Liver fibrosis associated with adipose tissue and liver inflammation in an obesity model

Wiwit Ananda Wahyu Setyaningsih, MSc, Dwi Cahyani Ratna Sari, PhD, Muhammad Mansyur Romi, MSc, Nur Arfian, PhD

Department of Anatomy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Indonesia

ABSTRACT
Introduction: Obesity, the main risk factor for type 2 diabetes mellitus (T2DM), affects the secretion of various hormones that lead to change in metabolism. Visceral adipose tissue accumulation may contribute to Non-alcoholic Fatty Liver Disease (NAFLD) and induce liver injury. This study was aimed to investigate the association between adipose tissue inflammation and liver fibrosis.

Materials and methods: Wistar male rats (3 months old, 160-230 grams) were divided into 4 groups that consisted of six rats in each group. The obesity model was induced through the administration of high-fat diet for a month (OB1), two months (OB2), and four months (OB4). Standard chow was provided for the control group for four months. After the specified date the rats were euthanized and the liver and retroperitoneal white adipose tissue (RWAT) were harvested. We performed RT-PCR to assess the mRNA expressions involved in proinflammatory mediators, fibrosis and anti-fibrosis signaling. Sirius red staining was performed to assess liver fibrosis. Data were analyzed with SPSS 23 for Windows with significance set as \( p < 0.05 \).

Results: Obesity-induced high-fat diet stimulated an increase of body mass index (BMI) in the OB groups \((p<0.05)\) compared to the control group. Increased BMI was followed by upregulation of proinflammatory mediators (MCP-1, CD68, TLR4, and NfKB) of the RWAT and liver in the obese groups \((p<0.05)\), which promoted hepatic fibrosis in triad portal areas and upregulation of TGFβ \((p<0.05)\) mRNA expression as well as downregulation of HGF and c-Met \((p<0.05)\). In addition, hepatic ppET1 and EDNRB mRNA level expressions \((p<0.05)\) were obviously upregulated in the obese groups followed by downregulation of eNOS \((p<0.05)\) mRNA expressions.

Conclusion: Obesity enhanced inflammation in RWAT and was associated with inflammation and fibrosis of liver.

KEYWORDS:
Obesity; retroperitoneal white adipose tissue; liver injury; inflammation; fibrosis

INTRODUCTION
Obesity is associated with an increased risk for type 2 diabetes mellitus (T2DM) which is characterized by insulin resistance and hyperglycemia. Around 80% of people with T2DM are also obese. The prevalence of obese people has significantly increased during every decade. It was reported that about 30% of Americans were obese. It is estimated that the prevalence of diabetes mellitus in adults aged 20-79 years has reached 285 million and will increase to 439 million people globally in 2030. In obese people, the adipose tissues undergo hypertrophy and hyperplasia resulting in increased secretion of leptin, glycerol, adiponectin, non-esterified fatty acids (NEFA), various proinflammatory cytokines and chemokines, which produce lipotoxicity.

Obesity is a condition characterized by the presence of excess adipose tissue, and additionally, it consists not only of adipocyte cells but also stromal vascular fractions. Adipose tissue is an endocrine organ that produces hormones which will affect metabolism. Hypertrophy of fat tissue causes an imbalance of hormone secretion and secretion of various proinflammatory cytokines with various factors that cause insulin resistance. Hypertrophy of adipocyte cells triggers an increase in leptin secretion and a decrease in adiponectin secretion. In obesity there is an increase in leptin receptors mainly in the brain. Increased receptors and ligands from leptin will induce an increase in fatty acid oxidation, increased corticotropin secretion, increased thermoregulation, and various changes in the metabolism of the body through several signal transduction pathways. Hyperleptinemia increases the ability of leptin to bind to the promoter side of Peroxisome Proliferator-Activated Receptor-β (PPARβ) and Sterol Receptor Element Binding Protein-1c (SREBP-1c) that affect mitochondrial work functions.

Metabolic alteration due to obesity affects the endothelial function. An increase of various hormones, such as resistin and leptin, stimulates an increase of reactive oxygen species (ROS) and inflammatory cytokines in in vivo models of endothelial cell culture which then leads to an elevation of endothelin-1 (ET-1) release and extrication of proinflammatory mediators. Moreover, obesity reduced nitrite oxide (NO) bioavailability in endothelial cells resulting in endothelial dysfunction. It is known that obesity causes cardiovascular disease due to an elevation of ET-1. It has been observed that upregulation of the ET-1 serum correlates with the development of liver fibrosis in patients with non-alcoholic steatohepatitis (NASH). However, the impact of obesity induced liver injury has not been clearly understood.

Liver is one of the organs that severely suffers in obesity. Hepatosteatosis is a common feature of histological changes...
Liver fibrosis associated with adipose tissue and liver inflammation in an obesity model

due to an increase of fatty acid oxidation (FAO) and release of fatty acid (FA). Hepatocyte growth factor (HGF) is a mitogen that contributes to cell growth, cell motility, and morphogenesis. In conditions of liver cancer HGF/c-Met plays a role in the process of liver regeneration and inhibits cellular senescence. However, the relationship between liver fibrosis due to an increase in sterile inflammation caused by adipocyte cells has not clearly understood.

MATERIALS AND METHODS

Animal experiments

Wistar male rats (3 months old, 160-230 grams) were divided into 4 groups that consisted of six rats in each group. The groups were: control (Control, n=6), Obesity 1 month (OB1, n=6), Obesity 2 months (OB2, n=6), and Obesity 4 months (OB4, n=6). The control group received AIN76A, while the obesity groups were fed High-Fat Diet (HFD) for 1 month (OB1), 2 months (OB2), and 4 months (OB4). After the specified date, the rats were injected using the lethal dose of ketamine (Ivanes, 250201) and euthanized. Then, their liver and retroperitoneal adipose tissue (RWAT) were harvested. Finally, the harvested organs were immersed in RNA Later in RNA assay (Ambion, 7021) and kept in Neutral Buffer Medium for paraffin making and histological examination.

The obesity criteria was determined according to the body mass index (BMI; weight (grams)/length (cm²)) and Lee Index formula (V/weight (grams)/nasoanl length (mm)). This research was based on the guidelines for animal care of the Universitas Gadjah Mada and we obtained a license from Ethical Committee of Medical Research and Health of Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada with ethical expediency number KE/FK/0490/EC/2018.

RNA Extraction, cDNA synthesis and Reverse transcriptase-PCR (RT-PCR)

Liver and RWAT was extracted using Genezol RNA Solution (GENEZol™, Cat. No. GZR100) based on the protocol from the manufacturer. The RNA concentration was quantified using nanodrop. The RNA was synthesized into cDNA using Revertra-Ace kit (Toyobo, Cat. No. TRT-101), random primer (TAKARA, Cat. No. 3801), and deoxyribonucleotide triphosphate (dNTP) (Takara, Cat. No. 4030), with PCR condition: 90°C for 10 min, 42°C for 60 min, and 99°C for 5 min.

Reverse Transcriptase-PCR was performed to examine these following genes with specific primer. The inflammatory mediators were assessed using cluster of differentiation 68 (CD68) (forward 5'- TGGTCCTTTCACACAGCAG-3' and reverse 5'- AAGAGAGACGATGCGCCGAG-3'), nuclear factor kappa-B (NFKB) (forward 5'- CACTCTCCTTTGAGGGT-3' and reverse 5'- TGGATAAGAGCCTTACG-3'), Toll-like receptor 4 (TLR4) (forward 5'- CAGGGACACGAGGCTCTAACC-3' and reverse 5'- CTGGTCCCTGTAGGCTTTG-3'), Monocyte Chemoattractant Protein-1 (MCP-1) (forward 5'- GCTTGAATTTTGTCAAAGCTC-3' and reverse 5'- ACAGAAGTGGCTGGTTGCT-3'), Fibrosis marker, Transforming Growth Factor Beta (TGFβ), was assessed using the following primers (forward 5'- CGAGGTGACCTGGCCACCACATC-3' and reverse 5'- GCTTCAACCTTGGGGTGACC-3'). The mRNA expression of Hepatocyte Growth Factor (HGF) was assessed using (forward 5'- ACAGCTTTTCTTCTGGAGC-3' and reverse 5'- TGGCGGATATTCCTTCGGC-3') and cMET (forward 5'- CCAAGCCGGATGTACAGTA-3' and reverse 5'- GCAGGCTCTAATGAAAACAGT-3'). The mRNA expression of endothelial nitrite oxide synthase (eNOS) (forward 5'- CCGGCGCTACAGAAGT-3' and reverse 5'- AGTCCAGGAGTGAAT-3'), ppET-1 (forward 5'- GTCGTCCTGTTATGGACAG-3' and reverse 5'- ACTGGCTGTCGCCCTTG-3'), and Endothelin receptor B (EDNBR) (forward 5'- TCTCAGGCTTTGTCCGAGC-3' and reverse 5'- CGCCGTTTTAGCTTGGCA-3') was performed to assess the endothelial dysfunction. The housekeeping gene, β-actin (forward 5'- GCAGATGTTGATCGAAGCAG-3' and reverse 5'- GGTGTAAGAGCAGGCTCGA-3'), was used to normalize the expression.

The cDNA was mixed with Taq Master Mix (Promega, GoTaq Green, MT122) and primers, then incubated in 94°C denaturation for 10 s, annealing at 60°C for 30 sec and extension 72°C for 1 min final extension phase end with the conditions of 72°C for 10 minutes for 35 cycles. The PCR products were separated using 2% agarose gel along with 100 bp DNA ladder (Bioron, Germany, Cat. No. 306009). The expression of the genes was quantified with a densitometry analysis using the ImageJ software.

Immunohistochemical (IHC) staining of CD68 and MCP-1

The liver and RWAT of the rats were embedded in paraffin blocks and cut into 4 µm in thickness. Then the slides were deparaffinized using xylene and rehydrated using 100%, 90%, 80%, and 70% alcohol, followed with antigen retrieval and blocking peroxidase using H2O2 3% in PBS solution. The slides were then incubated using background sniper, rabbit 1st monoclonal antibody MCP-1 (Abcam®, ab25124; 1:200) and mouse 1st polyclonal antibody anti-CD68 (Abcam®, ab955; 1:300), TrekAvidin-HRP, 2ndantibody Trekkie Universal Link (Biocare Medical®, STUHRP700L10), and diaminobenzidine tetrahydrochloride (DAB) (Biocare Medical®, STUHRP700L10). The macrophage number was quantified using ImageJ software, examined with light microscope (Olympus CX22®), and portrayed with the Opitolab software with 400x magnification.

Statistical analysis

The SPSS 23 software for windows was used for the analyses of data. Data normality test were conducted using Shapiro-Wilk and One-Way ANOVA for normal data distribution. The p-value less than 0.05 (p<0.05) was considered as statistically significant.

RESULTS

High-fat diet enhanced obesity and increased of SGOT level

We were able to show that high-fat diet feeding significantly induced obesity in the obese groups compared to the control group. The increased BMI was shown in the OB1 group (0.41±0.01; p=0.009), OB2 (0.57±0.02; p=0.047), and OB4 (0.61±0.03; p=0.028) compared to the control group. Meanwhile, determination of obesity according to the Lee Med J Malaysia Vol 76 No 3 May 2021
Index formula demonstrated that the obese groups which consisted of OB4 (2.48±0.04; \( p = 0.002 \)), OB2 (2.41±0.03; \( p = 0.001 \)), and OB1 (2.34±0.01; \( p = 0.000 \)) groups had higher scores compared to the control group (2.08±0.04). However, this alteration was not sufficient to induce an elevation of Serum Glutamic Oxaloacetic Transaminase (SGOT) level (\( p > 0.05 \)) in the obese groups.

**Long-term obesity induced retroperitoneal adipose tissue inflammation**

Regarding obesity stimulated inflammation of RWAT, we found that the mRNA expressions of MCP-1, CD68, TLR4, and NFkB mRNA expressions were significantly higher in accordance with an increase of BMI. The elevation of MCP-1, CD68, TLR4, and NFkB was obviously seen in the OB4 group (\( p<0.05 \)). Moreover, the alteration of MCP-1 and CD68 were followed by an upregulation of protein expression as shown by the immunohistochemistry staining (Figure 2).

**Obesity induced hepatic inflammation**

The increase of the inflammation in RWAT was followed by an increase of inflammatory mediators in the liver which was clearly shown in an elevation of the TLR4 and CD68 mRNA expressions compared to the control group (\( p<0.05 \)). The mRNA expression of TLR4 was markedly increased in the OB4 group (2.08±0.37; \( p=0.002 \)) which was then followed by OB2 (1.52±0.35; \( p=0.022 \)) and OB1 (1.39±0.05; \( p=0.019 \)) compared to the control group (0.62±0.20). In addition, the CD68 mRNA expression and the macrophage number were significantly upregulated in the obese groups which was significantly seen in the OB4 group with (2.12±0.09; \( p=0.006 \)) and (32.27±1.81; \( p=0.000 \)), respectively, compared to the control group (1.46±0.02) and (23±1.17), respectively.

**Liver fibrosis occurred in obese groups**

Subsequently, we assessed the histological changes which involved markedly increased areas of fibrosis in the portal
Liver fibrosis associated with adipose tissue and liver inflammation in an obesity model

**Fig. 3:** Obesity triggered hepatic inflammation. A-B. Upregulation of CD68 and TLR4 mRNA expressions using Reverse Transcriptase PCR (RT-PCR) in obese groups. C. Immunohistochemistry staining of CD68 protein expression in the liver with 400X magnification.

*\( p<0.05 \) vs Control, **\( p<0.01 \) vs Control, ‡ \( p<0.05 \) vs OB1, ∎∎ \( p<0.01 \) vs OB2.

**Fig. 4:** Obesity provoked liver fibrosis. A-B. Sirius Red staining in 400X magnification and fibrosis area fraction quantification. C-E. The representative picture and quantification of TGFβ, HGF, and c-Met mRNA expressions. *\( p<0.05 \) vs Control; † \( p<0.05 \) vs OB1. PV (portal vein), A (hepatic artery), BD (bile duct).
The obesity groups demonstrated significantly higher ET-1 and EDNRB mRNA expression in the liver compared to the control group that was followed by the reduction of eNOS mRNA expression. The ET-1 was known as a potent vasoconstrictor agent markedy increase in OB4 group (0.77±0.14; p=0.000) compared to the control group (0.32±0.09). Interestingly, the EDNRB was remarkably upregulated in the OB4 group (0.53±0.01; p=0.044) compared to the control group (0.31±0.09).

In addition, the eNOS mRNA expression was downregulated in the OB groups. However, this alteration was obviously seen in the OB4 group (p<0.05). In addition, the upregulation of vasoconstrictor agents promoted vasodilatation disturbance which was shown by downregulation of eNOS at the same time (p<0.05).

**DISCUSSION**

This study reveals that the upregulation of inflammatory mediators in adipose tissue during obesity which leads to liver fibrosis. Obesity has long been correlated with liver steatosis, insulin resistance, and inflammation. However, 4 months of high-fat diet feeding was not adequate to induce liver steatosis. The extrication of cytokines and chemokine released by enlarged white adipose tissue leads to sterile inflammation and insulin resistance. The inflammatory mediators released by adipose tissue were upregulated in the obese groups compared to the control group (Figure 2). Adipocyte tissue plays an important role as a bridge connecting between immunological properties and metabolic alteration. Macrophages are thought to contribute in an increase of inflammation, since they are recruited from the bone marrow and form crown-like structure (CLS). Then, macrophages surround the moribund adipocyte cells. Consequently, more than 50% of immune cells in adipose tissue are macrophages. Meanwhile, approximately less than 10% of immune cells in lean adipose tissue are macrophages.
Liver fibrosis associated with adipose tissue and liver inflammation in an obesity model

Our study revealed that upregulation of TLR4, NFκB, CD68, and MCP-1 signalling in adipocyte tissue that leads to low-grade chronic inflammation (Figure 2). The TLR4 in adipose and other tissues, including liver and muscles, recognized the free fatty acid (FFA) secreted by hypothyroid adipocyte cells, then caused binding to myeloid differentiation (MD2) and CD14 complex. Next, the dimerisation of this complex stimulates the adaptor protein, myeloid differentiation88 (Myd88) to activate the NFκB to produce various proinflammatory cytokines and chemokines, including MCP-1 and TNF-α. Infusion of lipids dramatically induced upregulation of NFκB, IL6, and MCP1 protein and mRNA expressions in adipose tissue as shown in LPS-induced activation of NFκB.20,21

We highlight that the upregulation of inflammatory mediators in the retroperitoneal adipose tissue which is in accordance with upregulation of TLR4 and CD68 in the liver. It has been known that obesity elevates hepatic NFκB activity that leads to hepatic inflammation due to an increase of FFA. In addition, it alters hepatic metabolism resulting in NAFLD. Kupffer cells play an important role in clearing damage-associated molecular patterns (DAMPs). FFA recognized by Kupffer cells triggers sterile inflammation and activates pattern recognition receptors (PRRs), such as TLR4 in the hepatocyte cells.22,23 Deficiency of TLR4 is associated with decrease of atherosclerosis and vascular inflammation.24 The number of macrophage-expressed CD68 was higher in the obesity groups compared to the control group. It seems that inflammation in the liver aligns with inflammation in RWAT, although we cannot establish direct evidence in this study. Shared circulation models, such as parabiosis may provide a much more complete study for elucidating the correlation between the two organs in the next future research. It obviously can be seen in the fibrotic liver induced by 4 months of high-fat diet feeding that aligns with RWAT.

As the Kupffer cells were activated due to inflammation, the hepatic stellate cells (HSCs) become activated which is mediated by TGFβ leading to liver fibrosis and increased secretion of extracellular matrix. Activation of the TGFβ has been considered to contribute to the development of fibrosis in the kidney, lungs, heart, and other organs. The adipocyte-derived hormone, such as leptin, contributes to sensitize the Kupffer cells producing collagen.25 We found that augmentation of TGFβ is followed by the downregulation of anti-fibrosis also assessed by the anti-fibrotic factor, HGF, and its receptor, c-Met during obesity. Our results demonstrate that obesity promotes augmentation of TGFβ mRNA expression and portal triad hepatic fibrosis which was followed by downregulation of HGF and c-Met as anti-fibrotic factors. HGF/c-Met plays an important role in contributing to lipid accumulation in the liver. HGF transgenic mice demonstrated low lipid accumulation through microsomal transfer protein (MTP) and apolipoprotein B (ApoB).26 In this research, the findings suggest that FFA which is produced by white adipose tissue and is transported to the liver through the hepatic artery and portal vein. Then, the circulating mesenchymal cells, cholangiocytes, resident fibroblast, or even endothelial cells may undergo myofibroblast formation that contributes to the development of triad portal fibrosis during obesity.27,28

The transformation of endothelial cells into mesenchymal cells known as endothelial to mesenchymal transition (EndoMT) is promoted by upregulation of TGFβ and perhaps by ET-1.29 The endothelin system is thought to contribute to the activation and proliferation of HSCs and collagen synthesis. The ET-1 was released by hepatic vascular smooth muscles, endothelial cells, stellate cells and mesenchymal cells during the injury process. Upregulation of serum ET-1 level was markedly correlated to the severity of liver fibrosis in NASH patients and this elevation was accomplished by an increase of ALT serum level.30 More than 80% of ET-1 was secreted abuminally toward the vessels, and in consequence, the ET-1 promotes endothelial dysfunction, cardiovascular disease, and portal hypertension.30,31 Interestingly, we found that in obesity, the mRNA expression of EDNRβ was upregulated. The EDNRβ expressed by vascular smooth muscle cells increased in cerebral ischemia, subarachnoid hemorrhage, and ischemia-reperfusion injury.32–34 The EDNRβ mRNA and protein expressions were upregulated in intestinal mucosa of obese rats due to inflammation, including in the liver.35 We hypothesize that upregulation of pET-1/EDNRβ promotes liver fibrosis in obesity-induced rats.

Imbalance of vasoconstrictors and vasodilators was markedly observed in the OB4 group which was shown by downregulation of eNOS. In obese patients there are decreasing bioavailability of NO in small vessels which promotes the generation of free radicals and pro-inflammatory mediators. Endothelial dysfunction promoted deterioration of eNOS due to decrease of phosphorylation of eNOS in serine 1177 in the blood vessels of obese patients which was accompanied by an increase of proinflammatory mediators. Therefore, we suggest that the downregulation of hepatic eNOS was associated with an elevated level of pro-inflammatory mediators. We highlight that adipose tissue inflammation may be associated with inflammation and fibrosis in the liver which may be mediated by ET-1 and TGF-B1 signalling.

We highlight here that adipose tissue inflammation directly promotes liver inflammation in high-fat diet feeding for 4 months and led to the development of liver fibrosis that was mediated by TGFβ and endothelial dysfunction.

CONCLUSION

In conclusion, our results indicated that chronic obesity contributes to the development of liver fibrosis through upregulation of TGFβ and downregulation of HGF that caused by sterile inflammation. Furthermore, this condition promotes endothelial dysfunction of the liver.

ACKNOWLEDGMENTS

The authors would like to extend our thanks to Mr. Mulyana for all of his help as the laboratory assistant. This research was funded by Hibah Peningkatan Kapasitas Dosen Muda, Universitas Gadjah Mada (3943/UN1/DITLIT/DITLIT/LT/2019), and Dana Masyarakat, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (UPPM//361/M/05/04/05.18).
REFERENCES


