TMAO Stimulate Inflammation and Lipid Accumulation via NF-κB Signaling Pathway in Nonalcoholic Fatty Liver Disease

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Research

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Abstract

Background

Nonalcoholic fatty liver disease (NAFLD) is a common disease and it is commonly associated with obesity. Trimethylamine N-oxide (TMAO) is a metabolite of intestinal flora generated in liver by flavin-containing monooxygenase 3 (FMO3), which has been widely studied in cardiovascular diseases and obesity. However, the mechanism of TMAO reacted on liver remains unclear. This study aimed to determine TMAO activated hepatitis inflammation and lipid accumulation which was associated with nuclear factor kappa B (NF-κB) signaling pathway in vitro.

Results

The present study showed that TMAO in 50μM markedly increased the LO2 cells function and decreased the cells inflammation. However, over the concentration of 200μM in TMAO, cells inflammation was increased and function was declined apparently. In addition, TMAO promoted lipid accumulation. Mechanistically, this change was accompanied by the activation of NF-κB signaling pathway. Furthermore, blocking NF-κB by SN50 was significantly increased in lipid accumulation and apoptosis. SN50 was markedly decreased the protein expression stimulating by TMAO.

Conclusions

Overall, the result suggested that TMAO promotes cells inflammation and lipid accumulation in hepatocytes and it might be associated with NF-κB signaling pathway.

Background

Nonalcoholic fatty liver disease (NAFLD), that has been recognized as an increasing public healthy burden with a morbidity of up to 20% in the worldwide, is characterized by lipid accumulation and inflammation deteriorated into fibrosis and cirrhosis [1, 2]. It is generally accepted that fibrosis is considered reversible, while the pathological process of end-stage cirrhosis is considered irreversible [3]. One of the causes of NAFLD is obesity [4]. In some treatments, patients were required to lose weight to reduce the level of obesity [5]. Obesity is become a healthy concern in the world and it always accompanies with unhealthy living habit, such as high-fat diet which promotes the fatty acid production [6]. Oleic acid (OA) was a kind of short-chain fatty acids. Over accelerating OA can contribute to the aggravation of the hepatic steatosis in vivo that deteriorates the progression of NAFLD [7]. In some studies, NAFLD model was used to establish by OA-stimulating method in vitro [8, 9].

In recent years, researches have indicated that the intestinal microbiota connect with multiple body organs and the association between microbiomes and obesity has been pointed out [10]. The gut-liver axis is established by portal vein which regulated the transport of intestinal metabolites to liver. Intestinal microbiota participates not only the immune system and energy metabolism, but also glucose and lipid

homeostasis of liver. For example, recent studies reveal that the complicated relationship between gut microbial organisms and host is associated with disruptions of choline metabolism [11]. Trimethylamine N-oxide (TMAO), is produced by dietary choline, betaine and L-carnitine which are metabolized to trimethylamine (TMA) as a carbon fuel source by intestinal microbiota. Then, TMA is transported by portal circulation to the liver and converted into TMAO by a family of enzymes, host hepatic flavin monooxygenases (FMOs) [12]. TMAO was confirmed to aggravate cardiovascular disease and related to obesity. However, there are few of researches to dig deeper into the connection between TMAO and NAFLD. TMAO As a representative metabolite, TMAO regulates obese-related diseases, such as NAFLD and insulin resistance [13]. TMAO also increases the inflammation response and injury in liver [14]. The previous studies suggest us that TMAO may regulate the liver inflammation and lipid metabolism. In addition, in vascular diseases, TMAO promoted vascular calcification and inflammation through NF-κB signaling pathway [15, 16]. In hepatocyte, NF-κB is a critical protein in the regulation of inflammation, especially p65, which promote proinflammatory generation [17].

In this study, we explored whether the effect of TMAO in LO2 cells is associated with cell apoptosis, inflammation and lipid accumulation. To verify whether the mechanism of TMAO regulated in fatty liver, we established through OA-stimulated in LO2 cells.

Results

TMAO is closely related to obesity in clinical samples

To understand the correlation between TMAO and obesity, 30 volunteers in different body mass index (BMI) were collected to test the TMAO concentration. According to WHO standard, BMI was classified into normal (20.0-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2), mild obesity (30.0-34.9 kg/m^2) and moderate obesity (35.0-39.9 kg/m^2). We observed that TMAO was increased significantly with the development of obesity in comparison to normal group, especially in moderate obesity (Fig. 1). It was indicated that increasing of TMAO concentration is related to the severity of obesity.

TMAO inhibited the proliferation of LO2 cells

In order to further investigate whether different concentration of TMAO affects the proliferation and migration of LO2 cells, we used cck-8 assay and wound healing assay to estimate the following indicators. LO2 cells were treated with TMAO in 0, 50, 100, 200, 400 and 800μM. Wound healing analysis had shown that TMAO in 50, 100μM had complete cells morphology but TMAO destroyed the cell shapes over 200μM (Fig. 2A-B). Also, TMAO in 50 and 100μM did not destroy the migration of LO2 cells, but it did destroy in 200, 400, 800μM in comparison to control group. Furthermore, 50μM TMAO group had better wound healing ability than that in other groups. The toxicity of TMAO was examined by CCK-8 assay. As illustrated (Fig. 2C), we found that 205μM was the half-maximal inhibition concentration (IC_{50}) of TMAO-treated LO2 cells after 24 hours. In particular, compared with control group, 50μM TMAO promoted cells proliferation, while other groups, such as 100, 200, 400 and 800μM, had significant inhibitory with the

increase of TMAO concentration. At the same time, the inhibition of cell proliferation in high concentration groups, 200, 400 and 800μM TMAO, was significantly higher than that in low concentration groups, 50 and 100μM TMAO, which was in comparison to control group. These results indicated that TMAO in 50μM significantly ameliorated LO2 cells proliferation and in 200, 400 and 800μM could destroy it.

TMAO inhibited LO2 cell cycle and promoted apoptosis, and inflammatory cytokines expression

To further verify the ability of TMAO to induce apoptosis and block cycle cycle, the flow cytometry assay was used. Apoptosis analysis of TMAO treated LO2 cells were stained with Annexin V-FITC and PI. TMAO had significant increase in 100, 200, 400 and 800μM concentrations of total apoptosis rates (including early apoptosis and late apoptosis), compared with the control group (Fig. 3A). Furthermore, the cell cycle analysis also had significant increase in 100, 200, 400 and 800μM concentrations in total cell cycle G0/G1 distribution (Fig. 3B). Meanwhile, IL-1β, IL-6, TNF-α, AST and ALT were detected. After exposure with TMAO, the levels of IL-1β, IL-6, TNF-α, AST and ALT was significant increased, especially in 400 and 800μM (Fig. 3C-D).

TMAO inhibited LO2 cells steatosis generation

According to the above, TMAO 100μM was lower damage than higher concentration groups so that we used 100μM TMAO and 40μM oleic acid to testify TMAO function in liver. Oleic acid, as a free fatty acid used into steatosis model, obviously promoted the apoptosis and increased in ALT and AST (Fig. 4A-B). It was found that compared with OA group, TMAO could inhibit the apoptosis in TMAO and OA-treated group. We investigated the effect of TMAO on OA-induced steatosis using the analysis of ALT and AST levels. The result has revealed a significant inhibition in steatosis compared with OA group. Next, we determined the levels of IL-1β, IL-6 and TNF-α. The result has showed that OA promoted the level of IL-1β, IL-6, TNF-α. Respectively, when TMAO was added, IL-1β was increased, but IL-6 was no significant change and TNF-α was decreased.

To further confirm whether TMAO on hepatitis steatosis were associated with autophagy, we detected the protein expression level of IκBα, NF-κB p65, ABCG5, SERBP1 and PPAR gamma through western blotting (Fig. 5). Compared with control group, both the protein expressions of IκBα, NF-κB p65, ABCG5, SERBP1 and PPAR gamma were significantly increased in TMAO and OA group. However, the protein expressions of NF-κB p65, ABCG5, PPAR gamma and SERBP1 were decreased when TMAO and OA group was added with SN50, an NF-κB p65 inhibitor, which could block the NF-κB signaling pathway.

TMAO reduced hepatitis steatosis and inflammation response through NF-κB signaling pathway

To further confirm TMAO decreased steatosis through NF-κB signaling pathway, we used the SN50 to stimulated LO2 cells. Compared with control or TMAO groups, SN50 added with TMAO and OA was marked increased lipid accumulation in LO2 cells (Fig. 6A). Moreover, we also found that TMAO could

significantly promote cells apoptosis in steatosis while SN50 was added (Fig. 6B). Expectantly, NF-κB signaling pathway was closed related to TMAO-stimulated in liver cells, especially in hepatic steatosis.

Discussion

NAFLD has the characteristics of hepatic steatosis in the liver that is often associated with obesity. The growing prevalence of NAFLD has a total of 25.2% and become the most common liver diseases in the world [18]. TMAO was widely studied in cardiovascular but it had barely research in liver. In the present study, we aimed to determine that TMAO produced effect on inflammation and lipid accumulation in fatty liver. Furthermore, we also examined the mechanism of TMAO-activated LO2 cells through NF-κB signaling pathway.

Dietary habits are a key immediate cause of NAFLD, especially long-term excess calorie intake, such as carbohydrates and fats which could result in lipid metabolism disorder [19]. In the recent years, gut microbes were confirmed with connected with multiple organs and played a critical role in metabolism and chronic diseases [20]. TMAO is oxidized in the liver, and its precursor, TMA, is generated by gut microbes [21]. TMAO was demonstrated that was related to various diseases, such as cardiovascular disease, obesity and type 2 diabetes [22]. However, the mechanism of the relationship between TMAO and NAFLD remains to be clarified. Luigi B et al. reported that the association between NAFLD and TMAO level in adult subjects was indicated that increased TMAO level was contributed to the identification of early NAFLD [23]. NAFLD is associated with chronic inflammation, such as IL-1β, IL-6 and TNF-α that are belong to the category of proinflammatory cytokines [24]. Hepatitis injury indicators, AST and ALT, increase with the degree of chronic inflammation [25]. Besides, lipid metabolism in NAFLD is also affected. NF-κB signaling pathway is activated during hepatocyte injury which can further promotes the generation of proinflammatory cytokines [26]. In addition, NF-κB signaling pathway also controls lipid accumulation [27, 28]. Chunxiao Li et al. reported that Allyl isothiocyanate ameliorated the lipid accumulation of mouse hepatocytes in virto through inhibiting the NF-κB signaling pathway [25]. Moreover, NF-κB signaling pathway is considered to play a key role in apoptosis that promote the release of inflammatory factors, including IL-6, TNF-α, IL-1β, CRP and PAI-1[29].

Numbers studies have suggested that TMAO has the accelerated effect in inflammation. Our finding has showed that different concentrations of TMAO have various effects and it contributed to the level of obesity. In addition, the further vitro study in LO2 cells was demonstrated that with the increasing concentration of TMAO, hepatitis inflammation was upregulated in IL-1β, IL-6, TNF-α and the hepatitis injury was also increased in AST and ALT. Intense inflammatory response leaded to block the cell proliferation and increase hepatocytes apoptosis. It meant that the higher concentration of TMAO was harm to hepatocytes. Of interest, the concentration of 50μM TMAO, which was a low concentration of the experimental group, was opposite experimental results. It was suggested that low concentration of TMAO could increases the activity of hepatocytes and enhance the ability of hepatocytes to resist the risk of inflammation and lipid accumulation.

Fatty liver is caused by excessive lipid accumulation in hepatocyte. In this study, we established the fatty liver disease model in vitro through OA induced. The result was showed that OA caused high apoptosis and hepatocytes injury but it caused low level of inflammation. Apoptosis is widely known as programmed cell death that occurs through the regulation of genes and their products in cells [30]. However, TMAO caused the inflammation and low level of hepatocytes apoptosis and injury. When hepatocytes were stimulated with TMAO and OA at the same time, the hepatocytes was decreased in apoptosis and liver injury than OA-induced in hepatocytes. Thus, TMAO was related to protect liver from free fatty acid damaged and strengthen liver defense ability. Moreover, OA-induced hepatocytes were significantly increased the lipid accumulation. When adding TMAO and OA in hepatocytes, cells steatosis was more serious, thereby lipid accumulation function of hepatocytes was aggravated by TMAO.

To further elucidate the potential mechanism based on TMAO acting on hepatocytes, we focused on NF-κB signaling pathway meditated inflammatory activation. ABCG5, PPAR gamma and SERBP1 are crucial proteins of lipid metabolism in liver so that they are widely used in hepatitis steatosis researches[31-33]. Herein, the protein expressions of ABCG5 and PPAR gamma were activated by TMAO or OA. Another experimental group was suggested that TMAO assisted with OA to aggravate the steatosis in hepatocytes, accompanied by the increase of ABCG5, SERBP1 and PPAR gamma. It was mentioned that TMAO was associated with lipid metabolism and could assist with free fatty acid, such as OA, to up-regulate steatosis in hepatocytes. Of interest, NF-κB p65 was activated in OA-stimulated group, which confirmed to the association with NF-κB signaling pathway and lipid accumulation in NAFLD. In our study, NF-κB p65 and IκBα was regulated by SN50. IκBα, an upstream protein of NF-κB signaling pathway, was demonstrated to be related with inflammation [34]. SN50 decreased the protein expressions of the NF-κB p65 and IκBα, and it also inhibited the expression of lipid metabolism protein in ABCG5, PPAR gamma and SERBP1. SN50 is an inhibitor of NF-κB signaling pathway, and it was verified to block the NF-κB signaling pathway about inflammation response [35]. In addition, TMAO could intensify steatosis in hepatocytes through NF-κB signaling pathway because SN50 significantly decreased the function of lipid accumulation caused by TMAO. In general, our result was indicated that TMAO induced inflammation response and lipid accumulation through NF-κB signaling pathway.

To dig deeper into the association between TMAO and NF-κB signaling pathway, the animal experiment and further study about the TMAO's effect of inflammation should be taken. Animal experiment has better explanation of TMAO effect to liver in vitro. In addition, the detailed relation between TMAO and inflammation need to be explored.

Conclusion

Collectively, our study showed that TMAO could activate the inflammation response and lipid accumulation in LO2 cells. However, TMAO ameliorated cell apoptosis and injury in OA-treated LO2 cells. The TMAO effect in LO2 cells was associated with NF-κB signaling pathway. Our finding indicates that low concentration of TMAO protects liver from fatty liver diseases and TMAO accumulated in high levels may aggravate the progress of fatty liver.

Methods

TMAO concentration was measured in volunteers

In order to determine the correlation between obesity and TMAO, 30 volunteers were from Puning People's Hospital. All subjects including patients of outpatient clinic and healthy volunteers provided informed consent. This study was approved by the Hospital Ethics Committee.

30 subject samples were measured the concentration of TMAO in blood by TMAO assay kit (Mlbio, China). The OD value was measured with microplate reader at 450 nm. All the operations were carried out according to the manufacturer's instructions.

Cell Culture and Treatment

The normal human hepatocyte cell line LO2 purchased from the Type Culture Collection of the Chinese Academy of sciences (Shanghai, China) was cultured in Dulbecco's Modified Eagle Medium (HyClone, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (100U/ml penicillin and 100μg/ml 1% streptomycin) at 37°C in a 5% CO_2 humidified atmosphere.

Cell migration assay

Cells were incubated into 6-well plates and stimulated with TMAO in 0, 50, 100, 200, 400 and 800μM in 24 hours. The cells in other plates were stimulated with TMAO and/or OA in 24 hours. Straight scratches were drawn using a 200-μL sterile pipette tip in center of wells. Then, Images were captured at 0, 6, 12 and 24 hours.

Cell proliferation assay

The cells were seeded into 96-well plates in triplicate and stimulated with TMAO in 0, 50, 100, 200, 400 and 800μM. The cells in other plates were stimulated with TMAO and/or OA in 24 hours. The Cell Counting Kit-8 (CCK-8, KeyGEN, China) solution was added to each well and detected at 450nm. CCK-8 assay was used in LO2 cells of IC_{50} and cytotoxic testing.

Biochemistry

To further characterize hepatitis function and inflammation, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were determined using commercial assay kits (Mindary, China). The proinfammatory cytokines, including interleukin- 1β (IL-1β), interleukin- 6 (IL-6) and tumor necrosis fator-α (TNF-α), was measured using ELISA kits (Neobioscience, China). The OD value was measured with microplate reader at 510 nm. All the experiments were performed according to the manufacturer's instructions.

Cell Apoptosis

After 24 hours exposure to TMAO, cells were harvested and resuspended in 500μL of binding buffer containing with 5μL annexin V-FITC and propidium iodide (PI, KeyGEN, China), then detected by flow cytometry (FCM).

Cell Cycle Analysis

After 24 hours exposure to TMAO, cells were harvested, washed with precooled phosphate buffered saline (PBS), and fixed in precooled 70% ethanol overnight. Then, the cells were wash with PBS and added in staining buffer with propidium iodide supplemented with RNase A (KeyGEN, China) at room temperature in the dark for 30 min. The samples were detected by FCM.

Western Blot Analysis

LO2 cells were extracted with RIPA buffer and centrifuged at 1,4000×g for 30 min by centrifuge (JiDi Instrument, China). Sediments were discarded and the protein concentrations were measured by BCA protein assay. Equal amounts of proteins were separated via 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel and transferred onto a polyvinylidene fluoride membranes. The membranes were blocked and primary antibodies specific were used included anti-NF-κB p65, anti-the ATP-binding cassette subfamily G member 5 (ABCG5), anti-sterol regulatory element binding protein 1 (SREBP1), anti-peroxisome proliferator activated receptor gamma (PPAR gamma) and anti-GAPDH (Cell Signaling Technology, USA) antibodies.

Oil Red O Staining

LO2 cells were incubated overnight in 6-well plates and treated with TMAO and/or OA. After 24 hours, the cells were washed with PBS, fixed with 70% ethanol, and stained with 0.5% Oil red O (Phygene, China). The stained lipid droplets within cells were visualized by light microscope and photographed under a light microscope.

Statistical analysis

All statistical analyses were expressed using GraphPad Prism 6.5 and were presented as the mean ± standard error of at least three independent experiments. To examine the statistical significance, the data were performed using a Student's unpaired *t*-test. Differences with P values < 0.05 were considered significant.

Declarations

Ethics approval and consent to participate

This reseach protocol was approved by the Ethics Committee of Puning People Hospital. All studies were conducted in accordance with relevant regulations. Ethics number: No.2021-03

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interest.

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Authors' contributions

ZC and JZ developed the idea, designed the study, analyzed the data and drafted the manuscript. JZ, QC, XC, JC and RX collected the samples. TL and XW designed the study and analyzed the results. ZC, JC and JZ reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Consent for publication

All authors of this manuscript provided consent for publication.

Competing interests

The authors declared no competing interests.

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Figures

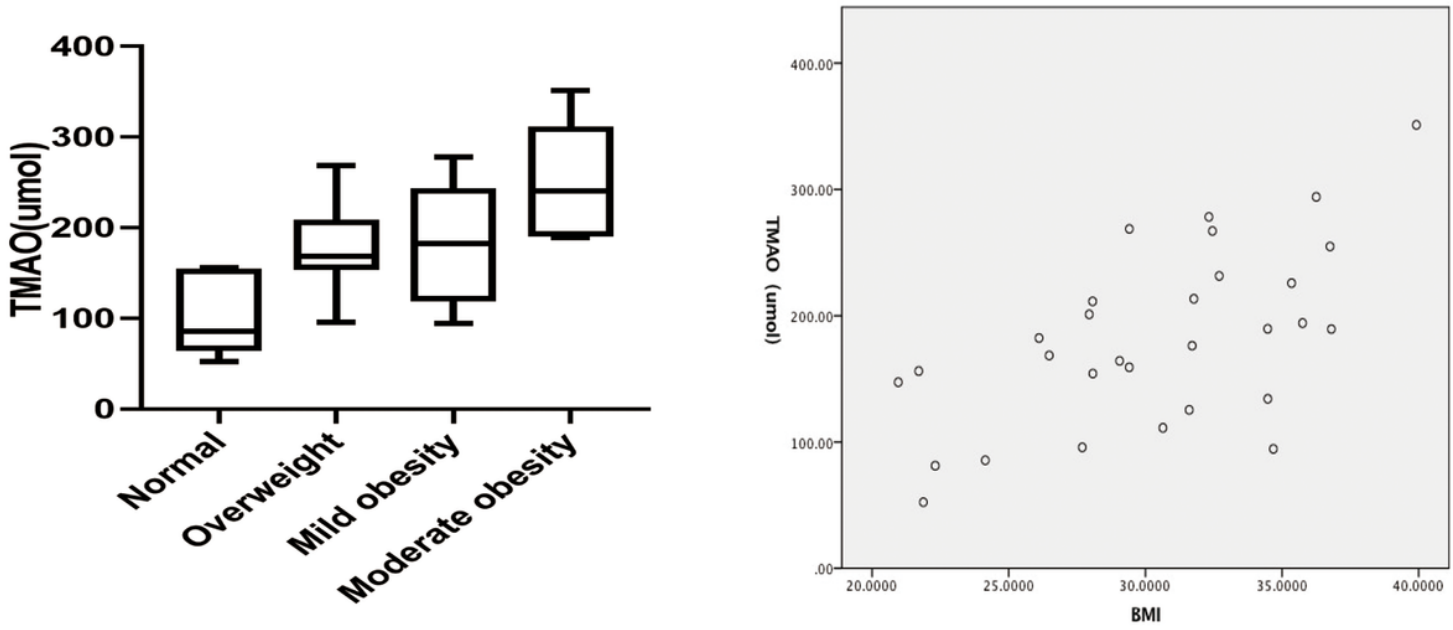

Figure 1

30 volunteers in different BMI had tested the content of TMAO. The experiment was performed in triplicates.

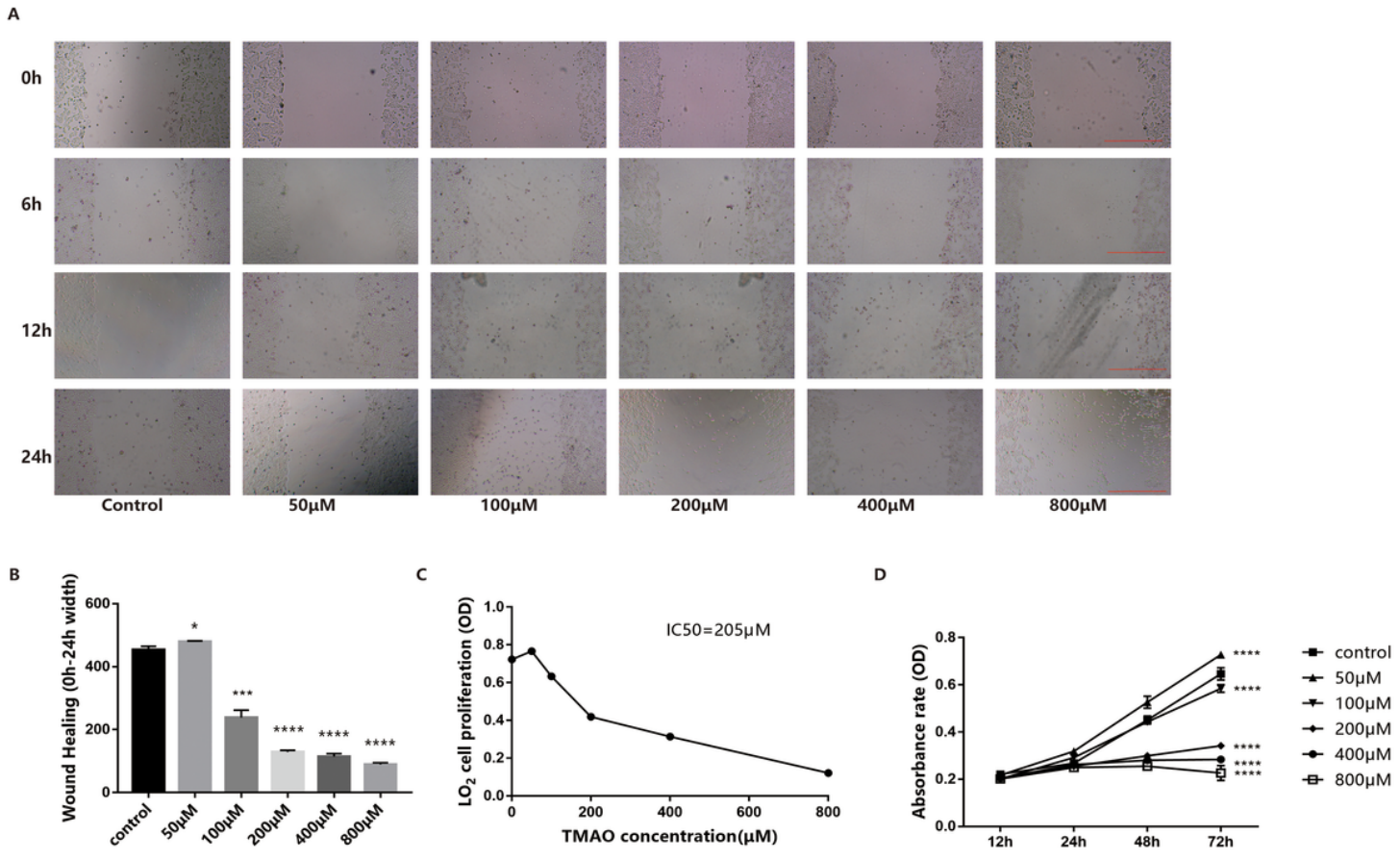

Figure 2

The higher concentrations of TMAO were harmful to LO2 cells. A-B Cell repair function was performed using wound healing assay. Representative images (up panel, original magnification ×100) and the quantification (down panel) are shown. C IC50 of TMAO. D Cytotoxic effect of TMAO against LO2 cells. *p < 0.05, ***p < 0.001, ****p < 0.0001 versus the control group.

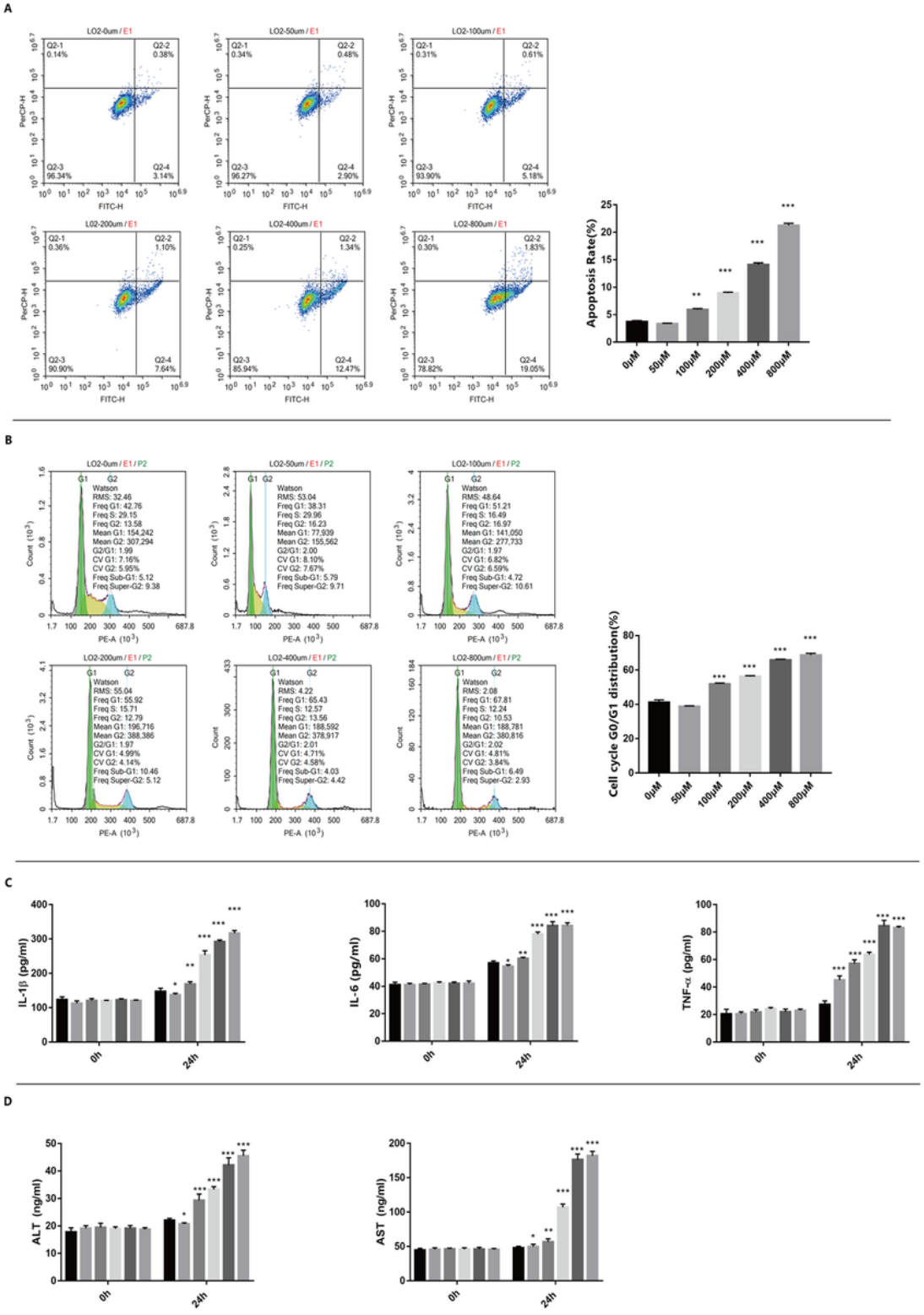

Figure 3

TMAO promoted LO2 cells apoptosis, cell cycle inhibition and inflammatory cytokine expression. A Effect of TMAO on apoptosis of LO2 cells stained with Annexin V-PI. B Effect of TMAO on cell cycle progression in LO2 cells. The indicated percentages are of the cells in G0/G1 phases. C-D ELISA was used to detect the levels of IL-1β, IL-6, TNF-α, ALT and AST. *p < 0.05, **p < 0.01, ***p < 0.001 versus the control group.

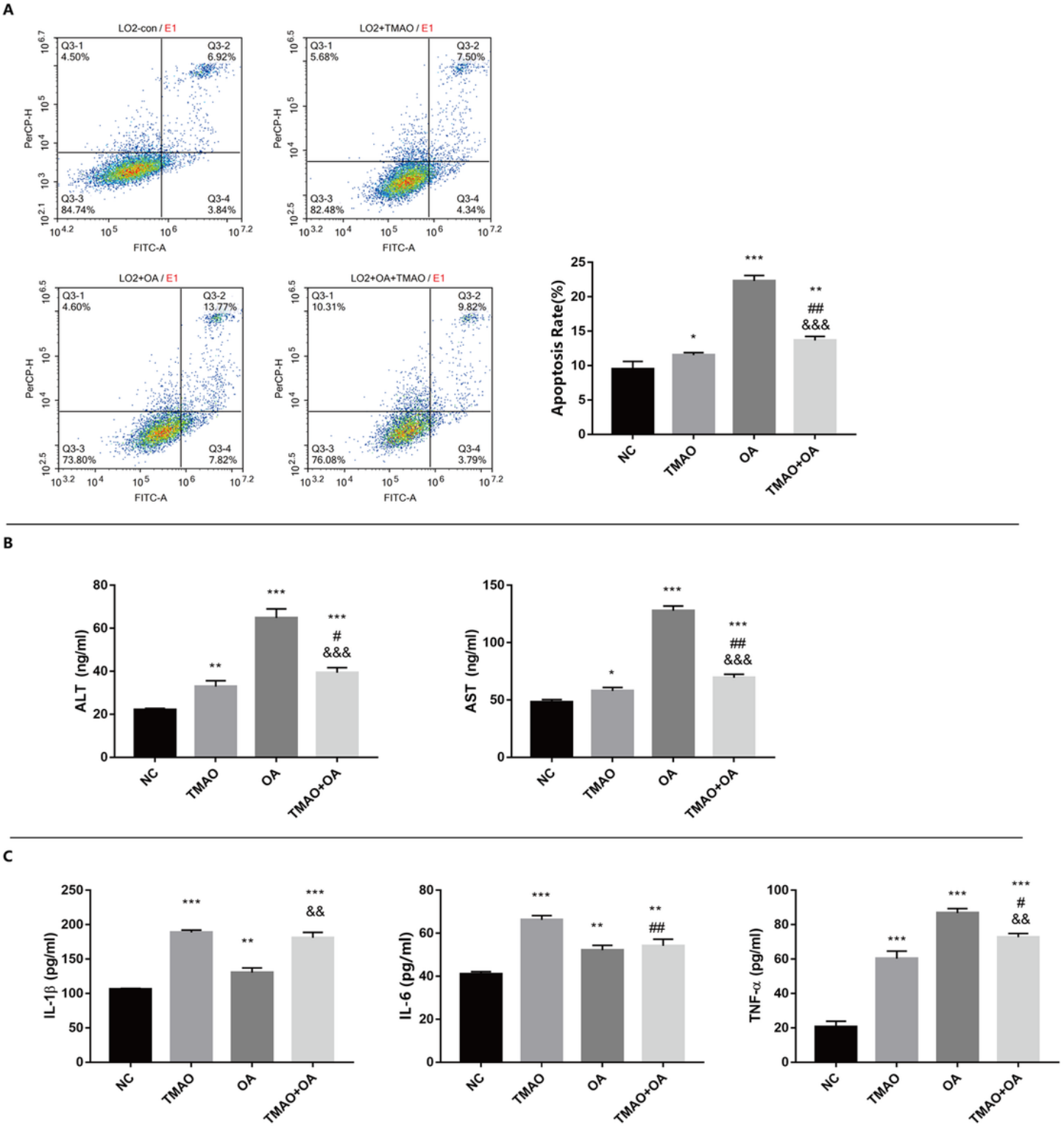

Figure 4

TMAO caused the suppression on OA-induced steatosis LO2 cells model. A Apoptosis ratio stained with Annexin V-PI. B-C ELISA was used to detect the concentrations of ALT, AST, IL-1β, IL-6 and TNF-α. *p < 0.05, **p < 0.01, ***p < 0.001 versus the control group, #p < 0.05, ##p < 0.01, versus the TMAO group, &&p<0.01, &&&p<0.001, versus the OA group.

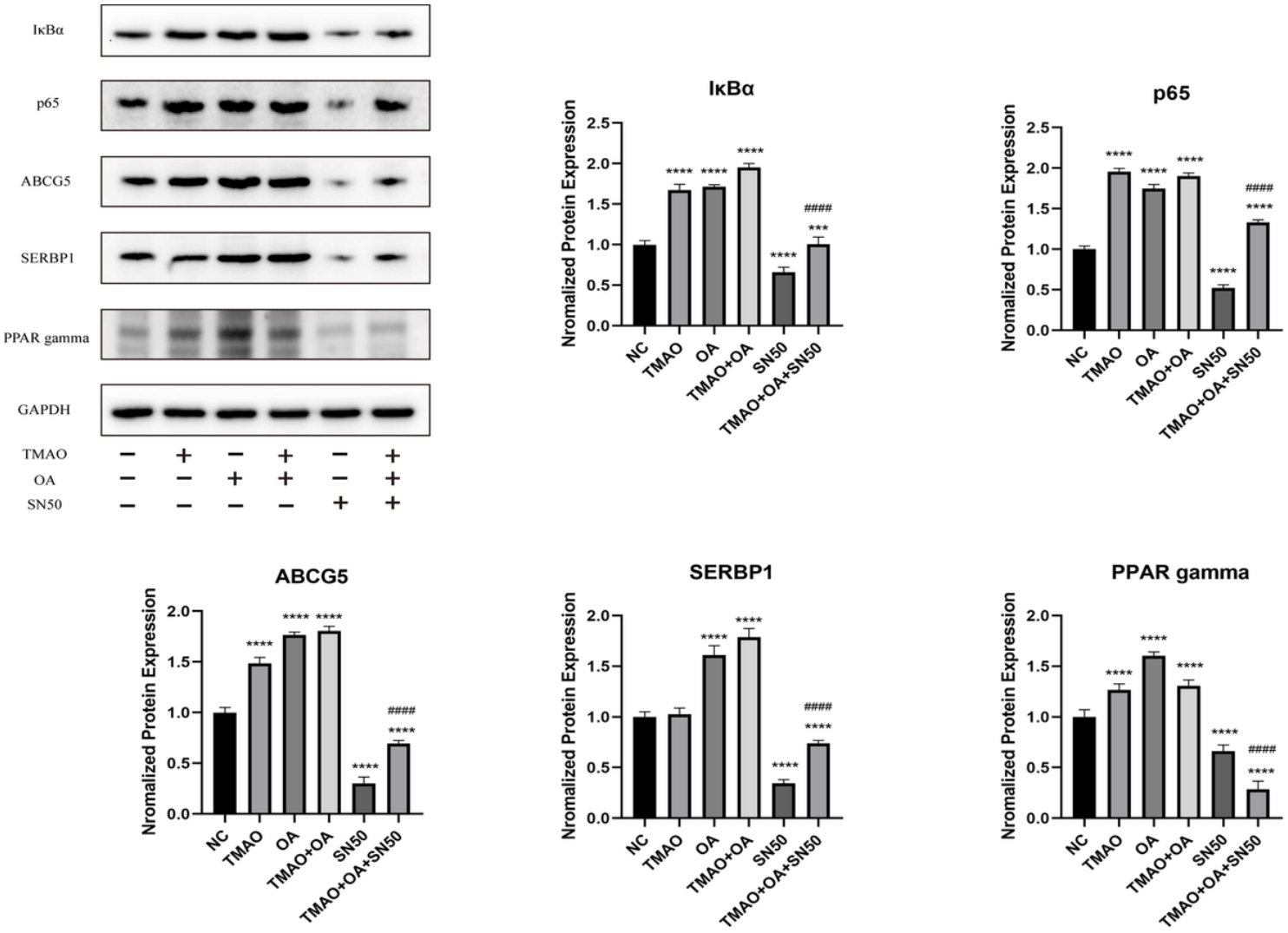

Figure 5

TMAO assisted with OA to increase the protein expressions of lipid metabolism through NF-κB signaling pathway. ***p < 0.001, ****p < 0.0001 versus the control group, ####p < 0.0001, versus the TMAO+OA group.

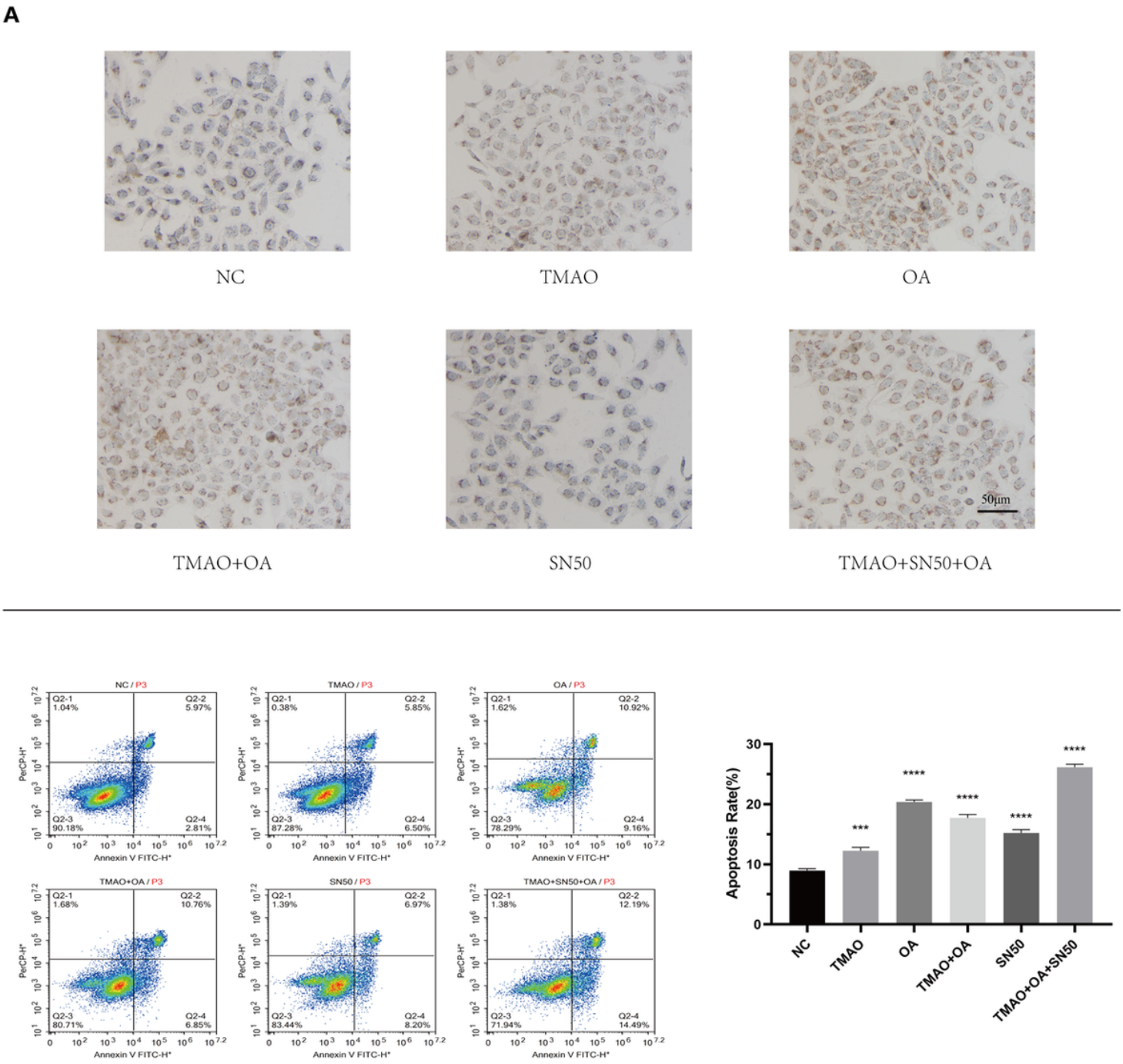

Figure 6

TMAO inhibited the LO2 cells steatosis through NF-κB signaling pathway. A Oil red O staining of activated LO2 cells treated with TMAO, OA and SN50. B Apoptosis ratio of LO2 cells treated with TMAO, OA and SN50.