

## Original Research Article

# Aspirin inhibits proliferation of gastric cancer cells via IL-6/STAT3 signaling pathway

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Sent for review: 16 January 2022

Revised accepted: 11 July 2022

### Abstract

**Purpose:** To study the effect of aspirin on the proliferation and apoptosis of gastric cancer cells, and its key molecular mechanism of action.

**Methods:** Gastric cancer SGC7901 cells were treated with aspirin at concentrations of 0, 1, 2 and 4 mmol/L. Cell proliferation was measured using cell counting kit (CCK)-8 assay, while messenger ribonucleic acid (mRNA) expressions of interleukin (IL)-6, B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax) were assessed by reverse transcription-polymerase chain reaction (RT-PCR). Cell apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Furthermore, the protein expression levels of the signal transducer and activator of transcription 3 (STAT3), phosphorylated STAT3 (p-STAT3), Bcl-2 and Bax were evaluated by Western blotting.

**Results:** Compared with control group, 1, 2 and 4 mmol/L aspirin groups showed lower cell proliferation, and decreased mRNA expressions of Bcl-2 and Bax and IL-6 release at 24, 48 and 72 h ( $p < 0.05$ ). Cell apoptosis in the aspirin groups was higher than in the control group. Also, compared with the control group, 1 mmol/L aspirin group did not exhibit significant changes in the expressions of STAT3 and p-STAT3 at 72 h. On the other hand, the 2 mmol/L aspirin group at 72 h and the 4 mmol/L aspirin group exhibited significant increases in the expressions of STAT3 and p-STAT3 ( $p < 0.05$ ). Furthermore, the levels of Bcl-2 and Bax declined in the aspirin groups when compared with the control group ( $p < 0.05$ ).

**Conclusion:** Aspirin inhibits the proliferation of gastric cancer SGC7901 cells, and induces their apoptosis *in vitro* in IL-6/STAT3 signaling pathway. The results of the current study may provide new insight into the treatment of gastric cancer.

**Keywords:** Aspirin, Gastric cancer cells, Cell apoptosis, IL-6/STAT3

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## INTRODUCTION

Gastric cancer is the most common cancer of the digestive tract, and although its morbidity and mortality have been well controlled in recent

years, comprehensive treatment of gastric cancer has progressed slowly in the past few decades [1]. Therefore, there is an urgent need to study more effective comprehensive treatment methods for gastric cancer. Aspirin is a non-

selective cyclooxygenase inhibitor with strong antipyretic and analgesic effects, and it is widely used for anti-inflammatory and anti-rheumatic diseases [2]. Small-dose aspirin prevents the onset of cardiovascular and cerebrovascular diseases, as well as transient ischemic attack [3].

In recent years, many studies have identified the anti-cancer effects of aspirin. It has been reported that people who regularly take aspirin or other non-steroidal anti-inflammatory drugs have a lower risk of colon cancer, gastric cancer, esophageal cancer, lung cancer and breast malignancy [4-7]. Non-steroidal anti-inflammatory medications suppress the synthesis of prostaglandin and block prostaglandin-induced immunosuppression to enhance immune responses and induce cell apoptosis [8]. The purpose of this study was to explore the influences of aspirin on the proliferation and apoptosis of gastric cancer cells through the interleukin (IL)-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway.

## EXPERIMENTAL

### Materials

Gastric cancer SGC7901 cells were purchased from the Cell Bank (Shanghai, China). Aspirin was purchased from Sigma (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 and fetal bovine serum (FBS) from Gibco (Rockville, MD, USA). STAT3, phosphorylated STTA3 (p-STAT3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies and horseradish peroxidase-labeled goat anti-rabbit/rat secondary antibodies were all from Abcam (Cambridge, MA, USA); Cell counting kit (CCK)-8 was purchased from Dojindo (Kumamoto, Japan); and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

### Cell culture and grouping

Gastric cancer SGC7901 cells were first cultured using 10 % FBS + RPMI1640 in an incubator with 5 % CO<sub>2</sub> and saturated humidity at 37 °C and sub-cultured for 2 - 3 d. The cells in logarithmic growth phase was then harvested, and the cell concentration was adjusted to 1.2 × 10<sup>5</sup> cells/L. Subsequently, no drug was administered in control group, while 1, 2 or 4 mmol/L aspirin was added to experiment groups based on previous experimental studies [9,10], with duplicate set in each group.

### Determination of gastric cancer cell proliferation

Following aspirin treatment, the cells at the adjusted concentration of 1.2 × 10<sup>5</sup> cells/L in each group were inoculated into a 96-well plate, and the cells in control group contained only medium, while those in experimental groups contained 1, 2 or 4 mmol/L aspirin. After culture for 24, 48 and 72 h, absorbance (A) was measured at 450 nm using a microplate reader. Inhibition (H) was computed as in Eq 1.

$$H (\%) = (Ae/Ac)100 \dots\dots\dots (1)$$

where Ae and Ac are the absorbance of experimental and control groups, respectively.

### Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. Real-time PCR was performed using a FastStart Universal SYBR Green Master kit (Roche, Basel, Switzerland) under the following conditions: 95 °C for 7 min, 95 °C for 12 s and 60 °C for 40 s for 40 cycles. The relative expression levels of genes were calculated using 2<sup>-ΔΔCt</sup> method.

**Table 1:** Primer sequences used in PCR

| Gene  |         | Primer sequence             |
|-------|---------|-----------------------------|
| IL-6  | Forward | 5'-ATGCGCTACGTGCTAGC-3'     |
|       | Reverse | 5'-TACGCGATGCACGATCG-3'     |
| Bcl-2 | Forward | 5'-GTCATTGTACAGTA-3'        |
|       | Reverse | 5'-CAGTAACATGTCAT-3'        |
| Bax   | Forward | 5'-TGTAGTCAACTTCGAG-3'      |
|       | Reverse | 5'-ACATCAGTTGAAGCTC-3'      |
| U6    | Forward | 5'-TCCGATCGTGAAGCGTTC-3'    |
|       | Reverse | 5'-GTGCAGGTCGAGGGGT-3'      |
| GAPDH | Forward | 5'-ACAAGCCTCAAGATCATCAGC-3' |
|       | Reverse | 5'-CACGCCACAGTTTCCCGGAG-3'  |

### Determination of IL-6 level

After the cell supernatant was collected, the experimental samples were added to the microtiter plate following the ELISA kit manufacturer's instructions, and incubated in a horizontal shaker flask for 2 h at room temperature. The plate was then washed with washing solution. After washing, enzyme-labeled detection antibody was added to each well and incubated on a horizontal shake flask for another

2 h at room temperature. IL-6 levels were determined by ELISA.

### TUNEL apoptosis assay

After 72 h of culture, the cells were fixed, washed, and permeabilized with 0.1 % Triton X-100 following the instructions of TUNEL Apoptosis Detection Kit manufacturer's. TUNEL-positive cells were observed under a fluorescence microscope. The TUNEL-positive cell rate was calculated based on 10 visual fields represent apoptosis rate of gastric cancer cells.

### Western blotting

Total proteins were extracted from each group of cells, and their concentration was determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Then, the prepared sodium dodecyl sulphate (SDS) protein loading buffer was mixed and boiled at 95 °C for 3 min. Subsequently, an equal volume of proteins was isolated *via* electrophoresis using 8 – 10 % polyacrylamide gel (Beijing Applygen, Beijing, China), transferred onto nitrate films, sealed using 10 % skim milk and incubated with the primary antibodies at 4 °C overnight. After the proteins were washed using tris-buffered saline with Tween-20 (TBST) for 3 times, they were incubated with the corresponding secondary antibodies for 1 h, washed with TBST 3 times, and added with electrochemiluminescence (ECL) solution for exposure and image development. Finally, the relative expression of the target

protein was analyzed using ImageJ software (NIH, Bethesda, MD, USA).

### Statistical analysis

SPSS statistical analysis software (version 26.0) was used for statistical analysis, and measurement data are presented as mean ± standard deviation. Differences between the two groups were analyzed using the Student's t-test. Comparison between multiple groups was done using one-way ANOVA test followed by post-hoc test (least significant difference).  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effect of aspirin on gastric cancer cell proliferation

Compared with that in the control group, the inhibition of cell proliferation rose in 1 mmol/L and 2 mmol/L aspirin groups ( $p < 0.05$ ), as well as in 4 mmol/L aspirin group ( $p < 0.01$ ). Besides, aspirin inhibited gastric cancer cells in a time- and concentration-dependent manner (Table 2).

### MRNA expressions of IL-6, Bcl-2 and Bax

Compared with that in the control group, the mRNA expressions of IL-6, Bcl-2 and Bax were decreased in 1 mmol/L and 2 mmol/L aspirin groups ( $p < 0.05$ ), and declined obviously in 4 mmol/L aspirin group ( $p < 0.01$ ) in a time- and concentration-dependent manner (Table 3).

**Table 2:** Comparison of influence of aspirin on gastric cancer cell proliferation (mean ± SD, n = 3)

| Group            | 24 h (%)                | 48 h (%)                | 72 h (%)                |
|------------------|-------------------------|-------------------------|-------------------------|
| Control          | 0 ± 0                   | 0 ± 0                   | 0 ± 0                   |
| 1 mmol/L aspirin | 13.18±2.82 <sup>a</sup> | 15.84±2.66 <sup>a</sup> | 32.44±9.51 <sup>a</sup> |
| 2 mmol/L aspirin | 16.57±3.37 <sup>a</sup> | 22.38±3.15 <sup>a</sup> | 47.19±5.14 <sup>b</sup> |
| 4 mmol/L aspirin | 29.87±3.08 <sup>b</sup> | 42.25±5.08 <sup>b</sup> | 77.27±8.29 <sup>b</sup> |

**Note:** <sup>a</sup> $P < 0.05$  and <sup>b</sup> $p < 0.01$  vs. control group

**Table 3:** Comparison of IL-6, Bcl-2 and Bax mRNA expressions in gastric cancer cells among all groups (mean ± SD, n = 3)

| Time | Group            | IL-6 mRNA              | Bcl-2 mRNA             | Bax mRNA               |
|------|------------------|------------------------|------------------------|------------------------|
| 24 h | Control          | 1.00 ± 0.02            | 1.00 ± 0.02            | 1.00 ± 0.02            |
|      | 1 mmol/L Aspirin | 0.78±0.82 <sup>a</sup> | 0.79±0.22 <sup>a</sup> | 0.81±0.12 <sup>a</sup> |
|      | 2 mmol/L Aspirin | 0.67±0.37 <sup>a</sup> | 0.69±0.87 <sup>a</sup> | 0.67±0.30 <sup>a</sup> |
|      | 4 mmol/L Aspirin | 0.33±0.08 <sup>b</sup> | 0.45±0.16 <sup>b</sup> | 0.48±0.90 <sup>b</sup> |
| 48 h | Control          | 1.00 ± 0.02            | 1.00 ± 0.02            | 1.00 ± 0.02            |
|      | 1 mmol/L Aspirin | 0.64±0.66 <sup>a</sup> | 0.69±0.56 <sup>a</sup> | 0.70±0.16 <sup>a</sup> |
|      | 2 mmol/L Aspirin | 0.58±0.15 <sup>a</sup> | 0.66±0.76 <sup>a</sup> | 0.61±0.05 <sup>a</sup> |
|      | 4 mmol/L Aspirin | 0.25±0.08 <sup>b</sup> | 0.35±0.91 <sup>b</sup> | 0.37±0.12 <sup>b</sup> |
| 72 h | Control          | 1.00 ± 0.01            | 1.00 ± 0.01            | 1.00 ± 0.01            |
|      | 1 mmol/L Aspirin | 0.50±0.51 <sup>a</sup> | 0.61±0.93 <sup>a</sup> | 0.69±0.59 <sup>a</sup> |
|      | 2 mmol/L Aspirin | 0.41±0.14 <sup>b</sup> | 0.51±0.14 <sup>b</sup> | 0.52±0.19 <sup>b</sup> |
|      | 4 mmol/L Aspirin | 0.17±0.29 <sup>b</sup> | 0.25±0.01 <sup>b</sup> | 0.21±0.97 <sup>b</sup> |

**Note:** <sup>a</sup> $P < 0.05$  and <sup>b</sup> $p < 0.01$  vs. control group

### Expression levels of IL-6

Compared with the control group, 1 mmol/L aspirin group showed no obvious differences in the expression level of IL-6 at 24 and 48 h ( $p > 0.05$ ), and a decline at 72 h ( $p < 0.05$ ). The 2 mmol/L aspirin group had decreased IL-6 ( $p < 0.05$ ), and 4 mmol/L aspirin group exhibited a decline in the expression level of IL-6 at 24 h, and obvious decreases at 48 and 72 h ( $p < 0.01$ , Table 4).

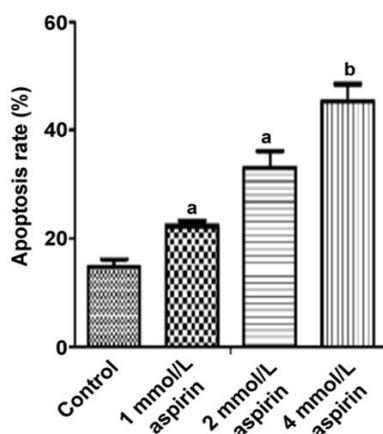
**Table 4:** Influence of aspirin on IL-6 expression level in each group of gastric cancer cells (pg/mg, mean  $\pm$  SD, n = 3)

| Group            | 24 h                          | 48 h                          | 72 h                          |
|------------------|-------------------------------|-------------------------------|-------------------------------|
| Control          | 58.18 $\pm$ 5.64              | 62.61 $\pm$ 5.91              | 78.47 $\pm$ 7.56              |
| 1 mmol/L aspirin | 53.61 $\pm$ 5.42 <sup>a</sup> | 56.15 $\pm$ 3.65 <sup>a</sup> | 67.49 $\pm$ 7.50 <sup>b</sup> |
| 2 mmol/L aspirin | 45.57 $\pm$ 4.39 <sup>b</sup> | 34.94 $\pm$ 3.18 <sup>b</sup> | 47.84 $\pm$ 4.19 <sup>c</sup> |
| 4 mmol/L aspirin | 30.82 $\pm$ 3.26 <sup>b</sup> | 25.24 $\pm$ 2.17 <sup>c</sup> | 18.97 $\pm$ 1.22 <sup>c</sup> |

**Note:** <sup>a</sup> $P > 0.05$ , <sup>b</sup> $p < 0.05$  and <sup>c</sup> $p < 0.01$  vs. control group

### Gastric cancer cell apoptosis

Compared with the control group, 1 mmol/L and 2 mmol/L aspirin groups had raised apoptosis ( $p < 0.05$ ), and 4 mmol/L aspirin group showed evidently increased apoptosis ( $p < 0.01$ , Figure 1).

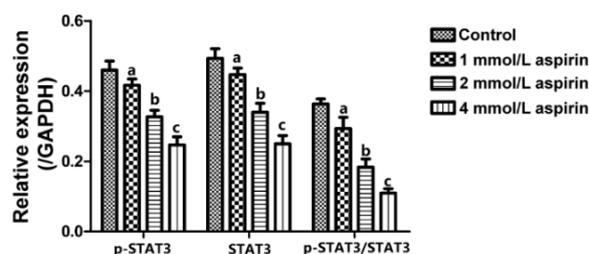


**Figure 1:** Comparison of TUNEL staining results (magnification: 200 $\times$ ) and apoptosis rate of cells in each group. **Note:** <sup>a</sup> $P < 0.05$ , <sup>b</sup> $p < 0.01$  vs. control group

### STAT3, p-STAT3, Bcl-2 and Bax expression levels

Compared with those in the control group, the expression levels of STAT3 and p-STAT3 did not obviously change in the 1 mmol/L aspirin group

( $p > 0.05$ ), but were lowered in the 2 mmol/L aspirin group ( $p < 0.05$ ), and distinctly decreased in the 4 mmol/L aspirin group ( $p < 0.01$ , Figure 2).



**Figure 2:** Protein expression levels of STAT3, p-STAT3, Bcl-2 and Bax in each group of cells. **Note:** <sup>a</sup> $P > 0.05$ , <sup>b</sup> $p < 0.05$ , <sup>c</sup> $p < 0.01$  vs. control group

### DISCUSSION

Gastric cancer, one of the most common fatal cancers, is associated mainly with *Helicobacter pylori* infections. Aspirin has protective effects on gastrointestinal cancer due to its anti-inflammatory and antiplatelet functions, including inhibition of gastric cancer development, apoptosis and angiogenesis of gastric cancer cells [11].

Kim *et al* [12] found through a large-scale population-based retrospective cohort study that the duration of regular use of low-dose aspirin is substantially correlated with a decline in incidence of gastric cancer in patients with hypertension or type 2 diabetes. Moreover, administration of low-dose aspirin for a long time, especially for not longer than 3 years, can notably lower the incidence of gastric cancer. A correlation study on the use of aspirin once or twice weekly and the incidence of gastric cancer, showed that aspirin dramatically reduces the risk of gastric cancer [13]. A meta-analysis demonstrated that aspirin use for longer than 5 years can reduced the risk of colorectal cancer [14]. The leading mechanism by which aspirin and other non-steroidal anti-inflammatory drugs prevent cancers is related to blockage of the COX pathway that affects inflammation, thrombosis, angiogenesis, and cell apoptosis, proliferation and migration. Compared with that in normal gastric tissues, COX-2 has a prominently high expression in gastric cancer, and such an increase is associated with multiple factors for the onset of gastric cancer. For example, it promotes cell proliferation and inhibits cell apoptosis [15].

According to a study, STAT3 may promote the proliferation of epithelial cells and inhibit their

apoptosis to exert its effects, and it protects cells from apoptosis stimuli as well as accelerates the cell cycle progression, thereby mediating tumorigenesis [16]. Griveikov *et al* [17] and Bollath *et al* [18] held that there is growing literature on the important role of persistently active STAT3 in carcinogenesis, and that the mechanism of consecutive STAT3 activation includes autocrine and paracrine IL-6 induced-STAT3 phosphorylation. Besides, current studies have established that STAT3-dependent tumorigenesis is mediated by IL-6 signals in the tumor microenvironment, and in fact, STAT3 activation in human tumors tend to be observed at the invasive front of tumors adjacent to inflammatory cells.

Chronic inflammation promotes tumor development and progression, and IL-6 is highly expressed in tumor microenvironment and serves as a major inflammatory mediator. IL-6 also directly stimulates the proliferation, survival and invasion of tumor cells, and induces the production of pro-inflammatory and pro-angiogenic IL-8 and VEGF. IL-6 acts on the immune and non-immune cells in tumor microenvironment in an autocrine and paracrine manner, thereby exerting its vital effects on different cancers [19,20].

Thus, IL-6, a potent pleiotropic inflammatory cytokine, mediates multiple physiological functions, including the development and differentiation of lymphocytes, cell proliferation, cell survival and improvement of apoptosis signaling, and directly activates STAT1 and STAT3 to participate in tumor progression. Although the existence of IL-6 is not aberrant in tissues, IL-6-induced chronic inflammation is closely associated with multiple cancers [21]. STAT3 is usually excessively activated in tumor-infiltrating immune cells to negatively regulate neutrophils, natural killer cells, effector T cells and dendritic cells, suggesting that the activation of STAT3 in immune cells probably weakens the anti-tumor immunity of STAT3 [22]. Moreover, STAT3 proactively regulates regulatory T cell and myeloid derived suppressor cell populations. Overall, these effects are conducive to highly immunosuppressive tumor microenvironment, and the IL-6/STAT3 signaling contributes to the growth and progression of tumors.

The findings of this study indicate that the aspirin groups exhibited increase in the inhibition of cell proliferation and decline in the release of IL-6 at 24, 48 and 72 h. Cell apoptosis at 72 h in the aspirin groups was higher than that in the control group. Compared with those in the control group, the expressions of STAT3 and p-STAT3 in cells

did not show obvious changes in the 1 mmol/L aspirin group at 72 h, but were raised in the 2 mmol/L aspirin group at 72 h, as well as in the 4 mmol/L aspirin group at 72 h. Therefore, aspirin repressed the proliferation of gastric cancer cell SGC7901 cells and promoted cell apoptosis *in vitro* via IL-6/STAT3 signaling pathway.

## CONCLUSION

This study demonstrates that aspirin inhibit the proliferation and induce apoptosis of gastric cancer cell SGC7901 through IL-6/STAT3 signaling pathway *in vitro*. The findings of this study further clarifies the mechanism of action of aspirin from the perspective of molecular biology, and also enhances the theoretical evidence for the clinical use of aspirin.

## DECLARATIONS

### Acknowledgements

None provided.

### Funding

None provided.

### Ethical approval

None provided.

### Availability of data and materials

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

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Shunyu Tang and Yun Liu contributed equally to this work.

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