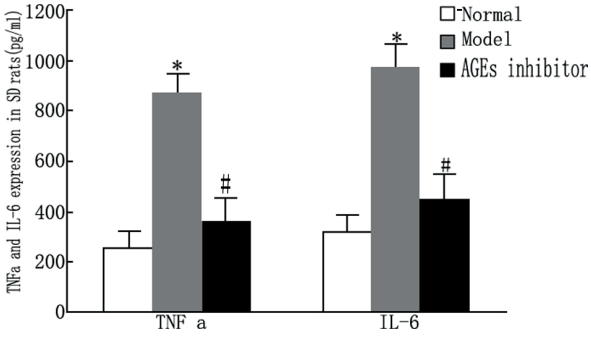

Figure 4. Serum TNF-α and IL-6 levels. *, $p<0.05$ compared to control group; #, $p<0.05$ compared to model group. TNF-α: tumor necrosis factor α, IL-6: interleukin 6.

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.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Qin XD, Qian Z, Vaughn MG, Trevathan E, Emo B, Paul G, Ren WH, Hao YT, Dong GH. Gender-specific differences of interaction between obesity and air pollution on stroke and cardiovascular diseases in Chinese adults from a high pollution range area: a large population based cross sectional study. Sci Total Environ 2015; 529: 243-248.
2) Chang CJ, Jian DY, Lin MW, Zhao JZ, Ho LT, Juan CC. Evidence in obese children: contribution of hyperlipidemia, obesity-inflammation, and insulin sensitivity. PLoS One 2015; 10: e0125935.
3) King WC, Chen JY, Bond DS, Belle SH, Courcoulas AP, Patterson EJ, Mitchell JE, Inabnet WB, Dakin GF, Flum DR, Cook B, Wolfe BM. Objective assessment of changes in physical activity and sedentary behavior: Pre- through 3 years post-bariatric surgery. Obesity (Silver Spring) 2015; 23: 1143-1150.
4) Federico A, Dallio M, Masarone M, Persico M, Loguercio C. The epidemiology of non-alcoholic fatty liver disease and its connection with cardiovascular disease: role of endothelial dysfunction. Eur Rev Med Pharmacol Sci 2016; 20: 4731-4741.
5) Xanthakos SA, Jenkins TM, Kleiner DE, Boyce TW, Mourya R, Karms R, Brandt ML, Harmon CM, Helmrath MA, Michalsky MP, Courcoulas AP, Zeller MH, Inge TH, Teen-LABS Consortium. High prevalence of nonalcoholic fatty liver disease in adolescents undergoing bariatric surgery. Gastroenterology 2015; 149: 623-634.
6) Pacifico L, Bonci E, Di Martino M, Versacci P, Andreoli G, Silvestri LM, Chiesa C. A double-blind, placebo-controlled randomized trial to evaluate the efficacy of docosahexaenoic acid supplementation on hepatic fat and associated cardiovascular risk factors in overweight children with nonalcoholic fatty liver disease. Nutr Metab Cardiovasc Dis 2015; 25: 734-741.
7) Celebi G, Genc H, Gurel H, Sertoglu E, Kara M, Tapan S, Acikel C, Karslioglu Y, Ercin CN, Dogru T. The relationship of circulating fetuin-a with liver histology and biomarkers of systemic inflammation in nondiabetic subjects with nonalcoholic fatty liver disease. Saudi J Gastroenterol 2015; 21: 139-145.
8) Luckhoff HK, Kruger 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.
9) Chou CY, Yang YC, Wu JS, Sun ZJ, Lu FH, Chang CJ. Non-alcoholic fatty liver disease associated with increased arterial stiffness in subjects with normal glucose tolerance, but not pre-diabetes and diabetes. Diab Vasc Dis Res 2015; 12: 359-365.
10) Basaranoglu M, Canbakan B, Yidiz K, Ceylan B, Baysal B, Uysal O, Senturk H. Fatty liver disease might increase the risk of abdominal operation in patients with fatty liver and the prevalence of cancer in firstdegree relatives. Turk J Gastroenterol 2014; 25 Suppl 1: 138-141.
11) Teichert T, Hellwig A, Pebler A, Hellwig M, Vossoughi M, Sugiri D, Vierkotter A, Schulte T, Freund J, Roden M, Hoffmann B, Schikowski T, Luckhaus C, Kramer U, Henle T, Herder C. Association between advanced glycation end products and impaired fasting glucose: results from the SALIA Study. PLoS One 2015; 10: e0128293.
12) Zhao DC, Lu HW, Huang ZH. Association between the receptor for advanced glycation end products gene polymorphisms and cancer risk: a systematic review and meta-analysis. J BUON 2015 20: 614-624.
13) Rempel LC, Finco AB, Maciel RA, Bosquetti B, Alvarenga LM, Souza WM, Pecoits-Filho R, Stinghen AE. Effect of PKC-beta signaling pathway on expression of MCP-1 and VCAM-1 in different cell models in response to advanced glycation end products (AGEs). Toxins (Basel) 2015; 7: 1722-1737.
14) Sebekova K, Stumer M, Fazeli G, Bahner U, Stab F, Heidland A. Is vitamin d deficiency related to accumulation of advanced glycation end products, markers of inflammation, and oxidative stress in diabetic subjects? Biomed Res Int 2015; 2015: 958097.
15) Chen PM, Gregersen H, Zhao JB. Advanced glycation end-product expression is upregulated in the gastrointestinal tract of type 2 diabetic rats. World J Diabetes 2015; 6: 662-672.
16) Chheda TK, Shivakumar P, Sadasivan SK, Chanderasekharan H, Moolemath Y, Oommen AM, Madanahalli JR, Marikunte VV. Fast food diet with CCl4 micro-dose induced hepatic-fibrosis--a novel animal model. BMC Gastroenterol 2014; 14: 89
17) Orman D, Vardi N, Ates B, Taslidere E, Elbe H. Aminoguanidine mitigates apoptosis, testicular seminiferous tubules damage, and oxidative stress in streptozotocin-induced diabetic rats. Tissue Cell 2015; 47: 284-290.
18) Kandel-Kfir M, Almog T, Shaish A, Shlomai G, Anafi L, Aviv C, Barshack I, Grosskopf I, Harats D, Kamari Y. Interleukin-1alpha deficiency attenuates en-

doplasmic reticulum stress-induced liver damage and CHOP expression in mice. J Hepatol 2015; 63: 926-933.

19) Tan DY, Shi HY, Li CP, Zhong XL, Kang M. Effect of nuclear factor-kappaB and angiotensin II receptor type 1 on the pathogenesis of rat non-alcoholic fatty liver disease. World J Gastroenterol 2015; 21: 5877-5883.

20) Praveen Raj P, Gomes RM, Kmar S, Senthilnathan P, Karthikehyan P, Shankar A, Palanivelu C. The effect of surgically induced weight loss on nonalcoholic fatty liver disease in morbidly obese Indians: «NASHOST» prospective observational trial. Surg Obes Relat Dis 2015; 11: 1315-1322.

21) Ganji SH, 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.

22) Gurel H, Genc H, Celebi G, Sertoglu E, Cicek AF, Kayadibi H, Ercin CN, Dogru T. Plasma pentraxin-3 is associated with endothelial dysfunction in non-alcoholic fatter liver disease. Eur Rev Med Pharmacol Sci 2016; 20: 4305-4312.