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Tolectin *in vitro* effects on TRP53 gene expression level in gastric adenocarcinoma cells

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Abstract: Tolectin is a non-steroid anti-inflammatory drug (NSAID) which has a clinically analgesic and antipyretic activity. Although the mechanism of its action is not known, tolectin inhibits prostaglandin synthetase *in vitro* and lowers the plasma level of prostaglandin E in humans. Recently, the anticancer effects of tolectin has been reported in *in vitro* studies. The aim of this study was to investigate the *in vitro* tolectin effects on antitumor TRP53 gene expression level in gastric adenocarcinoma cells. The gastric adenocarcinoma (AGS) cells were divided to control group and groups treated with 0.0012, 0.0023, 0.0049, 0.0097, 0.0194, 0.0389 mmol/mL. MTT assay method was used to determine the cell viability. Relative TRP53 gene expression level was evaluated by quantitative Real Time PCR. Data were analyzed using ANOVA and student's t-test. Cell viability significantly decreased in all groups of tolectin treated AGS cells compared to control group; however, expression level of the TRP53 gene did not significantly changed in cells treated with effective concentration of tolectin (0.0131 mmol/mL) compared with control group. Our findings indicated that tolectin has a significant cytotoxic effects on gastric adenocarcinoma cells, however, its effective cytotoxic concentration has not significant impact on antitumor TRP53 gene expression level.

Keywords: Tolectin, Cytotoxic effect, TRP53, AGS

1 Introduction

Gastric cancer is the second most common cancer worldwide. Despite the decreasing worldwide incidence, gastric cancer accounts for 3% to 10% of all cancer-related deaths. The

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substantial mortality associated with gastric cancer has prevailed despite technical advances in surgery and the use of adjuvant therapy. Gastric adenocarcinoma comprises 95% of the total number of malignancies (Van Cutsem et al., 2016; Smyth et al., 2020). Curative therapy involves surgical resection, most commonly a total or subtotal gastrectomy, with an accompanying lymphadenectomy. Endoscopic mucosal resection, chemotherapy, radiation therapy, chemoradiation, targeted therapy and immunotherapy are other treatment options for gastric adenocarcinoma. Cancer treatments can cause serious side effects. Side effects are problems that occur when treatment affects healthy tissues or organs. The important risk factors of the causes of gastric cancer are H. pylori, obesity, smoking, red meat, alcohol, and low socioeconomic status (Thrift and El-Serag, 2020; Machlowska et al., 2020). Recently association of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, ibuprofen and diclofenac, with gastric cancer prevention has been argued, however, the findings are controversial (Cai et al., 2020).

NSAIDs are medicines that are widely used to relieve pain, reduce inflammation, and bring down a high temperature. The main types of NSAIDs include: ibuprofen, naproxen, diclofenac, celecoxib, mefenamic acid, indomethacin, aspirin and tolectin. Tolectin (tolmetin sodium) is a nonsteroidal anti-inflammatory drug of the heterocyclic acetic acid derivative class. It is used primarily to reduce hormones that cause pain, swelling, tenderness, and stiffness in conditions such as osteoarthritis and rheumatoid arthritis (Parolini, 2020). Preclinical and clinical studies have clearly shown a benefit of nonsteroidal NSAIDs use in reducing cancer risk (Cha and DuBois, 2007). In the past few decades, there is a growing body of research on the use of NSAIDs in cancer treatment and prevention, whereas the relationship between chronic inflammation and cancer has long been discovered (Smith et al., 2000). While some studies found no significant association between NSAID use and cancer metastasis, other studies have demonstrated that NSAIDs are associated with reduced risk of metastasis and even with reduced cancer incidence (Zhao et al., 2017). NSAIDs are also further recommended as the primary drug for prevention of colorectal cancer (Bibbins-Domingo and US Preventive Services Task Force, 2016). Tolectin has been also reported to cause cell death in cervical cancer cells (Norouzi et al., 2020). Although a number of studies are reporting anticancer effects of NSAIDs on cancer cells, there have been several reports on the association between NSAID use and increased risk of renal cancer (Cho et al., 2011; Capitanio et al., 2019).

Studies have shown that NSAIDs have an impact on Tumor protein P53 (TRP53) gene in cancer cells (Janssen et al., 2008). TRP53, also known as cellular tumor antigen p53, or transformation-related protein 53 is a regulatory protein that is often mutated in human cancers. The p53 proteins are crucial in vertebrates, where they prevent cancer formation (Rose Li et al., 2020; Janssen et al., 2008). Despite a number of studies investigating the effects of NSAIDs on genes associated with cell death and tumor progress or prevention, few investigations have been carried out to study the effects of tolectin on cancer, in particular, gastric adenocarcinoma cancer cells. The present study was carried out to investigate the tolectin *in vitro* effects on TRP53 gene expression level in gastric adenocarcinoma cells.

2 Materials and Methods

2. 1. Cell line and cell culture

AGS cells (a human gastric adenocarcinoma cell-line) were obtained from the National Cell Bank of Iran (NCBI). The cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (Penicillin-streptomycin). Cultured cells at 70-80% confluency were washed with PBS and detached from the flask using trypsin-EDTA. The cell suspension was eventually centrifuged and the cell pellet was re-suspended in fresh culture medium to be used in the experiments.

2. 2. Cytotoxicity assay

To prepare tolectin solution, pure powder of tolectin was dissolved in 100 µL of sodium hydroxide solution. For better dissolution, 1000 µL of phosphate buffer solution (PBS) was added and the solution was sterilized by filtration through a 0.22-µm filter (Millipore; USA). 9 mL of DMEM culture medium (containing fetal bovine serum, penicillin and streptomycin) was added. Different concentrations (0.0012, 0.0023, 0.0049, 0.0097, 0.0194, 0.0389 mmol/mL) of the drug were prepared by serial dilutions. These concentrations were selected on the basis of the results of a pilot study performed in our laboratory. For each experiment, drug solutions were freshly prepared from the stock solution. In order to measure the cytotoxicity, the MTT colorimetric assay was used. Briefly, cells were seeded into 96-well plates containing DMEM medium [Dulbecco's Modified Eagle Medium] supplemented in 10 % FBS. After 24 hours of incubation at 37ºC and at a density of 1×10^4 cells in a well, the culture medium was changed. The cells were treated with tolectin (0.0012, 0.0023, 0.0049, 0.0097, 0.0194, 0.0389 mmol/mL). After 24 hours, the media was removed and the cells were washed with PBS. MTT (0.05 mg/well) [DOBIO Biotech, Shanghai, China] was added to each well and the cells incubated for another 3 hours at 37°C. During this incubation period, water-insoluble formazan crystals were formed and dissolved by adding of 100 µl /well DMSO (Sigma. Finally, the absorbance of the solution was calculated by a microplate reader (Bio-Rad, Hercules, CA) at a wavelength of 570 nm. Three wells containing only complete medium were used as blank controls for nonspecific dye reduction.

2. 3. Evaluation of TRP53 gene expression level

Quantitative Real Time PCR (qRT-PCR) method was used for evaluation of TRP53 gene expression level. AGS cells were seeded into 6-well plates (5×10^5 cells/well) and incubated for 24 hours. After treatment of cells with the effective concentration of tolectin (0.0131 mmol/mL) for 24 hours, total RNA was extracted. The cDNA synthesis was then performed using PrimeScriptTM First-Strand cDNA Synthesis Kit (Takara, Tokyo, Japan) according to the manufacturers' protocol. The PCR reaction was completed using10µl Power SYBR Green PCR Master Mix (2X), 1µl of each specific primer (2µM; table 1 shows the primer sequences), 1µl cDNA (100ng), and 7µl double-distilled water followed by an initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute by a real-time PCR thermal cycler machine. The expression of genes was calculated based on $2^{-\Delta\Delta CT}$ method and was

normalized to the loading control, GAPDH.

Table 1. Primer sequences used in qRT-PCR

Gene	Primer Sequences
P53	Forward:5´- CATCTACAAGCAGTCACAGCACAT-3´
	Reverse:5´- CAACCTCAGGCGGCTCATAG-3´
GAPDH	Forward:5´- CCCACTCCTCCACCTTTGAC-3´
	Reverse:5´- CATACCAGGAAATGAGCTTGACAA-3´

2. 3. Statistical analysis

Data were expressed as means ± SD. Data were analyzed using ANOVA and student's t-test. Statistical significance was defined at $p< 0.05$. SPSS software, version 21 was used for data analysis.

3 Results and Discussions

3. 1. Cytotoxic effect of tolectin on AGS cells

Figure 1 shows the cytotoxic effect of tolectin on AGS cells. The data obtained from the MTT assay test showed that the viability of AGS cells significantly decreased when treated with 0.0012, 0.0023, 0.0049, 0.0097, 0.0194, 0.0389 mmol/mL of tolectin compared with the control group.

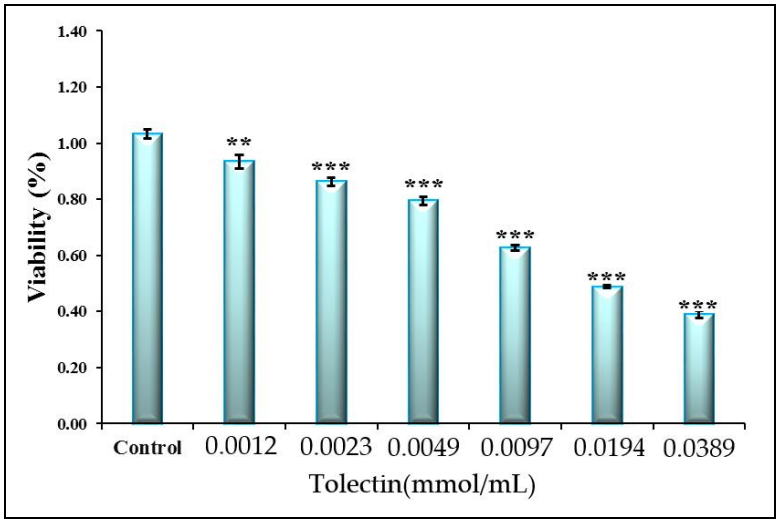

Figure 1. Viability of AGS cells treated with different concentrations of tolectin. * indicates significant difference compared with control group (**: $p<0.01$, ***: $p<0.001$).

3. 2. The effect of tolectin on TRp53 gene expression level in AGS cells

The results of qRT-PCR showed that the relative expression level of TRp53 did not significantly changed in AGS cells treated with effective concentration of tolectin compared with control group (Figure 2).

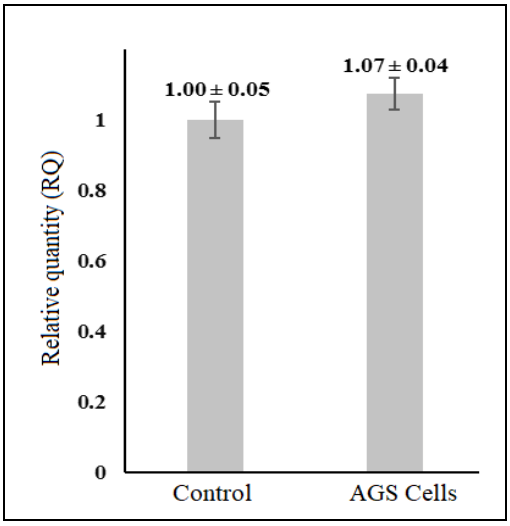

Figure 2. Relative quantitative (RQ) expression level of TRP53 gene in AGS cells treated with effective concentration of tolectin compared with control group.

Our findings indicated that tolectin had a significant *in vitro* cytotoxic effects on gastric adenocarcinoma cells. The cytotoxic effects of tolectin has been reported in our recent studies. We have shown that tolectin has cytotoxic and apoptotic effects on cervical cancer cells *in vitro* (Norouzi et al., 2020). In a study tolmetin hydrazide and a novel series of tolmetin hydrazide–hydrazones 4a–l were synthesized and it was revealed that tolmetin hydrazide–hydrazones has a potent apoptotic effects on colon cancer cells (Küçükgüzel et al., 2015). Recently, it has been shown that the novel synthesized tolmetin have cytotoxic effects on human colon cancer cells *in vitro* (Kassab et al., 2021). Tolmetin hydrazide derivatives also have shown anticancer effects against human prostate and human colon cancer cell lines *in vitro* (Dadaş et al., 2015).

We have shown that the effective cytotoxic concentration of tolectin had not significant impact on antitumor TRP53 gene expression level, showing that the drug is unlikely to be able to modulate the preventive effects on AGS cells. To our knowledge, this is the first report associated with tolectin effects on TRP53 gene expression level in cancer cells. Further research are required to investigate the effects of tolectin on antitumor genes expression level in cancer cells to reveal the exact mechanism of antitumor action of tolectin on cancer.

4 Conclusion

The findings of the present study shows that tolectin has cytotoxic effects on gastric adenocarcinoma cell *in vitro*; however, effective cytotoxic concentration of tolectin has no significant impact on antitumor TRP53 gene expression level in gastric adenocarcinoma cells.

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Conflict of interests

The authors state that there are no conflicts of interest regarding the publication of this paper.

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