Yiqi Tongluo capsule has protective effects against non-alcoholic fatty liver disease via regulating PI3K/AKT signaling

Mei-Yan Li1, Cheng-Xun He1, Ling-Yu Wang1, Die Rong Zhang1, Yu Chen2, Run-Chun Xu1*

1 School of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China. 2Department of Cardiovascular Medicine, Guangyuan Hospital of Traditional Chinese Medicine, Guangyuan 628000, China.

*Corresponding to: Run-Chun Xu, School of Pharmacy, Chengdu University of Traditional Chinese Medicine, No.1166, Liutai Avenue, Wenjiang District, Chengdu 611130, China. E-mail: 19972016@cductcm.edu.cn.

Abstract

Background: Oxidative stress is one of the key elements in the progression of non-alcoholic fatty liver disease (NAFLD), and Yiqi Tongluo capsule (YTC) have a variety of physiological activities which include antioxidant. The purpose of this investigation was to discover the potential mechanisms of YTC ameliorates NAFLD. Methods: In this investigation, a high-fat diet (HFD) was adopted to establish a NAFLD mouse model. Liver samples were stained for oil red O and hematoxylin and eosin staining. The levels of total cholesterol (TC), triglyceride (TG), malondialdehyde (MDA), and superoxide dismutase (SOD) in the tissues were also detected. Network pharmacology was analyzed to filter out the key ingredients and targets of effect of YTC for the therapy of NAFLD. Subsequently, free fatty acids (FFA) was applied to induce Aml12 cells for in vitro experiments, and the cell samples were stained with oil red O and assayed for TC, TG, MDA, and SOD contents. At last, the Western blot technique was used to illuminate the pathway by which YTC plays a protective role against NAFLD. Results: Histopathological results demonstrated that YTC ameliorated tissue damage in the HFD-induced mouse model. At the same time, it also reduced the contents of TC, TG, MDA and increased the expression of SOD in the liver tissue of NAFLD mouse model. All of these findings demonstrate that YTC can play a role in the treatment of NAFLD by ameliorating oxidative stress. Network analysis of YTC ameliorates NAFLD mainly by modulating the PI3K-Akt signaling pathway. Follow-up in vitro experiments revealed that FFA caused lipid accumulation in Aml12 cells, which was dramatically reduced by YTC. Meanwhile, YTC could remarkably reduce the FFA-induced elevation of TC, TG, and MDA contents, and reverse the FFA-induced reduction of SOD contents. Western blot was verified for the PI3K-Akt signaling pathway. It was found that FFA could remarkably decrease the expression of p-PI3K, p-Akt, and p-GSK-3β proteins, which could be significantly increased after YTC treatment. Conclusions: The combination of network analysis prediction and experimental verification was used to identify the therapeutic effect of YTC on NAFLD. The protective effect was achieved by YTC through upregulation of PI3K-Akt-GSK-3β pathway.

Keywords: Yiqi Tongluo capsule; non-alcoholic fatty liver disease; oxidative stress; PI3K-Akt-GSK-3β; Aml12 cells
**Introduction**

Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome of abnormal accumulation of lipids in hepatocytes caused in addition to alcohol and other specific liver damage [1]. The pathological process is separated into simple fatty liver, non-alcoholic steatohepatitis, fatty liver fibrosis and cirrhosis, and even evolution to hepatocellular carcinoma [2-4]. In the recent years, the constant renewal of the Internet has indeed enhanced the standard of living of human beings. However, its popularity has led to a change in the global structure of diet. Changes in nutritional patterns have pushed up to 30% of the general population at risk for NAFLD, which is increasing [5, 6]. NAFLD is not merely a single-target lesion and is frequently associated with cardiovascular disease, obesity, and type II diabetes. Its pathogenesis is comprehensive, involving multiple theories of “second strike”, “third strike” and “multiple strikes”. The etiology of the disease involves many factors, which include oxidative stress, apoptosis and autophagy, insulin resistance, etc [7-9]. All in all, there is an urgency for new clinical approaches to resist NAFLD owing to the prevalence of NAFLD and the lack of clarity on the mechanisms of NAFLD progression.

Yiqi Tongluo capsule (YTC) is composed of 12 medicines, which include the roots of Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao, the roots of Angelica sinensis (Oliv.) Diels, and the flower of Carthamus tinctorius L., et al. According to the preliminary study, YTC can improve myocardial ischemia by activating oxidative stress mediated the PI3K/Akt signaling pathway [10]. In NAFLD pathological conditions, reactive oxygen species production and scavenging in mitochondria are interrupted, which leads to oxidative stress and lipid peroxide formation, which may further induce mitochondrial dysfunction, consequently leading to hepatocyte injury and apoptosis. In the meantime, overaccumulation of reactive oxygen species can trigger the NF-kB-mediated inflammatory route in liver cells, which ultimately promotes the progression of simple steatohepatitis to non-alcoholic steatohepatitis [11]. There is also documented evidence that PI3K-Akt signaling pathway can inhibit hepatic oxidative stress and inflammation to ameliorate NAFLD [12]. Hence, we made the assumption whether YTC might have the potential to activate the PI3K/Akt signaling pathway to inhibit oxidative stress and thereby ameliorate NAFLD? The workflow is shown in Figure 1.

**Materials and methods**

**Materials and reagents**

All medicines were supplied by Guangyuan Hospital of Traditional Chinese Medicine (Guangyuan, China). The assay kits for malondialdehyde (MDA) and superoxide dismutase (SOD) were purchased from the Suzhou Michy Biomedical Technology Co., Ltd. (Suzhou, China). Total cholesterol (TC) and triglyceride (TG) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Oleic acid (OA) and palmitic acid (PA) were purchased from Sigma-Aldrich (USA). Fetal bovine serum and Dulbecco’s modified eagle medium were acquired from Gibco Co., Ltd. (Grand Island, NY, USA). SDS-PAGE preparation kit, bicinchoninic acid protein assay reagents, penicillin-streptomycin mixture and tryptophan were acquired from Roster Biological Technology Co., Ltd. (Pleasanton, CA, USA). Cell counting kit-8 (CCK-8) were acquired from Bioground Technology Co., Ltd. (Chongqing, China). Monoclonal antibodies for PI3K, p-PI3K, Akt, p-Akt, GSK-3β, p-GSK-3β were obtained from the ABclonal Technology Co., Ltd. (Wuhan, China).

![Figure 1 The workflow of this study](https://www.tmrjournals.com/im/ARTICLE/20248-e24001/IMD202408001)

Submit a manuscript: https://www.tmrjournals.com/im
Animals and administration
All animal experimental protocols used in this study were conducted in accordance with the standards of the Medical Ethics Committee of Guangyuan Hospital of Traditional Chinese Medicine (NO. 2023.004). C57BL/6 male mice were bought from Beijing Sibei Fu Laboratory Animal Technology Co., Ltd. (Beijing, China). Five-week-old mice weighing 18–22 g were housed in an artificial environment. Mice had unlimited drinks and access to food. Also, the ambient temperature was 22–24 °C, the humidity range was controlled at 60–70% and the light/dark cycle (12 h).

Within 25 weeks, C57BL/6 male mice were given a high-fat diet (HFD) (Research Diets Inc., New Brunswick, NJ, USA) to build a NAFLD pathology model. C57BL/6 male mice were equally distributed into 6 groups (n = 6/group): (1) normal diet group; (2) HFD group; (3) Positive (Atorvastatin: 20 mg/kg) group; (4) HFD + YTC (150 mg/kg) group; (5) HFD + YTC (300 mg/kg) group and (6) HFD + YTC (600 mg/kg) group. All treatment groups were weighed and given the appropriate drugs by gavage at the 5th week, and distilled water was given to the normal diet and HFD groups. Mice were fasted overnight and given free drinking water before sample collection.

Histopathologic analysis
Tissues which were fixed in 4% paraformaldehyde were removed, as well as the liver were then cut into 5 μm for hematoxylin and eosin (H&E) staining. When stained with oil red O, frozen liver sections were cut and rinsed with distilled water, followed by staining with oil red O for 6–10 min. Following staining, the slices were cleaned in water and soaked in 60% isopropyl alcohol. Sealing with glycerin gelatin at the end [13].

Biochemical indicators analysis
The levels of TC and TG in the liver tissues of mice in all groups were examined in accordance with the description of the TC and TG kits.

Evaluation of oxidative stress indicators
The MDA content and SOD level in the liver tissue of mice were assayed by the instructions of the MDA and SOD kits.

YTC active ingredient and related target screening
On the basis of the preliminary investigation of this topic, we screened the main components of YTC using UPLC-QTOF-MS technology, screening a total of 32 compounds. These 32 chemical components were searched individually by logging into Traditional Chinese Medicines Systems Pharmacology Database and Analysis Platform (TCMSP) (https://tcmsp.com/tcmsp.php) to obtain information on the active ingredient targets of the compound [14].

NAFLD target screening
NAFLD targets were filtered from GeneCards (https://www.genecards.org) and Online Mendelian Inheritance in Man (OMIM) (https://www.omim.org) databases using the term “NAFLD” as the keyword.

Construction and analysis of protein-protein interaction (PPI) networks
The intersection targets were introduced into the STRING library (https://string-db.org). The data were saved as “tsv” files to introduce Cytoscape 3.9.1 software to analyze the PPI results and construct the network [15].

Construction of “active ingredient-target-disease” network
YTC effective components, NAFLD and their cross-targets were integrated and introduced into Cytoscape 3.9.1 software to construct an “effective component-target-disease” network.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis
GO and KEGG pathway analysis of the acquired cross-targets were performed by the Metascape database (https://metascape.org), while the GO and KEGG pathway enrichment results were summarized by using Bioinformatics (http://www.Bioinformatics.com.cn) [16].

Cell cultivation
The mice liver cell line Aml12, bought from Shanghai Yubo Biotechnology Co., Ltd. (Shanghai, China). The cultures were kept in F12 medium (Gibco Co., Ltd. Grand Island, NY, USA) with 10% fetal bovine serum, as well as 1% dual antibody at 37 °C in a humid climate with 5% CO2.

CCK-8 assays
To determine cell viability, the CCK-8 was used following the manufacturer’s instructions. Aml12 cells in logarithmic growth phase were removed and made into cell suspension using trypsin digestion. 1 × 104 cells were inoculated in a 96-well culture plate and were processed with different levels of YTC. FFA (500 μmol/mL oleic acid + 250 μmol/mL palmitic acid) was applied to induce damage, and the absorption of the wells at 450 nm was determined using a zymography.

Assessment of cellular biochemical indicators
Aml12 cells were cultivated in 6-well plates. After treatment, TC and TG levels in the cells were assayed according to the kit instructions.

Assessment of oxidative stress indicators in cells
Aml12 cells were cultured in 6-well plates. Following treatment, MDA and SOD activities in the cells were determined according to the kit instructions.

Oil red O staining
Aml12 cells were cultured and the cells were stained according to the kit instructions, and the distribution of lipid droplets in the cells was viewed under a microscope.

Western blot assays
Preconfigured radioimmuno precipitation assay cracking liquid was used to extract the total protein in the organization. Protein samples were centrifuged for 5 min and the level was determined using a bicinchoninic acid kit. The protein solution was completely combined with the buffer solution and heated to denature the sample for 5 min. The samples were isolated by electrophoresis on a 10% SDS-PAGE gel then shifted to a Polyvinylidene fluoride membrane and closed with 5% bovine serum albumin for 1 h. They are cultured with the primary antibody overnight at 4 °C: p-Akt (1:500), Akt (1:500), p-Pi3K (1:500), Pi3K (1:500), p-GSK-3β (1:1000), GSK-3β (1:1000), GAPDH (1:5000). The rabbit or mouse secondary antibodies were subsequently incorporated for an additional 1 h of incubation at room temperature. ECL solution was applied to visualize the membranes. GAPDH was considered as an endo-reference, and the grayscale was compared with the internal reference. To get the expression of protein [17, 18].

Statistics analysis
Quantitative data are shown as mean ± standard deviation. Multiple group comparisons were determined by one-way analysis of variance. For all analyses, significant differences were accepted when P < was 0.05 using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Results
Histopathologic changes in the liver of mice
H&E staining revealed that the structure of liver lobules in normal mice was clearly defined, the cells were neatly arranged, with normal hepatic sinusoids. Hepatocytes of mice in the HFD group appeared to be disordered, and there was a large amount of lipid vacuoles (Figure 2). Hepatocyte morphology gradually recovered and lipid droplet

Submit a manuscript: https://www.tmrjournals.com/im
vacuoles were markedly reduced in the positive group and all YTC dose groups. As shown by the results of oil red O staining (Figure 3), the staining was intense in the liver organization of the HFD group. This indicated the existence of stronger lipid invasion in the hepatocytes. The positive group and all YTC dose groups improved and lipid droplets diminished.

As shown by the results of oil red O staining (Figure 3), the staining was intense in the liver organization of the HFD group. This indicated the existence of stronger lipid invasion in the hepatocytes. The positive group and all YTC dose groups improved and lipid droplets diminished.

As shown in Figure 4A and 4B, the concentrations of TC and TG in the liver organization in the HFD group was obviously elevated in comparison with the normal diet group ($P < 0.05$). The levels were markedly reduced in the positive group and in all dose groups of YTC than in the HFD group.

Figure 2 Representative images of H&E staining of liver tissue from NAFLD mice by YTC. H&E, hematoxylin and eosin staining; NAFLD, non-alcoholic fatty liver disease; YTC, Yiqi Tongluo capsule; NC, normal control.

Figure 3 Representative images of oil red O staining of liver tissue from NAFLD mice by YTC. NAFLD, non-alcoholic fatty liver disease; YTC, Yiqi Tongluo capsule; NC, normal control.

Figure 4 Influence of YTC on TC, TG, SOD, and MDA contents in liver tissues of NAFLD mice. Results were expressed as mean ± standard deviation ($n = 6$). $^* P < 0.05$, $^{**} P < 0.01$ vs. the Model group. YTC, Yiqi Tongluo capsule; TC, total cholesterol; TG, triglyceride; SOD, superoxide dismutase; MDA, malondialdehyde; NAFLD, non-alcoholic fatty liver disease; NC, normal control.
SOD and MDA changes in liver tissues of mice
As shown in Figure 4C, the SOD levels in the liver organization of mice in the HFD group were obviously decrease (P < 0.05) in comparison with the normal diet group. Compared with the HFD group, the SOD content was obviously increased in the positive group and all dose groups of YTC (P < 0.05). The Figure 4D shows that the MDA was dramatically increased in the HFD group in comparison with the normal group (P < 0.05). In comparison with the HFD group, the MDA level was remarkably lower in the positive group and in all dose groups of YTC (P < 0.05).

Acquisition of potential targets for YTC and NAFLD
According to the previous investigation of this project, UPLC-QTOF-MS was adopted to filter the major ingredients of YTC, which resulted in the screening of 32 chemical components (Table 1) [19]. Search the target information of each chemical component from TC MSP database to acquire the drug target. Screening disease targets from GeneCards, OMIM database. The YTC component targets and NAFLD targets were taken as intersections, which were imported into the venny online graphing software platform to draw a Venn graph. As Figure 5A show, there were 26 critical targets of YTC for the therapy of NAFLD. The intersecting targets were submitted to the String online platform and topologically analyzed by Cytoscape 3.9.1 to reconstruct the PPI network in order of degree value. The network consists of a total of 26 nodes and 119 straight lines. Nodes indicate proteins and straight lines indicate the presence of mutual relationships. The higher the degree value of the node, the deeper the color and the larger the area in the graph, meaning that more proteins are interacting with it. This means that the most important the role of this protein in the network (Figure 5B, 5C). The main key proteins in the network include epidermal growth factor receptor, estrogen receptor, GSK-3β. The 32 active ingredients in YTC intervention in the progression of NAFLD via 26 targets. The active ingredient-target relationship is imported into the Cytoscape platform for visualization, which together form the ingredient-target-disease relationship network (Figure 5D). Analysis indicates luteolin, formononetin, etc., may be major chemical components in the therapy of NAFLD.

GO functional annotation and KEGG pathway enrichment analysis
The Metascape data platform was used to enriched 26 key targets for GO and KEGG pathway analysis. The GO enrichment obtained a total of 462 entries. There are 406 biological processes including cellular response to organic nitrogen compounds, and others. There are 21 cellular compositions including transcription regulator complex, receptor complex, etc. There are 35 molecular functions, including kinase-bound, protein kinase-bound, etc. KEGG pathway enrichment obtained a total of 93 pathways containing pathways in cancer, PI3K-Akt signaling pathway, etc (Figure 6).

<table>
<thead>
<tr>
<th>Table 1 The active ingredients and their properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mol ID</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>13</td>
</tr>
<tr>
<td>14</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>16</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>18</td>
</tr>
<tr>
<td>19</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>22</td>
</tr>
<tr>
<td>23</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>25</td>
</tr>
<tr>
<td>26</td>
</tr>
<tr>
<td>27</td>
</tr>
<tr>
<td>28</td>
</tr>
<tr>
<td>29</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>31</td>
</tr>
<tr>
<td>32</td>
</tr>
</tbody>
</table>
Figure 5 Venny, PPI and potentially active compound-target-disease network construction and analysis. (A) Venn diagram of compound targets of YTC and NAFLD-related targets. (B) Cross-targeted PPI network bar diagrams. (C) PPI network of crossover targets. Node, target protein; Line, interaction between. (D) Network analysis of potentially active compound-target-disease. The outside ring is the active ingredient and the inside is the target point. PPI, protein-protein interaction; NAFLD, non-alcoholic fatty liver disease; YTC, Yiqi Tongluo capsule.

Figure 6 Analysis of GO and KEGG. (A) Biological processes. (B) Cellular components. (C) Molecular function. (D) KEGG pathway. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Influence of YTC on the viability of cell
Aml12 cells were processed with various concentrations of YTC (20, 40, 60, 80, 100 and 120 μg/mL) over 24 h. As in Figure 7A, as compared to the normal cell, the cell viability of the model group induced by FFA dropped to nearly 20% (P < 0.05). A concentration-dependent enhancement in viability was observed after 24 h of YTC (20, 40, and 60 μg/mL) treatment comparing with the model group (P < 0.05).

Influence of YTC on TG and TC in Aml12 cells
As shown in Figure 7B and 7C, the intracellular TG and TC contents were remarkably elevated within the model group compared to the normal group. YTC administration at all doses significantly decreased TC and TG levels as compared to the model group.

Influence of YTC on SOD and MDA in Aml12 cells
As in Figure 7D and 7E, the SOD content in Aml12 cells was...
dramatically decreased after FFA stimulation as compared to the normal group ($P < 0.05$). Comparing to the model group, SOD levels were obviously elevated in the YTC middle, high dose groups ($P < 0.05$). MDA content in Aml12 cells was remarkably increased after FFA stimulation when compared with the normal group ($P < 0.05$). MDA level was significantly lower in all dose groups of YTC than the model ($P < 0.05$).

Influence of YTC on intracellular lipid accumulation in Aml12 cells

After FFA stimulation, a large amount of accumulated lipids could be seen by oil red O staining. In comparison with the model group, YTC significantly decreased the lipid accumulation phenomenon in Aml12 cells ($P < 0.05$) (Figure 8).

Mechanism of action of YTC on FFA-induced Aml12 cells

We evaluated the outcome using western blot. As shown in Figure 9, p-PI3K, p-Akt, and p-GSK-3β protein expression were diminished as compared to the normal group. Nevertheless, the YTC medium and high dose groups reversed these variations ($P < 0.05$).
Discussion

NAFLD is a kind of metabolic liver disease which is not caused by alcoholism, but is gradually evolving into an end-stage liver disease culprit. A large amount of data indicates the beneficial role of traditional Chinese medicine in the treatment of NAFLD [20–22]. YTC has a promising antioxidant effect as a clinically used drug [10]. Thus, we used HFD to establish a mouse NAFLD model, the results of oil red O staining and H&E staining revealed the problem of mice with more lipid droplets and lipid deposition, which was significantly reduced after YTC treatment. At the same time, we assayed the level of TC, TG, MDA, and SOD in the tissues, which indicated a significant increase in the content of TC, TG, and MDA as well as a decrease in the content of SOD in the mice of the model group. The therapy of YTC has significantly alleviated the problem. These findings demonstrate that YTC may ameliorate NAFLD through an antioxidant effect. However, the underlying mechanism of action is still unclear.

Network analysis is characterized by completeness and systematicity, which establishes a link for the study of interactions between different drugs and is well suitable for the study of traditional Chinese medicine [23]. For further searching the potential mechanism of action of YTC to ameliorate NAFLD, we conducted a network pharmacology analysis. In this investigation, we screened 32 major chemical constituents of YTC by UPLC-QTOF-MS technique on the basis of previous experiments. These components were uploaded into the TCMSP online database to find drug targets, with disease targets collected from the OMIM and GeneCard databases. Compound active ingredient-target-disease network analysis indicates that luteolin, formononetin, etc. may be the major chemical ingredients for the therapy of NAFLD. It may be associated with genes such as AMPK, ADH1B, and CHRM3. KEGG analysis indicated that the cancer pathway, PI3K-Akt signaling pathway may be the underlying mechanism of role. GSK-3β is a serine/threonine kinase which acts as a multifunctional kinase responsible for the phosphorylation of more than 20 substrates, and it also receives regulation by Akt [24]. So subsequently we adopted FFA-induced Aml12 cells to create an in vitro model for validation. The outcome of oil red O indicated that YTC treatment could dramatically reduce the phenomenon of lipid droplet accumulation caused by the induction of FFA. Meanwhile FFA also caused an increase in TC, TG, and MDA content and a decrease in SOD content in Aml12 cells, which was significantly reversed by YTC treatment. At last, western blot was applied to confirm the corresponding pathway. The outcome showed that p-PI3K, p-Akt, and p-GSK-3β protein levels were reduced in FFA-induced Aml12 cells as compared to normal cells. In comparison the model group, YTC treatment dramatically enhanced protein expression.

Conclusion

In the present study, we first validated the effect of YTC on the protection of HFD-induced NAFLD mice model by in vivo experiments. Network pharmacology predicts that YTC may ameliorate NAFLD via the PI3K-Akt-GSK-3β signaling pathway. The in vitro experimental results also validated the results predicted by the network analysis. To sum up, the present study elucidated that YTC may inhibit oxidative stress by up-regulating the PI3K-Akt-GSK-3β pathway, thereby ameliorating NAFLD.

References


Submit a manuscript: https://www.tmrjournals.com/im
http://doi.org/10.17179/excli2021-3815
8. Wang L, Xiao J, Tian Y, et al. Study of persimmon leaf flavonoids ameliorates liver steatosis by activating autophagy and antioxidant stress in NAFLD rats. *Tianjin Med J* 2023;51(11):1211–1216. Available at: https://kns.cnki.net/kcms2/article/abstract?v=ebKgZyeBkY42ooOUnh4tqGe77w-8T7zzn3_3_07q0zNgJaAHQJDWVqTc7R4bQ95SGo_yVtpDF34qWAT3GnGgQb0Vq0hIliMJ_VQJAYNQOOFb