AMPK-SIRT1 Pathway Modulates The Apoptosis, Proliferation and Migration of AR42J Cells By Regulating p53 and NF-κB

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Research Article

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Abstract

Background: Acute pancreatitis (AP) is an acute abdomen caused by abnormal activation of trypsin. AMPK-SIRT1 pathway has been reported to be related to various diseases, but the function in AP remains unclear. This study is designed to investigate the mechanism and effect of AMPK-SIRT1 pathway in AP.

Methods: An experimental AP model of AR42J cells was stimulated with caerulein after pretreated with compound C or metformin. The mRNA and protein expressions of genes were analyzed by qRT-PCR and western blot. Cell apoptosis, proliferation and migration were measured using flow cytometry, MTT and transwell assay.

Results: After pretreated with metformin, expressions of p-AMPKα, SIRT1 were elevated, ace-p53, ace-NF-κB were attenuated, cell apoptosis, proliferation, and migration were decreased. After pretreated with compound C, the reverse effects occurred. p-AMPKα and SIRT1 expressions were decreased, ace-p53 and ace-NF-κB were rasied, and cell apoptosis, proliferation, and migration were enhanced after caerulein induced in each group.

Conclusion: When AP happened, expressions of p-AMPKα and SIRT1 were reduced, resulting in up-regulation of acetylation levels of p53 and NF-κB, acceleration of cell apoptosis, proliferation and migration. It hinted that AMPK-SIRT1 pathway could modulate the apoptosis, proliferation, migration and inflammation reaction of AR42J cells by regulating p53 and NF-κB.

Introduction

Acute pancreatitis (AP) is a potentially life-threatening inflammatory disease of the pancreas, common causes of which include biliary tract diseases, heavy drinking, hyperlipidemia, drug use, autoimmune diseases [1]. The above causes may lead to auto-digestion of pancreatic tissue, damage, degeneration and necrosis of pancreatic parenchymal cell, and an intense inflammatory reaction in the pancreas [2, 3]. Despite most patients with AP present in a mild and self-limited condition disease course, about 5~10% of the patients develop severe acute necrotizing pancreatitis, which has high mortality due to systemic inflammatory response syndrome (SIRS) or multiple organ failure (MOF) [4]. At present the treatment methods for AP are mostly rely on fluid rehydration, prophylactic use of antibiotics and enteral nutrition support to alleviate illness [1]. Although it is generally believed that the abnormal activation of pancreatic enzyme in the pancreas is a key factor to cause the occurrence of AP, the pathogenesis of AP is not entirely clear [2, 3]. Therefore, AP remains a refractory and critical disease due to lack of specific treatment for the pathogenesis and effective treatment method.

Adenosine Monophosphate Activated Protein Kinase (AMPK) is a trimer composed of α□β□γ and other three subunits, each of which has its own isomer and binds different ligands [5]. The subunit is the catalytic reaction unit, and phosphorylation of Thr172 is a key way to regulate its catalytic activity [5]. AMPK is activated by energy stress in response to increased ATP consumption (such as exercise, cell

proliferation and anabolism) or decreased ATP production (for example, low glucose levels, oxidative stress and hypoxia), which are sensed as a low ratio of ATP to AMP and ADP [6]. Silent information regulator 2 (Sir2) family is a kind of NAD^+ dependent deacetylases, which consist of seven Sir2 homologues, namely SIRT1-SIRT7 in mammals [7]. SIRT1 is one of the most studied members in Sir2 family, which participates in a large number of biological processes including cell cycle regulation, DNA repair, apoptosis and inflammation [8]. SIRT1 can catalyze the deacetylation of lysine residues of histone proteins such as H1, H3, H4 and non-histone substrates, including p53, FoxOs, PGC1-a, PPAR-γ and NF-κB [8]. AMPK can activate nicotinamide phosphoribosyltransferase at the transcriptional level, up-regulate NAD+/NADH, and then induce the activation of SIRT1. The activated SIRT1 can act on downstream targeted genes and play an important role in the regulation of apoptosis autophagy, oxidative stress and inflammation of cells [9].

An increasing body of evidence had suggested that AMPK-SIRT1 pathway played a pivotal role in diabetes, fatty liver, tumor, and cardiovascular diseases [10, 11, 12, 13]. In hepatocellular carcinomas (HCC), experimental results had demonstrated that AMPK regulated the activity of SIRT1 by direct phosphorylation. After preconditioning of HCC cells with AMPK activator, the level of acetylated p53 was significantly reduced and the growth of HCC cells was inhibited. The deletion of AMPK promoted the growth of HCC cells [14]. In a recent study, it was found that Mogroside IIIE alleviated the inflammation, oxidative stress and apoptosis of mercury-induced podocyte by activating the AMPK-SIRT1 pathway, while Compound C significantly reversed the inhibitory effects of Mogroside IIIE by inhibiting AMPK-SIRT1 pathway [15]. Similarly, in oxygen glucosedeprivation (OGD)-induced myocardial cell injury model, activated AMPK/SIRT1 pathway by Arctigenin down-regulated NF-κB phosphorylation, while inhibited AMPK by the Compound C significantly attenuated Arctigenin-exerted protective effects on cardiomyocytes [16]. AICAR, an activator of AMPK, could suppress Sev-induced neuronal apoptosis and the activity of SIRT1 in vitro. Further animal studies also showed that AICAR treatment blocked the deleterious cognition and reduced the activity of AMPK-SIRT1 pathway in the cognition impairment rats induced by Sev [17]. Therefore, AMPK-SIRT1 pathway participated in various diseases and might be a potential therapeutic target.

However, the role of AMPK-SIRT1 pathway in AP has not been determined, which remains to be investigated. This study is designed to investigate the mechanism and effect of AMPK-SIRT1 pathway on inflammatory response, apoptosis, proliferation and migration of pancreatic acinar cells. Finally, it will provide experimental data and theoretical guidance for the diagnosis and treatment of AP.

Materials And Methods

Cell Culture and Treatment

Rat pancreatic acinar AR42J cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution, and grown at

37˚C with 5% CO_2. The cells were divided into 3 groups: control group (PBS), compound C group (AMPK inhibitor, 10 μM, TargetMol, Shanghai, China) and metformin group (AMPK activator, 10 Mm, Abcam, Shanghai, China) and pretreated with compound C or metformin for 24 h. Subsequently, cells were incubated with caerulein (100 nmol/L Solarbio, China) for 24 h to induce AP cell model in vitro.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from the AR42J cells with TRIzol reagent according to the manufacturer's protocols. A template including five microlitre of total RNA was used for the synthesis of cDNAs using cDNA synthesis kit (TaKaRa, Dalian, China). The synthesized cDNA samples were subjected to quantitative PCR (qPCR) using a SYBR® Green Quantitative PCR kit (TaKaRa Bio, Siga, Japan). The following primers were designed for qPCR amplification: 5'- ATGATGAGGTGGTGGAGCAGAGG -3' (AMPK forward) and 5'- GTTCTCGGCTGTGCTGGAATCG -3' (AMPK reverse), 5'- CAGTTCCAGCCATCTCTGTGTCAC -3' (SIRT1 forward) and 5'- GATTCCTGCAACCTGCTCCAAGG -3' (SIRT1 reverse), 5'- ATGACTCTACCCACGGCAAG -3' (GAPDH forward) and, 5'- GATCTCGCTCCTGGAAGATG -3' (GAPDH reverse). Then, PCR was applied under the following reaction conditions: 95˚C for 15 min, followed by 38 cycles of 95˚C for 20 s, 60˚C for 30 s and 72 ℃ for 30 s. GAPDH was regarded as an internal reference. The relative mRNA expression levels of genes were analyzed using the $2^{-\Delta\Delta CT}$ method [18].

Western blot analysis

The total proteins from AR42J cells were lysed in RIPA buffer. Then the lysate was centrifuged at 8000 g for 30 min to collect supernatant. The protein concentration was determined by BCA assay. Cellular proteins were subjected to 10% SDS/PAGE gel and transferred onto the PVDF membrane (Millipore, USA). The PVDF membranes were blocked by incubating with PBS supplemented with 5% bovine serum albumin for 2 h to reduce non-specific binding. Subsequently, membranes were incubated with the following primary antibodies: rabbit anti-AMPK, AMPKα, SIRT1 (Cell Signaling Teghnology, USA), anti-p53, acetylated p53 (ace-p53), NF-κB, acetylated NF-κB (ace-NF-κB), β-actin (Abcam, Shanghai, China). After wash three times with TBST buffer, the blots were incubated with the horseradish peroxidase–conjugated secondary antibodies at room temperature for 2 h. The protein bands were detected using ECL Plus detection System (Bio-Rad, USA) and relative protein levels were quantified using Image-J software.

Flow cytometry assay

The AR42J cells were seeded in 6-well plates and then pretreated with compound C or metformin. After pretreatment, cells were stimulated with caerulein for 24 h and harvested. Subsequently cells were digested with EDTA-free trypsin and stained with the annexin V-APC apoptosis kit (Sangon biotech, Shang-hai, China) according to the manufacturer's derections. Cell apoptosis was detected by CytoFLEX low cytometry, and the apoptosis rate was calculated by CytExpert analysis software.

MTT Assay

The AR42J cells were plated into 96-well plates, then pretreated with compound C or metformin, and subsequently incubated with caerulein for 24 h. Then, cells were incubated with 20 μL (5 mg/ml) of MTT reagent for 2 h at 37°C. The medium was removed and 200 μL of DMSO was added and incubated for 10 minutes at 37 °C. The absorbance was determined using a 96 well microtiter plate reader at 570 nm.

Transwell assay

AR42J cells were seeded in 24-well plates, then pretreated with compound C or metformin, and subsequently incubated with caerulein for 24 h. Then, cells were harvested and incubated in RPMI with 15% FBS for 24 h. Cell migration assay was performed in transwell chamber system (Sangon biotech, Shanghai, China) according to the manufacturer's instructions.

Statistical analysis

All data analyses were performed via SPSS 25.0 software. Results were expressed as mean ± standard deviation. The difference between two groups was assessed by Student's t test. The difference among three or more groups was compared using one-way ANOVA. P-value of <0.05 was regarded as statistically significant.

Results

The mRNA expression levels of AMPK and SIRT1 were decreased in AR42J cells after induced with caerulein

As shown in Fig. 1, qRT-PCR results revealed that after compound C pretreated, the expression levels of AMPK, SIRT1 were attenuated compared with control group. After metformin pretreated, the mRNA expression levels of AMPK and SIRT1 were incresed compared with control group. After stimulated with caerulein, the mRNA levels of AMPK and SIRT1 were significantly decreased ($p < 0.05$, Fig. 1A and 1B).

The protein expression levels of AMPK, SIRT1, ace-p53, ace-NF-κB were decreased in AR42J cells after induced with caerulein

The western blot results indicated that after compound C pretreated, the protein levels of p-AMPKα/AMPK, SIRT1 were reduced, while ace-p53, ace-NF-κB were increased compared with control group. After metformin pretreated, the protein levels of p-AMPKα/AMPK and SIRT1 were enhanced, while ace-p53, ace-NF-κB were decreased compared with control group. After stimulated with caerulein, the protein levels of AMPK and SIRT1 were significantly attenuated, while ace-p53, ace-NF-κB were increased ($p < 0.05$, Fig. 2A, 2B, 2C, 2D and 2E).

The apoptosis of AR42J cells was suppressed by activation of AMPK and raised by inbition of AMPK

Flow cytometry assay was conducted to detect the apoptosis of AR42J cells. Representative cell apoptosis images were shown as Fig. 3A, 3B, 3C, 3D, 3E and 3F. Apoptotic rate of control group was

15.80 ± 0.84% (Fig. 3A, 3G), the apoptosis rate of the compound C group was 32.01 ± 17.73% (Fig. 3B, 3G), and the apoptosis rate of the metformin group was 12.08 ± 1.99% (Fig. 3C, 3G). After caerulein-stimulated 24 hours, the apoptotic rate of the control group was 22.14 ± 1.38% (Fig. 3D, 3G), the apoptosis rate of the compound C group was 42.20 ± 17.73% (Fig. 3E, 3G), and the apoptotic rate of the metformin group was 20.64 ± 1.99% (Fig. 3F, 3G). After compound C pretreated, apoptosis rate of AR42J was enhanced, while pretreated with metformin, apoptosis rate of AR42J was reduced compared with control group. After stimulated with caerulein, apoptosis rate of AR42J was significantly enhanced in each group ($p < 0.05$, Fig. 3G).

The proliferation of AR42J cells was inhibited by activation of AMPK and enhanced by inbition of AMPK

As shown in Fig. 4, the value of OD_{570nm} was rasied after compound C pretreated, while pretreated with metformin, the value of OD_{570nm} was reduced compared with the control group. After caerulein-stimulated 24 h, the value of OD_{570nm} was significantly enhanced in each group ($p < 0.05$, Fig. 4). These results suggested that inbition of AMPK increased the proliferation of AR42J cells, while activation of AMPK decreased the proliferation of AR42J cells.

The migration of AR42J cells was repressed by activation of AMPK and elevated by inbition of AMPK

As shown in Fig. 5, transwell assay was performed to analyze the migration of AR42J cells. Representative cell migration images were shown as Fig. 5A, 5B, 5C, 5D, 5E and 5F. After compound C pretreated, migration rate of AR42J was rasied, while pretreated with metformin, migration rate of AR42J was decreased compared with control group. After stimulated with caerulein, migration rate of AR42J was significantly elevated in each group ($p < 0.05$, Fig. 5G).

Discussion

AP is a potentially life-threatening inflammatory disorder caused by excessive activation of trypsin in the pancreas, which lead to pancreatic edema, hemorrhage, and necrosis [1, 2, 3]. The incidence and death rate of AP are increasing in recent years. Despite the majority of AP are self-limited and mild, symptoms can become critical when local or systemic complications occur [2, 3]. Approximately 20 ~ 30 percent of AP may progress to severe acute pancreatitis (SAP). The mortality rate of SAP can be up to 30 percent, and even as high as 50% once complications of SAP occur [2, 3]. Despite great progress has been made in the diagnosis and treatment of AP in recent years, there is no specific targeted treatment methods based on the pathogenesis of AP. Therefore, it is very important to study the mechanism of AP for drug development and clinical treatment.

AMPK is a highly conserved serine/threonine protein kinase, which plays a crucial role in cell energy metabolism and cell survival [5, 6]. Due to its important role in energy control, AMPK has attracted widespread interest as a potential therapeutic target for a wide range of diseases, including aging, breast cancer, type 2 diabetes, and acute myeloid leukemia [19, 20, 21, 22]. SIRT1 is a downstream effector of

AMPK, and shares common characteristics with AMPK in metabolism and cell survival [9]. A large number of studies have indicated that AMPK-SIRT1 pathway is involved in a variety of diseases, including cerebral ischemic stroke, liver cancer, diabetic nephropathy, rheumatoid arthritis, sepsis, and Alzheimer's disease [14, 15, 23, 24, 25]. However, the role of AMPK-SIRT1 pathway in AP has not been determined, which remains to be investigated.

To further explore the mechanism and the crucial role of AMPK-SIRT1 in AP, AR42J cells stimulated by caerulein for 24 h were performed to establish an in vitro model of AP after pretreated with compound C (AMPK inhibitor, 10uM) and metformin (AMPK activator, 10mM). Results showed that when AMPK was inhibited by compound C, expressions of p-AMPKα, SIRT1 were attenuated (Figs. 1 and 2) while the acetylation level of p53 and NF-κB were elevated (Fig. 2), the apoptosis, proliferation, and migration of AR42J cells were rasied (Figs. 3, 4 and 5). The opposite data occurred when AMPK was activated by metformin.

Established facts has indicated that SIRT1 can be activated through the phosphorylation of AMPK [26]. SIRT1 can regulate the inflammatory responses and apoptosis through the deacetylation of NF-κB and p53 [27]. NF-κB and p53 both can be activated by acetylation. NF-κB activation has been implicated as a key inflammatory pathway in the pathogenesis of AP [28]. Furthermore, it has been reported that p53 acetylation could be capable of suppressing cell proliferation and survival, which would contribute to development of AP [29]. Related research has suggested that the activation of SIRT1 could inhibit the acetylation of p53 and repress the apoptosis of acinar cells, thus to alleviate the SAP model induced by caerulein [30]. In addition, another study indicated that resveratrol (SIRT1 activator) significantly reduced the severity of AP, effectively improved the survival rate, relieved the inflammatory response and decreased the acinar necrosis and apoptosis in a mouse model of L-arginine-induced acute necrotizing pancreatitis, which might be related to the enhancement of SIRT1-mediated deacetylation of p53 [27]. In our study, Novel insights showed that when AP occurred, p-AMPKα was significantly down-regulated in AP, leading to the decrease of SIRT1, and the decreased SIRT1 inhibited deacetylation of downstream targets, including p53 and NF-κB, which were both highly activated by acetylation. Moreover, activated AMPK could up-regulate SIRT1, ace-p53 and ace-NF-κB, inhibit the apoptosis, proliferation, and migration of AR42J cells. On the contrary, inhibited AMPK could down-regulate SIRT1, ace-p53 and ace-NF-κB, promote the apoptosis, proliferation, and migration of AR42J cells.

Conclusion

Generally speaking, the above findings manifested that AMPK could affect p53 and NF-κB signaling through regulating SIRT1 in pancreatic acinar cells. AMPK-SIRT1 pathway modulated the apoptosis, proliferation, migration and inflammation reaction of AR42J cells by regulating p53 and NF-κB. Our data might provide a novel insight into understanding the molecular mechanisms of AP and theoretical guidance for investigating a promising therapeutic target for AP.

Declarations

Funding

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Availability of data and material

All data included in this study are available upon request by contact with the corresponding author.

Code availability

Not applicable

Author' contributions

Wei-Li Yu: Conceptualization, Methodology, Software, Data curation, Writing- Reviewing and Editing, Funding acquisition. Xiao-Die Wang: Methodology, Software, Data curation, Writing-Original draft preparation. Fu-Gui Wang: Methodology, Software, Data curation. Zhong-Hua Lu: Methodology, Software, Data curation. Yun Sun: Methodology, Software, Investigation, Funding acquisition.

Ethics approval

The paper is exempt from ethical committee approval. Because the studies are not involve in human and animal subjects.

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

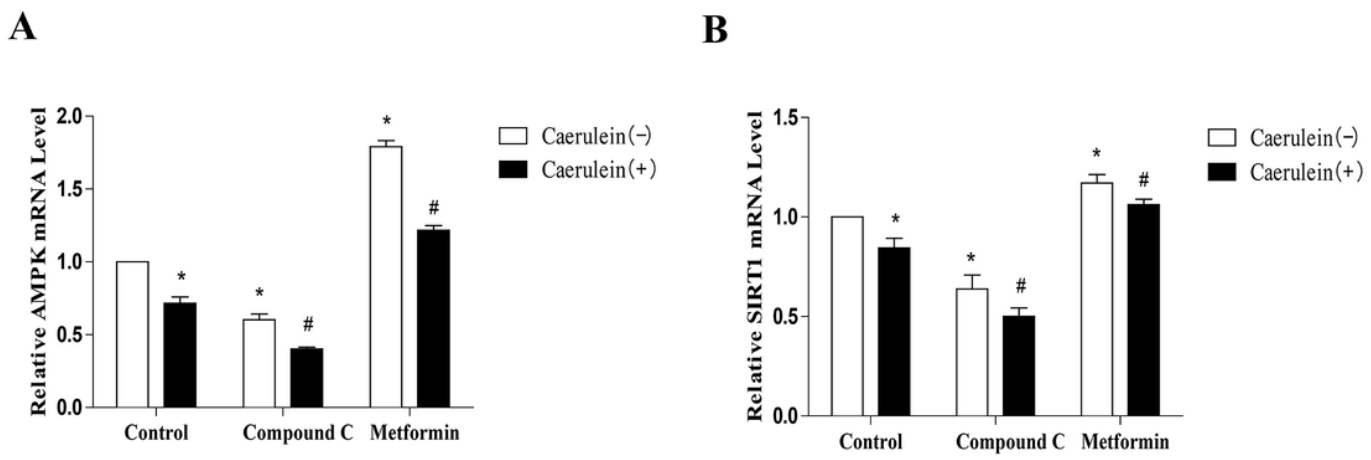

Figure 1

The relative mRNA levels of AMPK (A) and SIRT1 (B) in control and caerulein-stimulated group. *p< 0.05 compared with control group; #p < 0.05 compared with caerulein-stimulated group.

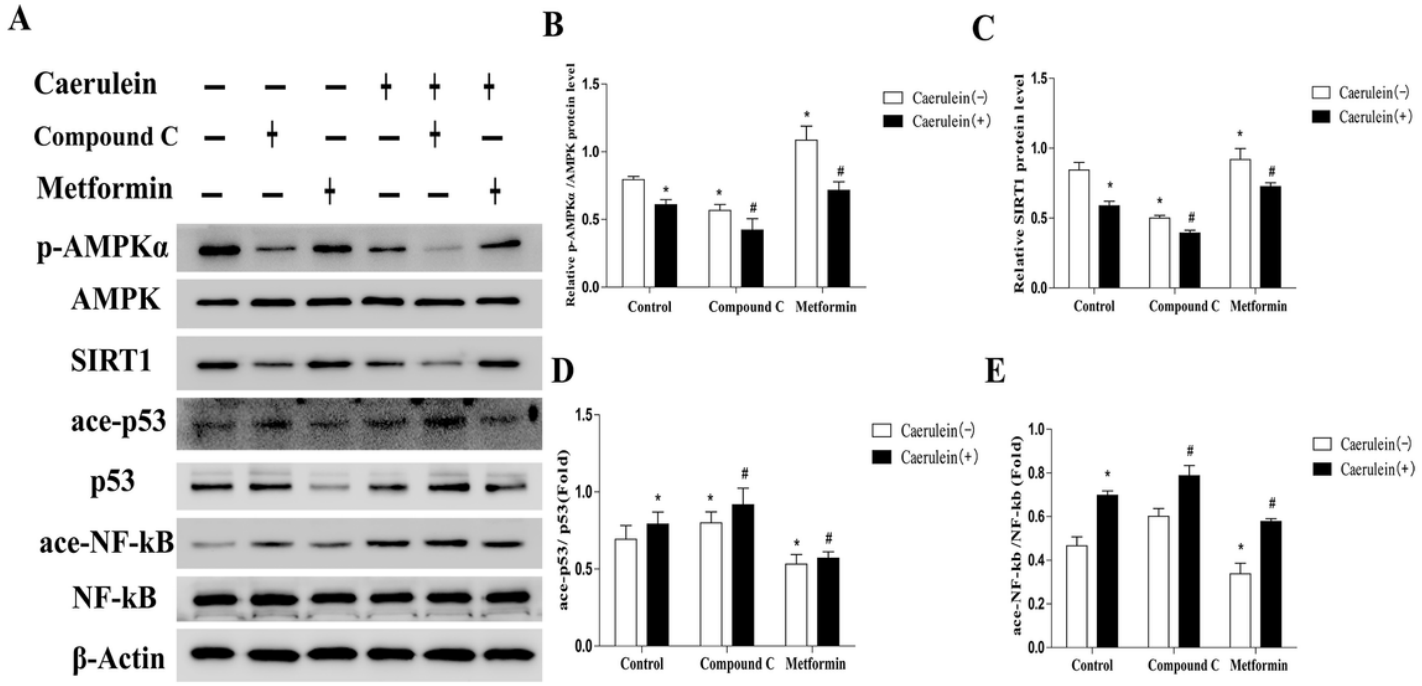

Figure 2

The protein levels of AMPK, SIRT1, ace-p53, ace-NF-κB in control and caerulein-stimulated group. (A) The representative protein images of AMPK, SIRT1, ace-p53, ace-NF-κB detected by western blot. (B) The relative protein levels of p-AMPKα/AMPK (B), SIRT1 (C), ace-p53 (D), ace-NF-κB (E) in control and caerulein-stimulated group. *p< 0.05 compared with control group; #p < 0.05 compared with caerulein-stimulated group.

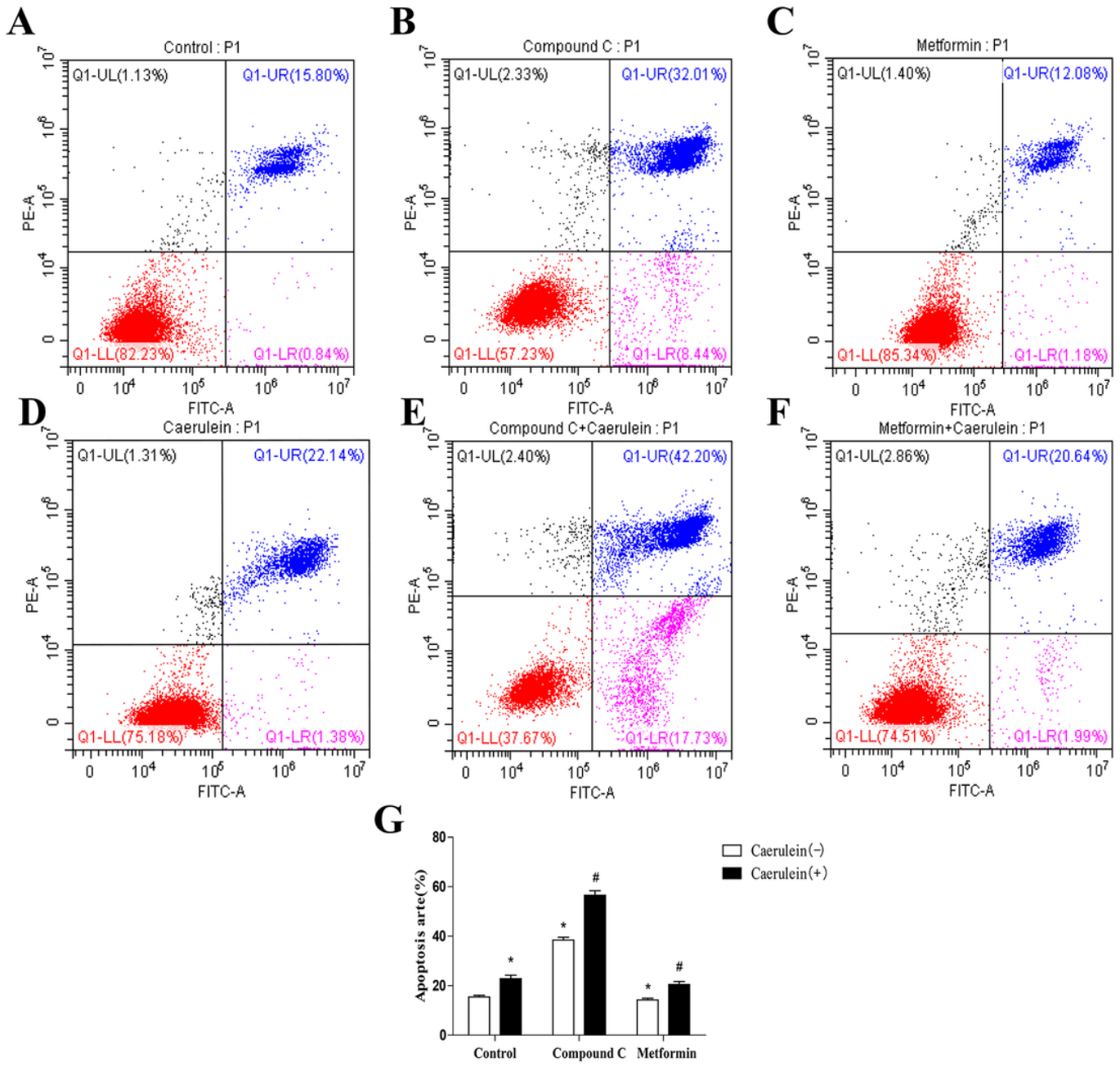

Figure 3

The apoptosis of AR42J cells in each group was determined by flow cytometry. Representative dot-plot images were shown for control group (A), compound C group (B), metformin group (C), control group + caerulein (D), compound C group+ caerulein (E), metformin group+ caerulein (F). (G) The quantitative results of apoptosis rate in each group were shown. *p< 0.05 compared with control group; #p < 0.05 compared with caerulein group.

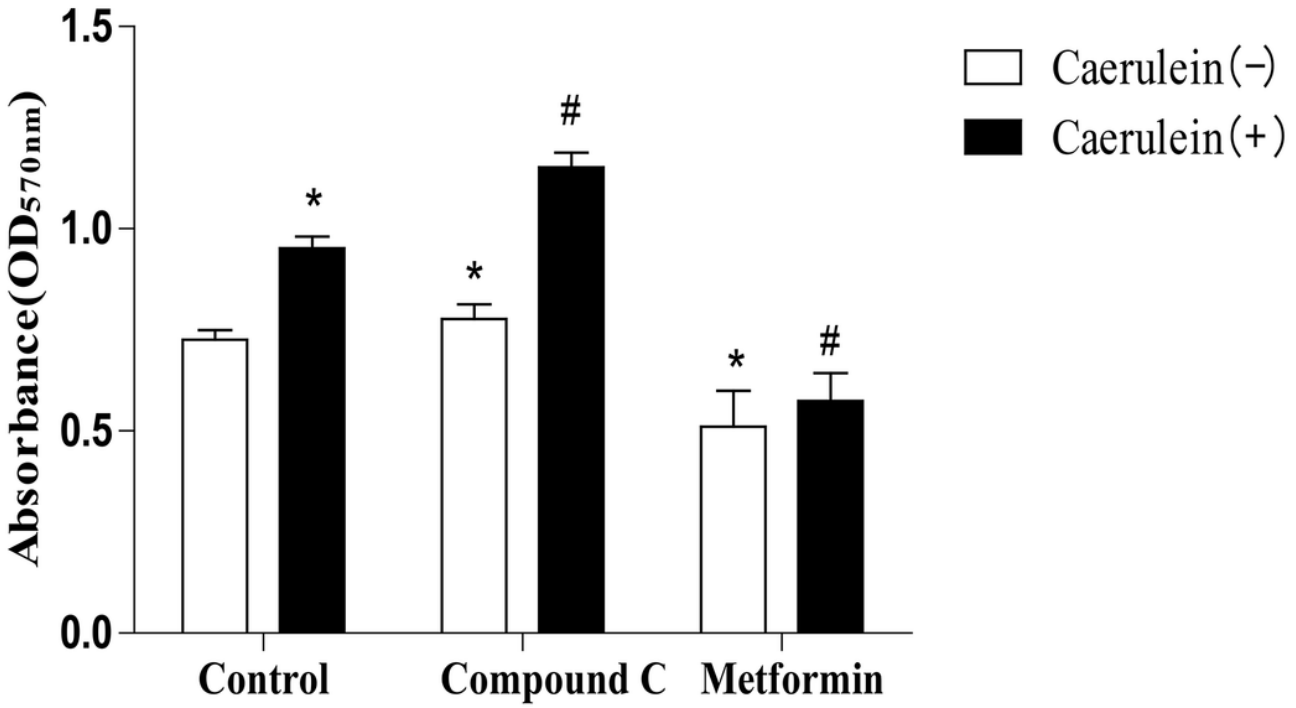

Figure 4

The cell proliferation was determined by MTT assay. *p< 0.05 compared with control group; #p < 0.05 compared with caerulein group.

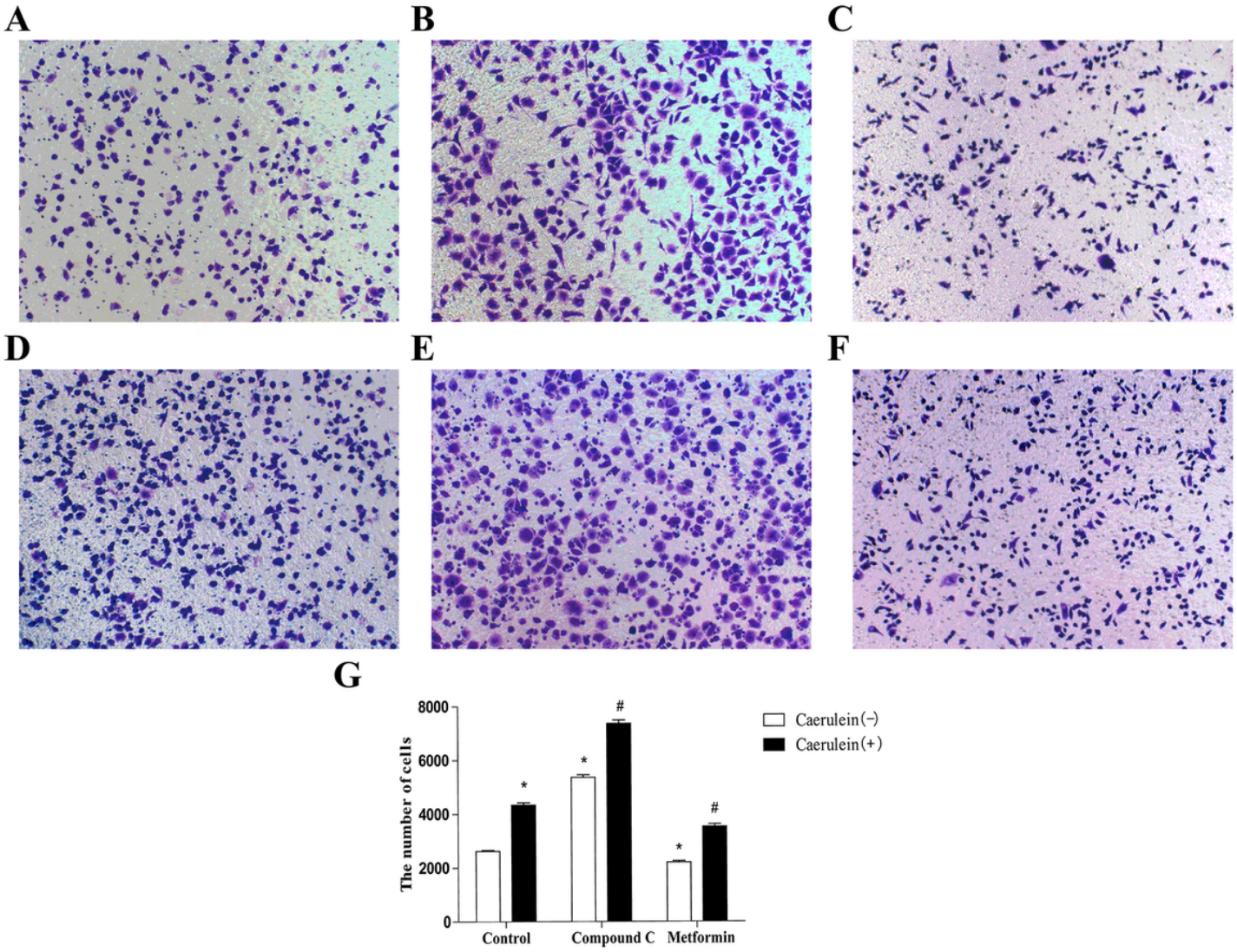

Figure 5

The migration of AR42J cells was determined by transwell assay. Representative dot-plot images were shown for the control group (A), the compound C group (B), the metformin group (C), control group + caerulein (D), compound C group+ caerulein (E), metformin group+ caerulein (F). (G) The quantitative results of migration rate in each group were shown. *p< 0.05 compared with control group; #p < 0.05 compared with caerulein group.