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# Apigenin suppresses mouse peritoneal fibrosis by down-regulating miR34a expression

Yiming Zhang[a,b,1], Qiaoling Sun[a,1], Xiang Li[b], Xiaofen Ma[b], Yang Li[b], Zhanfeng Jiao[b], Xiang-Dong Yang[a,*]

[a] Department of Nephrology, Qilu Hospital, Shandong University, Jinan, Shandong, 250012, China
[b] Department of Nephrology, Affiliated Hospital of Jining Medical University, Jining, Shandong, 272029, China

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ABSTRACT

Peritoneal fibrosis is a severe side-effect of chronic peritoneal dialysis in patients with end-stage renal disease, but not enough effective therapeutic drugs are currently available in clinics. The aim of this study was to evaluate the effects of apigenin and miRNA on the progression of peritoneal fibrosis. We treated isolated mouse mesothelial peritoneal cells (MMCs) with high glucose (HG), to induce fibrosis. We used qRT-PCR and Western blotting to measure the expressions of multiple epithelial-mesenchymal transition (EMT) biomarkers, like E-cadherin, transcription termination factor (TTF), N-cadherin and vimentin, as well as several apoptosis and autophagy biomarkers. We determined the IC50 of apigenin on MMC fibrosis. We also used qRT-PCR to assess the expressions of miRNAs in MMC fibrosis. In addition, we by used the CCK8 assay, Hoechest staining and flow cytometry, to measure cell viability and proliferation rates. We successfully induced fibrosis using high glucose (HG) treatment in MMCs. This was further validated by the observed changes in E-cadherin, TTF, N-cadherin and vimentin expression levels. We also observed highly elevated expression levels of miR34a during HG-induced MMC fibrosis. Apigenin treatment induced a significant decrease in miR34a expression levels in HG-treated MMCs. Moreover, both apigenin treatment and miR34a depletion, as well as their combination, significantly promoted proliferation and suppressed apoptosis of MMCs treated with high glucose. This was accompanied with a corresponding alteration in expressions of EMT, apoptosis and autophagy biomarkers. In summary, apigenin effectively inhibits mouse mesothelial peritoneal cell fibrosis induced by high glucose, and this is, at least partially mediated by the suppression of miR34a expression.

## 1. Introduction

Peritoneal dialysis (PD), a form of renal replacement therapy, is an effective method for treating patients with end-stage renal diseases, like acute and chronic renal failure, and acute drug and poison intoxication [1]. However, long-term use of peritoneal dialysis is greatly limited because of peritoneal fibrosis caused by the glucose added in the bioincompatible peritoneal dialysis solutions (PDF) [2]. Chronic exposure to glucose-containing bioincompatible peritoneal dialysis solutions usually leads to histological alterations in peritoneum, such as the loss of mesothelial cell monolayer, excessive extracellular matrix deposition and angiogenesis [3,4]. It is thus, clinically imperative to prevent peritoneal fibrosis during peritoneal dialysis.

MicroRNAs (miRNAs) are small non-coding RNA molecules, that regulate various physiological and pathological processes by modulating post-transcriptional gene expression [5,6]. Interestingly, miRNAs are also important players in development of multiple fibrosis-related diseases [7]. One recent study showed the miRNA-221/222 family exhibits anti-myocardial fibrosis activity in pressure-overloaded hearts, where their expression is suppressed [8]. In addition, miR-34a in alveolar type II epithelial cells regulates transforming growth factor (TGF)-β1 and bleomycin-induced epithelial-mesenchymal transition, which is an important mediator of pulmonary fibrosis [9]. Another miRNA, miR-351, was also recently shown to promote schistosomiasis-induced hepatic fibrosis by targeting the vitamin D receptor (VDR), which acts as an antagonist of the Mothers against decapentaplegic homolog (SMAD) signaling pathway [10]. Furthermore, miR144 and miR16 were found to be involved in human fibrotic liver and native cystic fibrosis, respectively [11,12]. More importantly, miRNAs have also been shown to be associated with peritoneal fibrosis [13], but their specific roles and the underlying mechanisms remain largely unknown.

Besides adding to the limited knowledge, an urgent need exists for

* Corresponding author at: Department of Nephrology, Qilu Hospital, Shandong University, No 107,Wenhuaxi Road, Lixia District, Jinan, Shandong,China.
*E-mail address:* yangxiangdongd@163.com (X.-D. Yang).
[1] These authors contributed equally to this work.

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effective preventive and therapeutic treatments for peritoneal fibrosis. Apigenin, a plant-derived secondary metabolite usually present in glycosylated form, is a major dietary flavonoid synthesized in a number of plant species such as celery, parsley, thyme, chamomile, onions, and even plant-derived beverages like tea, wine and beer [14,15]. The clinical studies suggest that apigenin has the potential to treat multiple diseases such as human cancers and neurodegenerative diseases (NNDs) [14,16,7]. For instance, apigenin inhibits the growth and proliferation of human head and neck squamous carcinoma cells by promoting the G2/M cell cycle arrest and enhancing reactive oxygen species (ROS) levels [17]. Moreover, apigenin has been shown to exhibit anti-tumorigenic effects in various other human cancer types including breast cancer, prostate cancer, colorectal cancer, pancreatic cancer, skin cancer, liver cancer, cervical and ovarian cancer [14,17,18,15]. Apigenin also exerts anti-inflammatory and immunomodulatory effects on autoimmune myocarditis [19].

More importantly, apigenin also showed promising therapeutic effects in a number of fibrosis-related diseases. For instance, apigenin treatment in rats revealed that strategically released apigenin from the polymeric carrier significantly inhibited progression of idiopathic pulmonary fibrosis by regulating expression of fibrosis-related cytokines [20]. A recent transcriptome-based analysis showed that apigenin is an effective anti-fibrotic agent in hepatic stellate cells, by potentially mediating the activation of a secreted adipocytokine complement C1q tumor necrosis factor-related protein 2 (C1QTNF2) [21]. In addition, apigenin was also reported to have general inhibitory roles on other fibrosis-related diseases such as renal fibrosis and pancreatic fibrosis [22,23]. However, the roles of apigenin in regulating progression of peritoneal fibrosis have not been previously addressed.

In the present study, we isolated mouse mesothelial peritoneal cells (MMCs) and induced fibrosis by treating with high glucose (HG), followed by analyzing the inhibitory role of apigenin on the fibrotic transformation in MMCs. Also, the expression levels of miR34a and the anti-fibrotic effects of miRNA inhibitors were further tested in MMCs. Our results provide a strong basis for the application of apigenin and miRNA-related strategies in the treatment of peritoneal fibrosis.

## 2. Material and methods

### 2.1. Isolation of mouse mesothelial peritoneal cells

The isolation of mouse mesothelial peritoneal cells from the mouse peritoneal cavity *in vivo* was carried out, as previously described to minimize lymphocyte and macrophage contamination [24]. Briefly, 8-week-old Balb/c mice, provided by the Beijing Vital River Laboratory Animal Technology, were used for mesothelial peritoneal cell isolation. After euthanizing with methoxyflurane, peritoneal cavities of the mice were surgically exposed and immediately washed with injection and removal of 10 ml PBS buffer, followed by injection of 10 ml 0.25% trypsin solution containing 0.02% EDTA solution, into the mice peritoneums. Then, the animal bodies were kept at 37℃ for 15 min, and PBS buffer was applied to keep the external peritoneal cavity moist, accompanied with periodical massaging to aid cell detachment. The trypsin solution was collected using needle and syringe, and fetal calf serum (FCS)-containing culture medium was again injected into the peritoneal cavity. After the peritoneal cavity was opened, the peritoneal walls were washed with culture medium. Cell-containing medium was then centrifuged for 5 min at 250 *g* for collection of detached cells, which were then resuspended with new culture medium and cultured for subsequent analysis.

### 2.2. Cell culture and treatment

Isolated mouse mesothelial peritoneal cells (MMCs) were cultured in RPMI 1640 medium supplemented with 15% FCS, L-glutamine, hydrocortisone, insulin, transferrin, selenium, 20 mM HEPES, 4 mg/l gentamicin, 120 mg/l benzylpenicillin and 2.5 mg/l amphotericin at 37 ˚C in a humidified culture chamber supplied with 5% $CO_2$. The culture medium was changed every three days until cells were confluent. Then, cells were washed with PBS buffer, digested with 0.007% EDTA solution containing 0.02% trypsin at 37 ˚C for 5 min, followed by addition of fresh medium for a new round of culture or subsequent analysis. The apigenin used for cell treatment was purchased from the Merck company (CAS: 520-36-5; Cat. No: 42,251), with a purity of over 99% by high performance liquid chromatography assay. For analysis of miR34a in MMC fibrosis, mouse mesothelial peritoneal cells were cultured in medium containing specific miRNA inhibitors against miR34a, provided by the TEHE-PNA company (Hangzhou, China).

### 2.3. Quantitative RT-PCR

RNA extraction from cultured cells was performed using Trizol solution (Cat.#9109; Takara), according to the manufacturer's instructions. Approximately 4.0 μg RNA from each sample was used for cDNA synthesis using the Bestar™ qPCR RT kit(Cat. #2220; DBI), according to the manufacturer's instructions. Finally, relative mRNA levels were measured by PCR using DBI Bestar® SybrGreen qPCR master Mix (Cat. #2043; DBI) following the manufacturer's instructions. β-actin was used as the internal standard, and at least three biological replicates were performed for statistical analysis of mRNA levels. The sequences of primer pairs used in this study are listed in Table 1.

### 2.4. Western blotting

Total protein was extracted from samples using M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) as per the manufacturer's instructions, separated by 10% SDS-PAGE, and transferred onto PVDF membrane. After being incubated with 5% lipid-free milk solution for 2 h, the PVDF membrane with cell proteins was then incubated with primary and secondary antibodies, and finally developed with Amersham ECL Select™ detection system (Amersham™) for analysis of protein levels. Primary antibodies used in this study are listed as follows: anti-E-Cadherin antibody (Cat.# ab15148; Abcam); anti-TTF1 antibody (Cat.# A01477; Biocompare); anti-*N*-cadherin antibody (Cat.# ab18203; Abcam); anti-vimemtin antibody (Cat.#RV202; Abcam); anti-Bcl 2 antibody (Cat.#GTX27973; GeneTex); anti-LC3B antibodies (Cat. #ab51520; Abcam); anti-P62 antibodies (Cat. #GTX28112; GeneTex); anti-GAPDH antibody (Cat.# ab9484; Abcam).

### 2.5. Cell counting Kit-8 (CCK8) assay

Mouse mesothelial peritoneal cells were seeded in 96-well plates and cultured at 37℃ for 4 h, and then mixed with either specified apigenin or miRNA inhibitors. After treatment for the specified time, about 10 μl CCK8 solution (MedChem Express) was added into each well, followed by incubation for another 1–4 h at 37℃. Finally, absorbance at 450 nm (OD450) was measured using a spectrophotometer, for comparison of the viabilities and proliferation rates of mouse mesothelial peritoneal cells. At least three biological replicates were performed for statistical analysis of MMC viability and proliferation rates.

### 2.6. Cell apoptosis assay

Hoechest staining was carried out using the Hoechst Staining Kit (Cat. #C0003) provided by the Beyotime company (Jiangsu, China), according to the manufacturer's instructions. For flow cytometry analysis, approximately $10^4$ cells cultured in medium were washed twice with PBS solution, digested with 1 ml 0.25% trypsin solution, and resuspended in fresh medium. After centrifuge at 1000 rpm for 5 min, cell pellets were resuspended in 200 μl annexin V/7-AAD solution and incubated for 15 min in the dark. The ratio of apoptotic cells were analyzed using flow cytometry.

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