COX-2 siRNA Strengthens The Anti-Proliferative Effects of Acid And Bile Salts on Human Esophageal Cells And Barrett Esophageal Cells

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Abstract

Aims: Investigating the effect and mechanism of COX-2 on viability, intestinal metaplasia, and atypia of human esophageal squamous and Barrett esophageal cell lines.

Methods: Human esophageal squamous and Barrett esophageal cell lines were transfected with COX-2 expression vector and COX-2 siRNA, then were treated with acid, bile salts, and a mixture of both. Cell viability, the expression of COX-2, NF-κB, CDX-2, MUC2 and BMP-4, and the morphology and microstructure of cells were observed.

Results: The viability of the COX-2 over expressed cells was significantly higher than that of the control cells, while the viability of the COX-2 siRNA-treated cells was significantly lower than that of the control cells. The intestinal metaplasia and atypia were observed in cells over expressing COX-2. Acid, bile salts, and their mixture inhibited the viability of the two cell lines, but the inhibitory effect of the mixture was stronger than single treatment with either. siRNA of COX-2 strengthened the anti-proliferative effects of the mixture on Het-1A and BAR-T cells. Expression of NF-κB, CDX-2 and BMP-4 was positively correlated with COX-2 expression.

Conclusion: COX-2 may influence the viability, atypia and intestinal metaplasia of human esophageal cells and Barrett esophageal cells. Activation of NF-κB, CDX-2, and BMP-4 signaling pathway by COX-2 may be part of the mechanisms.

Introduction

Barrett's esophagus (BE) is characterized by the replacement of normal squamous epithelium (SQ) by intestinal-type columnar epithelium in the distal esophagus. BE is predominantly relevant to gastroesophageal reflux disease (GERD), genetics, obesity, lifestyle, gender and race, among which GERD is the most important[1]. Gastroesophageal reflux, mainly acid and bile, plays an important role in the occurrence of BE[2,3]. BE is a precancerous lesion of esophageal adenocarcinoma (EAC)[4]. Whether BE is derived from esophageal squamous cells or stem cells remains controversial, but most studies have suggested that BE may be derived from esophageal squamous cells and EAC is derived from BE. Now the mechanism by which BE occurs and develops into EAC is largely unknown[5,6,7].

Cyclooxygenase-2 (COX-2) is not only a key enzyme to initiate the inflammatory response[8,9], but also participates in the initiation and development of a variety of inflammatory conditions by promoting cell proliferation, inhibiting apoptosis, promoting angiogenesis, increasing the invasion ability of tumor cells, and inhibiting the immune function of the body[10,11,12,13,14]. It was found that COX-2 expression in human BE tissues was significantly higher than that in surrounding squamous cells and control tissues[15,16,17], and was significantly higher in EAC tissues[18], suggesting that COX-2 may be involved in the occurrence and development of BE. Previous studies have shown that the high expression of toll-like receptor 4 in BE can promote the strong expression of COX-2 and lead to the transformation of BE cells into atypia[19]. COX-inhibitors such as indomethacin could inhibit the growth of esophageal

adenocarcinoma in nude mice and induce its regression[20]. These studies suggest that COX-2 may also play an important role in the occurrence of EAC.

It is speculated that COX-2 may play an important role in the initiation and development of BE. In this study, we aimed to determine the effect of COX-2 on viability, intestinal metaplasia, and atypia in a normal esophageal squamous cell line (Het-1A) and a Barrett esophageal cell line(BAR-T). In order to determine mechanisms, we also tested the expression of nuclear factor kappa B(NF-κB), bone morphogenetic protein-4(BMP-4), caudal-related homeobox transcription factor-2(CDX-2), muc-2, and c-myb. The expression of COX-2 and cell viability in the presence of acids and bile salts, which simulates the human microenvironment, were investigated.

Materials And Reagents

Mouse anti-human monoclonal antibody and fluorescently labeled sheep anti-mouse secondary antibody of COX-2, p-P65, BMP-4, CDX-2 and GAPDH were all purchased from Epitomics. MTT and DMSO were available from Sigma. RNA extraction Trizol kit, lipofectamine liposome 2000, RPMI1640 and fetal bovine serum were purchased from Invitrogen, USA. The bile salts media contained a mixture of conjugated bile salts, including glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, glycodeoxycholic acid, and taurodeoxycholic acid (Sigma) in a 20:3:15:3:6:1 molar concentration as previously described[21].

Cell culture

The human esophageal squamous cell line Het-1A was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Het-1A is a normal human esophageal epithelial cell line immortalized by transfection of the SV40 T antigen early region gene[22]. BAR-T is a human Barrett’s esophagus cell line. Het-1A and BAR-T cells were cultured as previously described[23,24].

Cell proliferation

Cell proliferation was analyzed using the 3-(4,5-Dimethylthiazol-2-yl) -2,5- Diphenyltetrazolium bromide assay, according to the manufacturer’s instructions as described previously[25].

COX-2 siRNA transfection of Het-1A and BAR-T cells

COX 2 siRNA were acquired from Santa Cruz Biotechnology. Each siRNA was transfected into cells using Lipofectamine reagent according to the manufacturer's protocol. When cells of Het-1A grew to account for 90% of the bottom of the culture flask, they were digested, centrifuged, re-suspended and transferred to the 24-well plate. Transfection was performed when the cells grew to about 60%~70% of the fusion area. The experiment was divided into four groups: blank control group (only Het-1A/BAR-T cells were inoculated without any treatment), negative control group (transfected with negative siRNA to Het-1A/BAR-T cells), COX-2 group (transfected with COX-2 to Het-1A/BAR-T cells) and COX-2 siRNA group

(transfected with COX-2 siRNA to Het-1A/BAR-T cells). The western blot was utilized to detect COX-2 expression to determine the transfection effect.

Western blot analysis

The cells to be tested were inoculated in 6-well plates, cultured for 48h, collected, and washed with ice in PBS 3 times. Cells were lysed with 1% Triton X-100 lysate. Total protein was collected and SDS-PAGE gel electrophoresis and membrane transfer were performed. The primary antibody was mouse anti-human COX-2, CDX-2, BMP-4, p-P65, and GAPDH monoclonal antibody, and the second antibody was a sheep anti-mouse antibody labeled with fluorescence. Fluorescence color rendering was performed and the gray value of the reaction band was measured. GAPDH served as the internal reference, the relative value was computed. The experiments were repeated 3 times.

Acid and bile salts exposure of Het-1A and BAR-T cells

For individual experiments, cells were cultured in one of four different experimental media: 1) control medium which consisted of neutral full growth medium (pH 7.0). 2) neutral bile salts medium (containing conjugated bile acid with a total concentration of 500μM at pH 7.0). 3) acidic rich growth medium (brought to a pH of 6.0 with HCl). 4) acidic bile salts medium (the same bile acid solution at pH 6.0). The medias were inserted for 10 minutes to equally seeded wells of cells, then removed and replaced with a neutral pH medium until the next treatment. Het-1A and BAR-T cells were treated with either experimental or control medium 3 times per day for 7 days (unless otherwise stated).

Electron microscopy

Transmission electron microscopy (TEM) was used to detect ultra-structural changes in Het-1A and BAR-T cells. Cells were attached with 3% glutaraldehyde in 0.1 mM cacodylate buffer. Samples were post set at 1% osmium tetroxide, dehydrated in a graded series of ethanols, and integrated into epoxy resin. Ultrathin sections were measured for morphological changes using a Japan Electron Optics Laboratory JEM-2010 transmission electron microscope.

Statistical analysis

SPSS 19.0 statistical software was used for statistical analysis, and all measurement data were present as mean ± standard deviation (SD). The methods used were factorial analysis of variance, repeated measurement analysis of variance, and one-way analysis of variance. LSD method was used for multiple comparisons between groups. Results were examined statistically significant at $P< 0.05$.

Results

Effects of over expression or gene silencing of COX-2 on proliferation and morphology of Het-1A and BAR-T cells

Cell proliferation was assessed by MTS, as shown in Fig. 1. On the second day and the third day after over expression or silencing of COX-2 gene, the proliferation rate of COX-2 group was significantly higher than that of control group ($P < 0.05$), while cellular proliferation of siCOX-2 group was significantly lower than that of control group ($P < 0.05$). Meanwhile COX-2 was over expressed in Het-1A cells for 3 days, and the increase of microvilli on the cell surface was observed by electron microscopy, and the adenoid cavity structure was observed, suggesting intestinal metaplasia of the cells, while siRNA of COX-2 showed no such intestinal metaplasia. Nuclear abnormality and autophagosome were observed after COX-2 over expression in BAR-T cells for 3 days, suggesting atypia of the cells. siRNA of COX-2 induced no such changes(Fig. 2A,2B).

Effects of COX-2 over expression and gene silencing on COX-2, CDX-2, BMP-4, p-P65, P65, muc-2 and c-myb in Het-1A and BAR-T cells

Protein expression levels of COX-2, P65, p-P65, CDX-2, and BMP-4 were assessed by western blot on the second day after COX-2 over expression or gene silencing in the two cell lines. As showed in Fig. 3, COX-2 over expression or knockdown effects were achieved in both cell lines. Expressions of BMP-4, p-P65 and CDX-2 were positively correlated with COX-2, while the expression levels of P65, MUC2, and c-myb remained unchanged.

Effects of acid, bile salts, and their mixture on the proliferation of Het-1A and BAR-T cells

Different concentrations of bile salts were tested on both cell lines (Het-1A: 0μmol/L, 400μmol/L, 800μmol/L, 1200μmol/L; and BAR-T: 0μmol/L, 800μmol/L, 1200μmol/L, and 1600μmol/L). MTS test results were shown in Fig. 4A. In this experiment, bile salts concentrations of 1200μmol/L were selected for both cells, and treatment time was set at 0, 30, 60, 90min. For the experiment presented in Fig. 4B. The concentration of 1200μmol/L of bile salts was selected, and the treatment time was set as 30min, 60min and 90min to detect the COX-2 protein expression level. The detection results were presented in Fig. 4C: when HET-1A was treated with bile salts for 90min and BAR-T was treated with bile salts for 60min and 90min, the COX-2 expression was substantially up-regulated.

The PH value of the medium was adjusted with hydrochloric acid, and the cells were cultured in the medium with PH values of 4.0, 5.0, and 6.0. In the blank control group, medium was not treated with hydrochloric acid. Cell viability was measured by MTS, as shown in Fig. 5A, and COX-2 protein expression level was detected by western blot, as shown in Fig. 5B. We found that after incubation with hydrochloric acid at PH6.0, 5.0, and 4.0 for certain time, and the cell activity and COX-2 expression were both up-regulated. Based on the above experimental results, PH6.0 was selected for the treatment of both cell lines with Het-1A being treated for 30min and BAR-T for 60min.

According to the above experimental results, four groups (bile salts and hydrochloric acid) were set, namely, the control group (0μmol/L, PH7), the bile salts group (1200μmol/L, PH7), the hydrochloric acid group (0μmol/L, PH6), the hydrochloric acid, and bile salts mixed group (1200μmol/L, PH6). Cell proliferation was detected by MTS after 30min treatment of Het-1A and 60min treatment of BAR-T. As

shown in Fig. 6, acid, bile salts and the mixture of the both inhibited the proliferation of the two cell lines, but the inhibitory effect of bile salts + hydrochloric acid was stronger than bile salts or hydrochloric acid treatments.

Effects of acid, bile salts, and their mixture on COX-2, CDX-2, BMP-4, and p-P65 expression in Het-1A and BAR-T cells

According to the above groups, protein expression levels of COX-2, P65, p-P65, CDX-2, and BMP-4 were detected after 30min treatment of Het-1A and 60min treatment of BAR-T. As shown in Fig. 7, the protein expressions of COX-2, CDX-2, BMP-4, and p-P65 in each group were increased compared with the normal group, and the expression of these proteins in bile salts and hydrochloric mixed group was strongest. However, the expression of P65 was not changed in all groups.

Effects of COX-2 gene silencing on the proliferation of Het-1A and BAR-T cells after acid and bile salts treatment

The cells were transfected with COX-2 siRNA, and treated with hydrochloric acid PH6.0 and bile salts of 1200μmol/L for 48h before sample collection. Het-1A cells were treated for 30min and BAR-T for 60min. Cell proliferation was detected by MTS, and the results are presented in Fig. 8. COX-2 siRNA silencing further enhanced the inhibitory effect of acid and bile salts mixture on the proliferation of Het-1A and BAR-T cells.

Effects of COX-2 gene silencing on expression of COX-2, CDX-2, BMP-4, and p-P65 in Het-1A and BAR-T cells after acid and bile salts treatment

The cells were transfected with COX-2 siRNA, and treated with hydrochloric acid (PH6.0) and bile salts (1200μmol/L) for 48h before sample collection. Het-1A cells were treated for 30min and BAR-T cells were treated for 60min. Protein expression levels of COX-2, P65, p-P65, CDX-2, and BMP-4 were identified by western blot. As shown in Fig. 9, the expressions of COX-2, CDX-2, BMP-4 and p-P65 proteins were up-regulated after treatment with acid and bile salts mixture, while they were down-regulated after COX-2 siRNA was transfected. The expression of P65 was not changed.

Effects of acid and bile salts mixture on the morphology of Het-1A and BAR-T cells before and after COX-2 gene silencing

After the mixture of acid and bile salts acted on the cells, nuclear inclusion bodies, autophagosome-like structures, and other cellular morphological manifestations were observed in Het-1A cells. Due to the damage of the cells induced by acid and bile salts, changes such as incomplete capsule, formation of vacuolar structure in cytoplasm, mitochondrial swelling, cavitation, and disappearance of the chute, and the intestinal metaplasia of the cells were not obvious. Heteromorphic changes such as nuclear heteromorphism was found in BAR-T cells. After gene silencing of COX-2 followed by treatment with a mixture of acid and bile salts, no such changes were seen in the two cell lines(Fig. 10A,10B).

Discussion

In this study, we showed that over expression of COX-2 in Het-1A cells could promote cell proliferation, accompanied by intestinal metaplasia, while COX-2 siRNA could inhibit cell proliferation and prevent the emergence of intestinal metaplasia. In BAR-T cells, over expression of COX-2 could promote cell proliferation, accompanied by cellular heteromorphism, while COX-2 siRNA could inhibit cell proliferation and the development of heteromorphism. These results suggest that COX-2 may play a major role in the occurrence and development of BE, which is consistent with our hypothesis.

As an essential transcription factor of inflammatory response, NF-κB is believed to play an important role in the development of cancer and participate in apoptosis of various cells and tissues[26, 27]. Studies have found that NF-κB is increased in BE and esophageal adenocarcinoma tissues, which may play a role by activating surviving, an anti-apoptotic factor[28]. Inhibition of NF-κB in esophageal squamous cells inhibited cell proliferation, accompanied by decreased COX-2 expression[29]. Inhibition of NF-κB expression in EAC cells reduced the expression of COX-2 and CDX-2, and enhanced apoptosis of EAC cells[30]. The above studies suggest that NF-κB plays a major role in the occurrence and development of BE, and NF-κB acts as the upstream molecule to regulate the expression of COX-2. Park et al. found that celecoxib, a COX-2 inhibitor in leiomyoma cells, could inhibit cell proliferation through the NF-κB pathway, suggesting that COX-2 could regulate NF-κB in leiomyoma cells[31]. In this study, we found that COX-2 could regulate the expression of NF-κB in Het-1A and BAR-T cells, and NF-κB may play a significant role in the effect of COX-2 on Het-1A and BAR-T cell proliferation and cell morphology changes.

Bone morphogenetic proteins (BMPs) are mainly expressed in embryonic development or disease states such as cancer tissue, and its family members can participate in cell proliferation, migration, apoptosis, and differentiation[32]. Studies have shown that BMP-4 was highly expressed in BE and EAC tissues, and its downstream signaling molecule ID2 was also highly expressed, suggesting that BMP-4 signaling pathway was activated in BE and EAC[33]. After recombinant BMP-4 treatment in vitro, normal squamous epithelial cells were transformed into columnar epithelial cells, and intestinal epithelial markers Villin and CDX-2 were detected[34]. BMPs signaling pathway could activate SOX9 and play an important role in the occurrence and development of BE[35]. In this study, it was shown that COX-2 could regulate the expression of BMP-4 in Het-1A and BAR-T cells, and BMP-4 may also play an important role in the effect of COX-2 on Het-1A and BAR-T cell proliferation and cell morphology.

As a member of the caudal homologous nuclear transcription factor family, CDX is a nuclear transcription factor specifically expressed during intestinal development and regulates the proliferation and differentiation of intestinal epithelial cells[36, 37]. CDX-2 plays a key role in intestinal metaplasia of BE, and its expression in the esophagus is an early explicit marker of intestinal metaplasia[38, 39, 40]. In the environment of acid or bile acid, demethylation of the promoter could promote the expression of CDX-2 in esophageal epithelium and promote intestinal metaplasia[41]. It could be involved in the carcinogenic mechanism of EAC by inhibiting the expression of the DNA repair enzyme and promoting the expression of CDX-2[42]. In this study, we found that COX-2 could regulate CDX-2 expression in Het-1A cells,

suggesting that COX-2 played a more prominent role in intestinal metaplasia of esophageal squamous cells. CDX-2 was highly expressed in digestive tract tumors and is involved in the occurrence of EAC[43, 44]. In this study, we found that COX-2 could regulate the expression of CDX-2 in BAR-T cells, suggesting a role in the occurrence and heteromorphism of BE.

MUC2 is mainly expressed in intestinal metaplasia and malignant lesions of BE[45, 46, 38]. C-myb is an intra nuclear oncogene, which is involved in cell proliferation and plays an important role in the proliferation regulation of numerous malignant tumor cells. Studies have shown that up-regulation of mRNA expression of c-myb was an early event in the process of BE turning to esophageal cancer[47, 48]. However, changes in COX-2 expression in Het-1A and BAR-T cells did not cause changes in the expressions of MUC2 and c-myb, possibly because the regulation of the two proteins was not dependent on COX-2.

The above studies indicate that COX-2 plays an important role in the process of intestinal metaplasia of esophageal squamous epithelial cells and atypia of BE cell, and its mechanism is to regulate the expression of p-P65, BMP-4, CDX-2, and other cytokines. To verify these conclusions, we used different concentrations of acid, bile salts and a mixture of the two to treat Het-1A and BAR-T cells by simulating human microenvironment of GERD, and we assessed the role and possible mechanism of COX-2 in this process.

Our study showed that the proliferation of Het-1A or BAR-T cells was inhibited under the action of acid, bile salts, and the mixture of the two, and the mixture of acid and bile salts had the strongest effect. These treatments resulted in increased expressions of COX-2, BMP-4, p-P65, and CDX-2. SiRNA of COX-2 could enhance the inhibitory effect of acid, bile salts and the mixture of the two on the proliferation of Het-1A and BAR-T cells, accompanied by the reduced expressions of COX-2, BMP-4, p-P65, and CDX-2. These observations suggest that in the environment of acid, bile salts, and the mixture of the two, the proliferation of Het-1A and BAR-T cells is closely related to the expression of COX-2, which further regulates cytokines such as p-P65, BMP-4, and CDX-2.

Conclusions

COX-2 plays an important role in the occurrence and development of BE, which can be used as a target for diagnosis and treatment of BE and EAC. Activation of NF-κB, CDX-2, and BMP-4 signaling pathway by COX-2 may be part of the mechanisms. In the process of BE occurrence and heteromorphism transformation, acid, bile salt and their mixture play a certain role, and the mixture of the two plays the strongest role. In the clinical treatment of BE and GERD, we should not only pay attention to the role of acid, but also pay attention to whether there is bile reflux.

Abbreviations

BE Barrett's esophagus

SQ squamous epithelium

GERD gastroesophageal reflux disease

EAC esophageal adenocarcinoma

COX-2 Cyclooxygenase-2

Het-1A esophageal squamous cell line

BAR-T Barrett esophageal cell line

NF-κB nuclear factor kappa B

BMP-4 bone morphogenetic protein-4

CDX-2 caudal-related homeobox transcription factor-2

TEM Transmission electron microscopy

Declarations

Availability of data and materials

All data generated or analysed during this study are included in this published article. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Jiangang Shen: Conceptualization, Methodology, Formal analysis, Investigation, Visualization; Nayoung Kang: Conceptualization, Methodology, Formal analysis, Writing – reviewing&editing; Hongfang Wang: Investigation, Formal analysis, Resources; Junda Li: Investigation, Formal analysis; Li Chen: Investigation, Formal analysis; Xuefeng Bai: Writing – original draft; Mingsong Li: Supervision, Project administration, Funding acquisition

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Figures

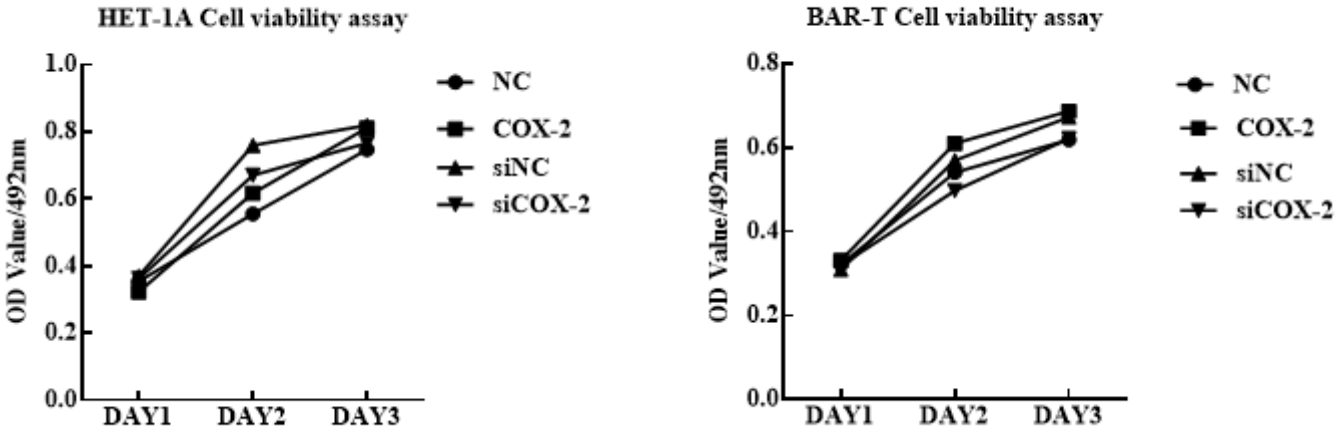


Figure 1

The overexpression and gene silencing of COX-2 were detected by MTS in Het-1A and BAR-T cells.

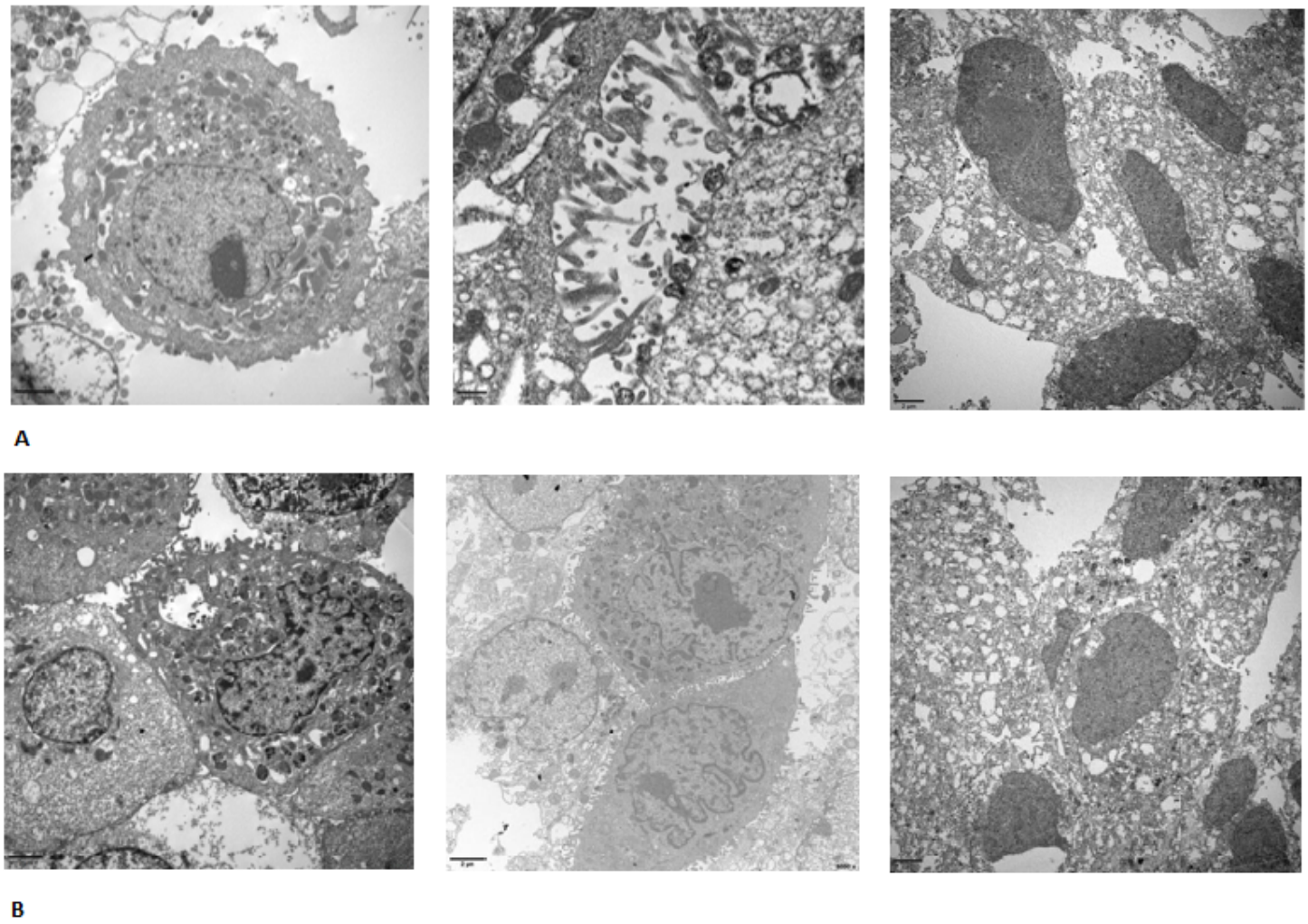


Figure 2

The three pictures were negative control group, COX-2 group and COX-2 siRNA group in Het-1A cells for 3 days. The three pictures were negative control group, COX-2 group and COX-2 siRNA group in BAR-T cells for 3 days.

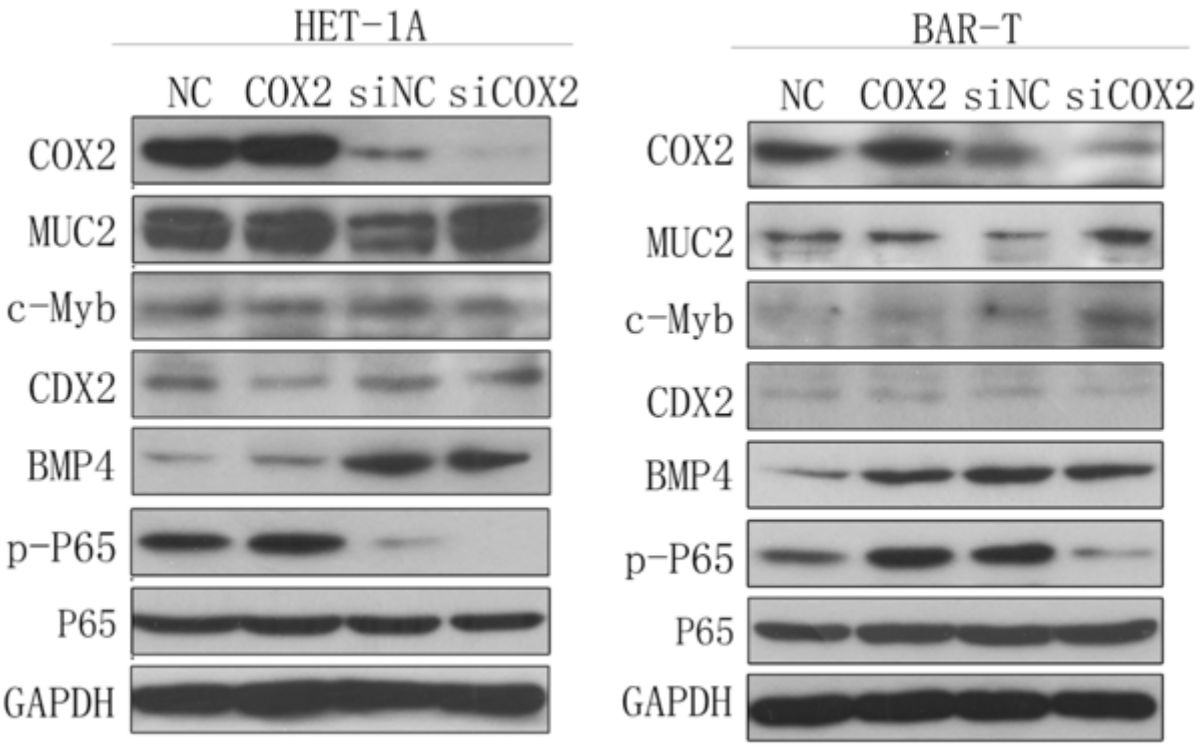


Figure 3

The expression levels of COX-2, CDX-2, BMP-4, p-P65, P65, muc-2 and c-myb were detected by western blot in Het-1A and BAR-T cells.

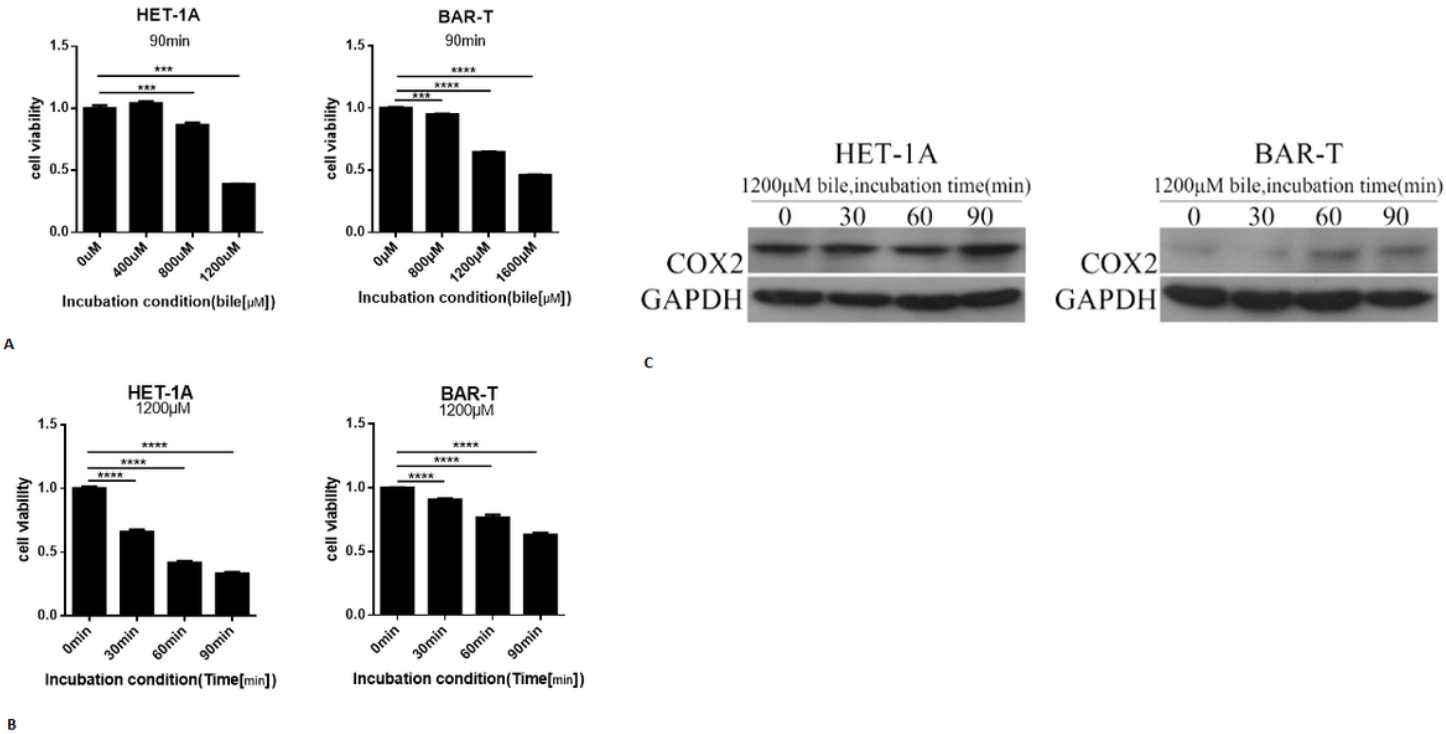
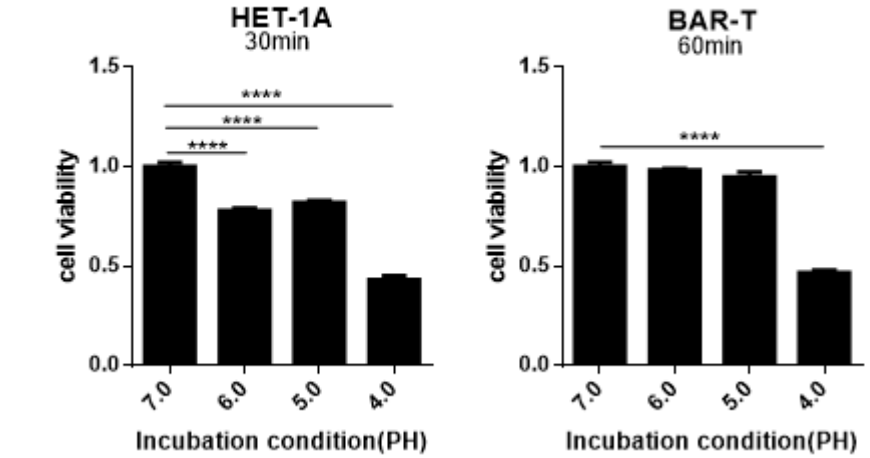
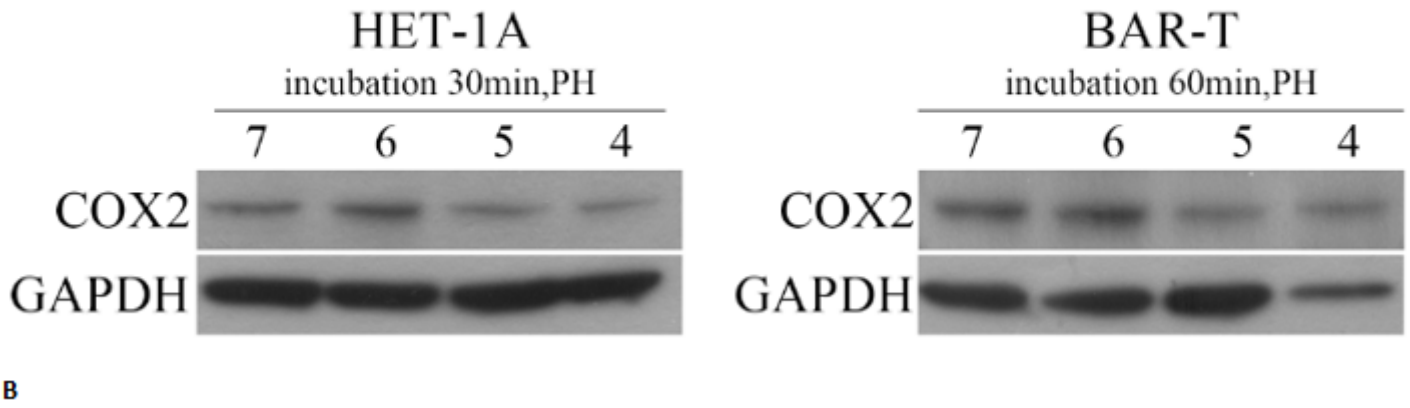


Figure 4

After 90 minutes, cell proliferation was assessed by MTS in different concentrations of bile salts in Het-1A and BAR-T cells. In the concentration of 1200μmol/L of bile salts, cell proliferation was assessed by MTS in different times in Het-1A and BAR-T cells. In the concentration of 1200μmol/L of bile salts, the expression levels of COX-2 were detected by western blot in different times in Het-1A and BAR-T cells.



A



B

Figure 5

After 30 minute treatment in Het-1A cells and 60 minutes treatment in BAR-T cells, cell proliferation was assessed by MTS in different concentrations of hydrochloric acids. After 30 minute treatment in Het-1A cells and 60 minutes treatment in BAR-T cells, the expression levels of COX-2 were detected by western blot in different concentrations of hydrochloric acids.

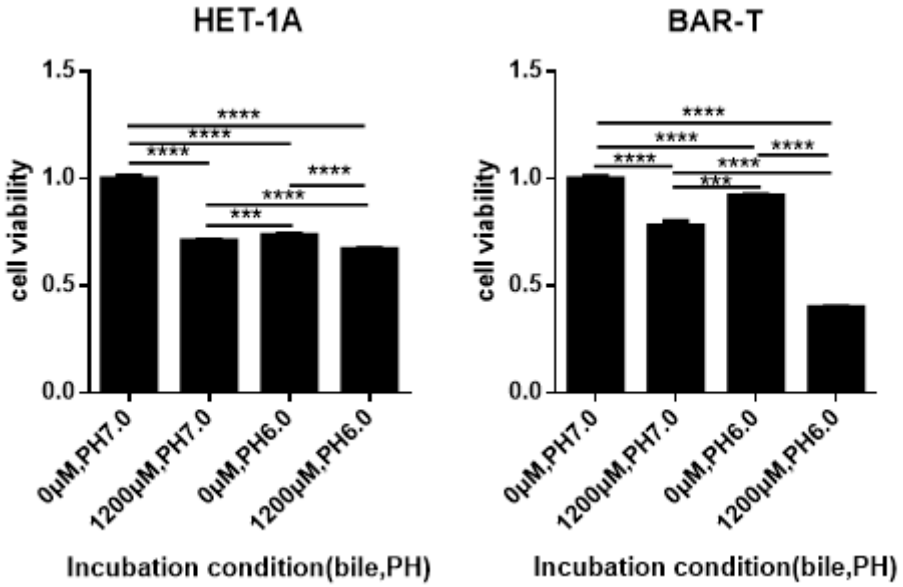


Figure 6

After 30 minute treatment in Het-1A cells and 60 minutes treatment in BAR-T cells, cell proliferation was detected by MTS in four groups.

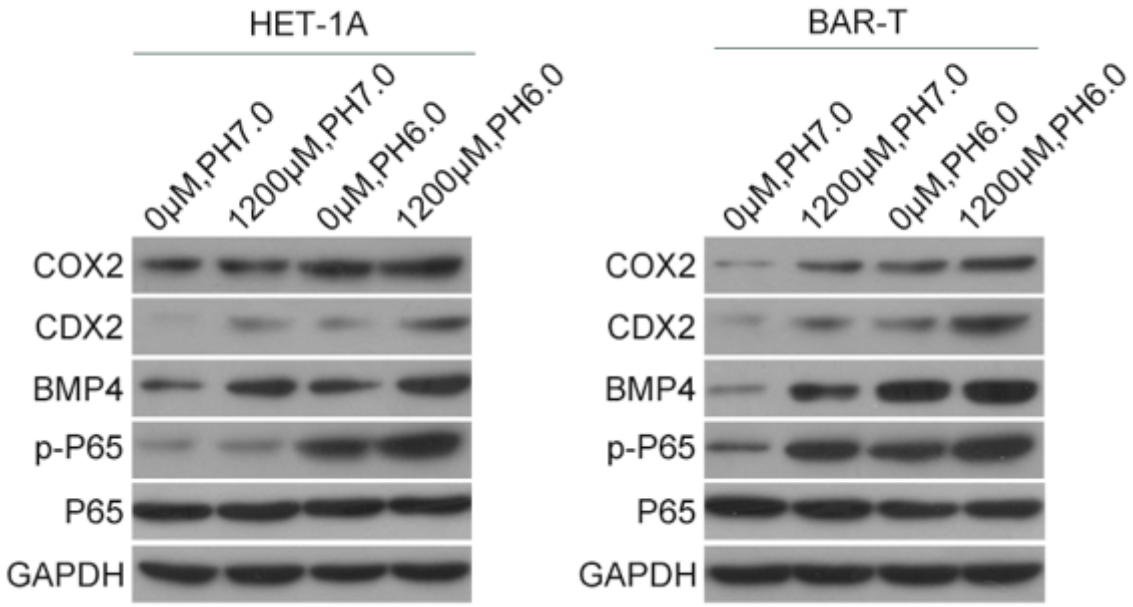


Figure 7

The expression levels of COX-2, CDX-2, BMP-4, p-P65, and P65 were detected by western blot in four groups in Het-1A and BAR-T cells.

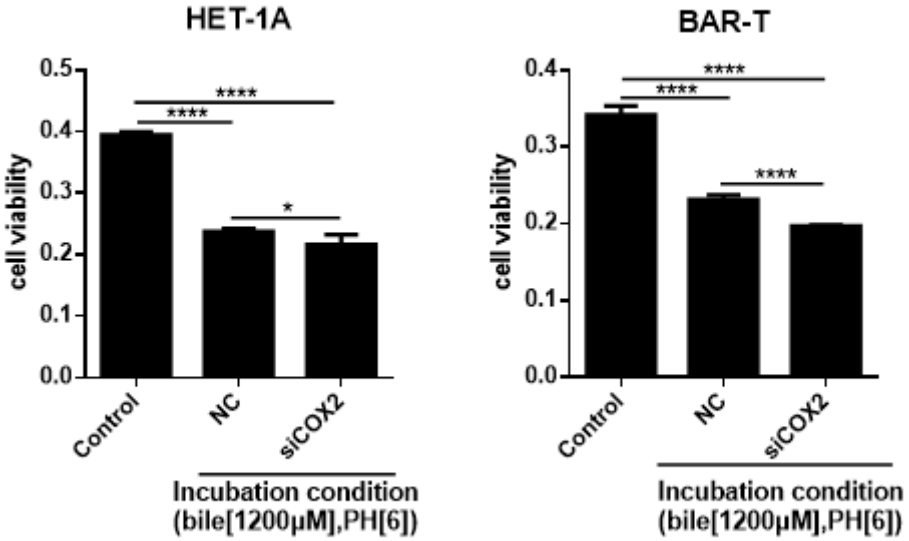


Figure 8

After acid and bile salts treatment and COX-2 gene silencing, cell proliferation was detected by MTS in Het-1A and BAR-T cells.

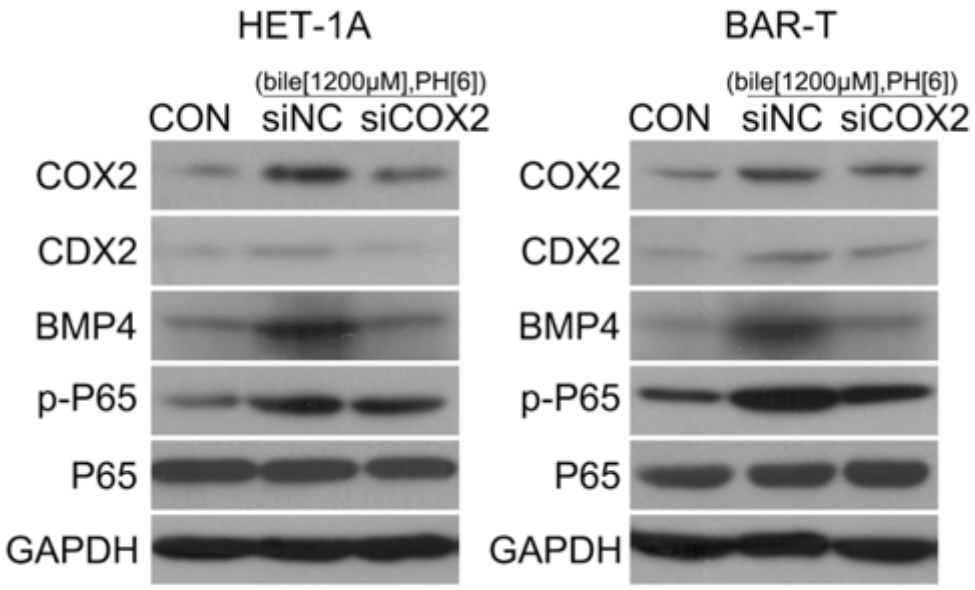


Figure 9

After acid and bile salts treatment and COX-2 gene silencing, the expression levels of COX-2, CDX-2, BMP-4, p-P65, and P65 were detected by western blot in Het-1A and BAR-T cells.

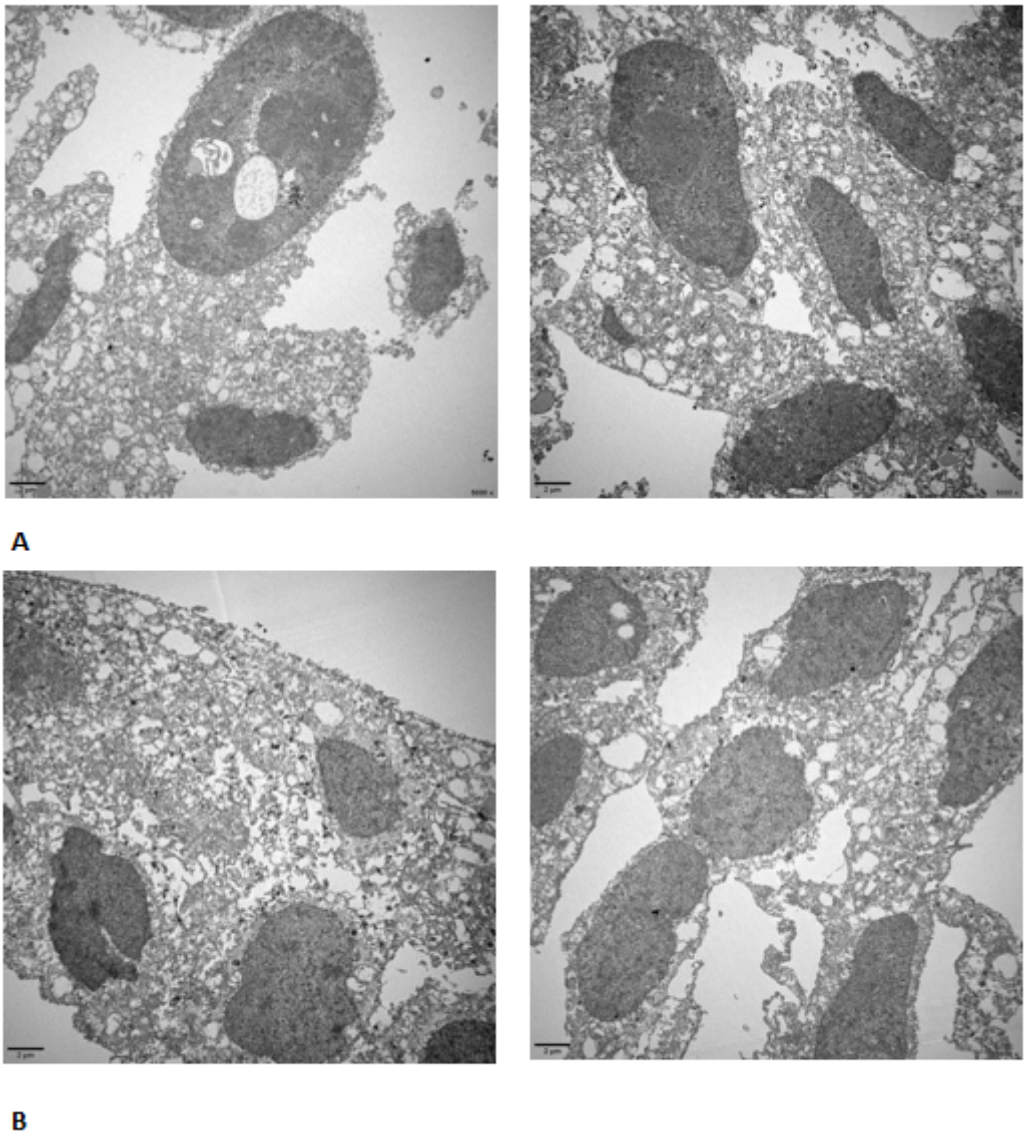


Figure 10

The pictures were the mixture of acid and bile salts groups before and after COX-2 gene silencing in Het-1A cells. The pictures were the mixture of acid and bile salts groups before and after COX-2 gene silencing in BAR-T cells.