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The effect of aspirin on caspase-8 and -9 activity level in cervical cancer cells *in vitro*

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Abstract: Caspases are key mediators of apoptosis. Caspase-8 mediates extrinsic, and caspase-9 initiates the intrinsic pathway of apoptosis. The literature shows that there is a relation between non-steroidal anti-inflammatory drugs (NSAIDs) and apoptosis in cancer cells. Accordingly, the current research investigated the effects of aspirin on caspase-8 and -9 activity level in cervical cancer (Hela) cells. In this *in vitro* study, Hela cells were divided to control untreated group and the groups treated with 0.0001, 0.001, 0.01, 1.0, 1 and 10 mg/ml of aspirin. MTT method was used to determine the cell viability. The caspase colorimetric assay method was used to detect the caspase activity level. The data were analyzed using ANOVA and Student's t-test. The results showed that the cytotoxic concentration of aspirin significantly increased the activity level of caspase-9 ($p<0.001$), however, did not significantly change the caspase-8 activity level. It can be concluded that aspirin is able to initiate the intrinsic pathway of apoptosis in cervical cancer cells.

Keywords: Aspirin, Cervical cancer, Caspase-8, Caspase-9

1 Introduction

Cervical cancer is one of the most common cancers among women in the world (Bruni et al., 2022). Cervical cancer is preventable and treatable, yet remains a significant global health burden. In 2020, it was the fourth most diagnosed cancer and the fourth leading cause of cancer-related deaths in women (Monk et al., 2022). Persistent human papillomavirus (HPV) infection is the most important factor in the development of cervical cancer (Cascardi et al., 2022). The

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incidence of cervical cancer seems to be related to the prevalence of HPV in the population (Gupta et al., 2022; Koh et al., 2013; Tsunoda et al., 2003). Over the last two decades, minimally invasive surgery has emerged as the surgical management of early-stage cervical cancer (D'Oria et al., 2022; Cohen et al., 2019; Crosbie et al., 2013; Huh et al., 2017).

Aspirin as the most commonly used nonsteroidal anti-inflammatory drug (NSAID) in the world (Hua et al., 2019). Aspirin (acetylsalicylic acid) is an old drug that is widely administered for preventing and treating cardiovascular and cerebrovascular diseases (Hua et al., 2019; Gilligan et al., 2019). After oral administration of aspirin, it is absorbed in the gastrointestinal system and then converted into salicylic acid, followed by metabolic processing. Liver is the primary organ for salicylic acid metabolism. After salicylic acid is coupled with glycine, it is converted into salicylic acid. Aspirin has been reported to reduce the risk and incidence of cancer and also prolongs survival when administered post diagnosis (Joharatnam-Hogan et al., 2019).

Caspases are a group of enzymes that belong to the family of proteases (Julien et al., 2017). A consensus view of caspases places them in two main groups. First are the cytokine activators (inflammatory caspases) related to caspase-1, probably including mouse caspase-11 and its orthologs caspase-4 and caspase-5 in humans (Villa et al., 1997). The second group constitutes the apoptotic caspases that transduce and execute death signals (Villa et al., 1997; Cohen et al., 1997). Caspase-8 is a member of the family of cysteine-aspartic acid proteases and is involved in the process of apoptosis. Roles for cell death in development, homeostasis, and the control of infections and cancer have long been recognized (Salvesen et al., 2014). Although excessive cell damage results in passive necrosis, cells can be triggered to engage molecular programs that result in cell death. Such triggers include cellular stress, oncogenic signals that engage tumor suppressor mechanisms, pathogen, and immune mechanisms (Tummers et al., 2017). Caspase-9 it is one of the initiator caspases and is an aspartic acid-specific protease that is associated with the process of "mitochondrial death" and is activated during programmed cell death (Li et al., 2017). As the most intensively studied initiator caspase, caspase-9 is a key player in the intrinsic or mitochondrial pathway which is involved in various stimuli, including chemotherapies, stress agents and radiation (Bhatia et al., 2004). Previous studies related to the effect of aspirin on cancer has shown that aspirin significantly enhanced the cisplatin-mediated inhibitions of cell proliferation, migration and invasion and the induction of apoptosis in colon cancer cells (Jiang et al., 2020). Aspirin has become a new focus of cancer prevention and treatment research at present, however, clinical studies found conflicting conclusions of its anticancer characteristics (Tran et al., 2019). Data has shown that aspirin regulates almost all the hallmarks of cancer (Zhang et al., 2019). Within tumor tissue, aspirin suppresses the bioactivities of cancer cells themselves and deteriorates the tumor microenvironment that supports cancer progression. Reports revealed that aspirin appears to have a favorable effect on the outcome of patients with colorectal cancer (Mädge et al., 2022; Huang et al., 2016). Data has been shown that in both *in vitro* and *in vivo* models, aspirin influences carcinogenesis leading to clinically relevant improvements in survival (Wield et al., 2018). Different reports lower risk of ovarian cancer among women who used aspirin at a daily dose continuously (Lo-Ciganic et al., 2012). Aspirin also may reduce cancer mortality patients with endometrial cancer (Verdoodt et al., 2017). Studies have revealed that a proposed mechanism behind decreased cancer mortality in

endometrial cancer may be a result of inhibition of metastasis via platelet inactivation and possible prostaglandin E2 suppression by aspirin (Takiuchi et al., 2018). Reports revealed that acetylsalicylic acid has antiproliferative and apoptotic effects *in vitro* (Arango et al., 2001). Data has shown that caspase-3 may play an important role in cervical cancer cells apoptosis (Lee et al., 2008; Im and Jang et al., 2012; Kim et al., 2003).

Caspase -8 and -9 are key mediators of apoptosis in cancer cells. The literature shows that there is a relation between NSAIDs and apoptosis in cancer cells. The present study was carried out to investigate the effects of aspirin on caspase-8 and -9 activity level in cervical cancer (Hela) cells *in vitro*.

2 Materials and Methods

2. 1. Aspirin

Pure aspirin powder was prepared from Pharmaceutical to prepare aspirin solution 1 g of the w aspirin was added to sodium hydroxide and PBS. The solution was filtered. 9 mL of DMEM culture was added to the filtered solution to achieve a volume of 10 ml. The obtained solution was used as a stock solution to prepare different concentrations of aspirin.

2. 2. Cell culture

Hela and Hek293 were purchased from the cell bank of the Pastor Institute of Iran and were kept frozen in a nitrogen tank at -196°C. Hela and Hek293 Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium; SIGMA, USA) with 10% fetal bovine serum (FBS) and 1% antibiotic (gentamicin). Cells were then incubated in a humidified atmosphere with 5% CO2 in a 37°C incubator. PBS and trypsin-EDTA were used to wash the cultured cells and separate the cells from the flask, respectively. A culture medium containing 10% FBS was added to neutralize excess trypsin-EDTA activity. The cell suspension was centrifuged, and the cell pellet was suspended in a fresh culture medium and prepared for use in subsequent experiments.

2. 3. MTT assay

Hela and Hek293 cells were divided into control (untreated) groups and groups treated with 0.0001, 0.001, 0.01, 0.1, 1 and 10 mg / mL of aspirin. MTT assay method was used to evaluate the aspirin cytotoxicity effect on the cells. 10^4 cells were cultured in each well of the 96-well plate and the plate was placed in incubator for 24 hours. The cells were treated with 0.0001, 0.001, 0.01, 0.1, 1 and 10 mg / mL of aspirin for 24. 100 λ of MTT solution was added to the. The plate was placed in the incubator for 4 hours, then the supernatant of the wells was removed and 100 λ of isopropanol was added to the wells. Finally, the resulting colored solution was measured for optical density using a microplate reader at a wavelength of 570 nm. Cell viability was calculated as the percentage of viable cells in the total population and aspirin inhibitory concentration (IC50) was calculated.

2. 4. Caspase activity measurement

To quantitatively measure the activity of caspases-8 and -9 in Hela and Hek293 cells treated

with IC50 concentration of aspirin, the caspases assay kit (Novex) was used.

2. 4. Data analysis

Data were analyzed by SPSS and Excel software. The Kolmogorov-Smirnov test was used to verify the normal distribution of data. Given normalization of data distribution, the data were analyzed using one-way analysis of variance (ANOVA) and Student's t-test.

3 Results and Discussions

Cell viability significantly decreased in Hela cells treated with 0.1, 1 and 10 mg/mL of aspirin compared to control group, however, aspirin did not show significant cytotoxic effects on Hek293 cells, indicating that although aspirin has anticancer effects against cervical cancer cells, it has not killing effect on normal cells (Figure1).

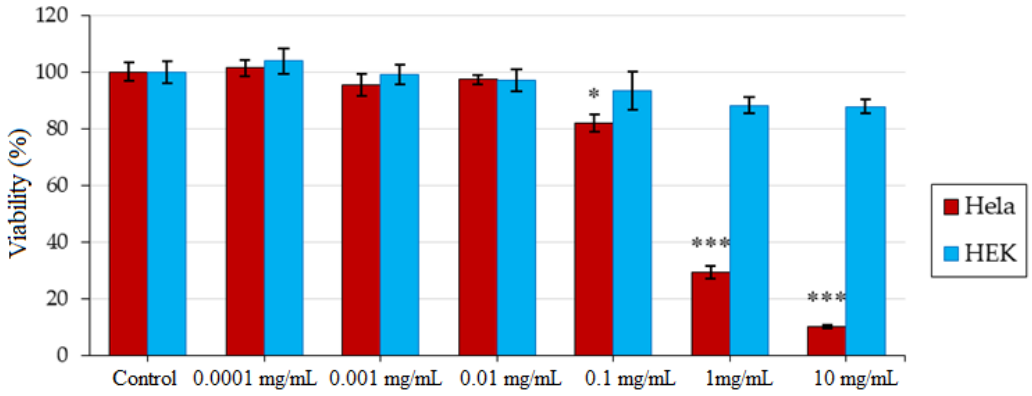

Figure 1. Viability of Hela and Hek293 cells treated with different concentrations of aspirin. * indicates significant difference (*: p<0.05 and ***: p<0.001).

Treatment of Hela cells with IC50 concentration of aspirin (0.43 mg/mL) resulted in significant increase in caspase-9 activity level, however, did not change caspase-8 activity level (Figure 2).

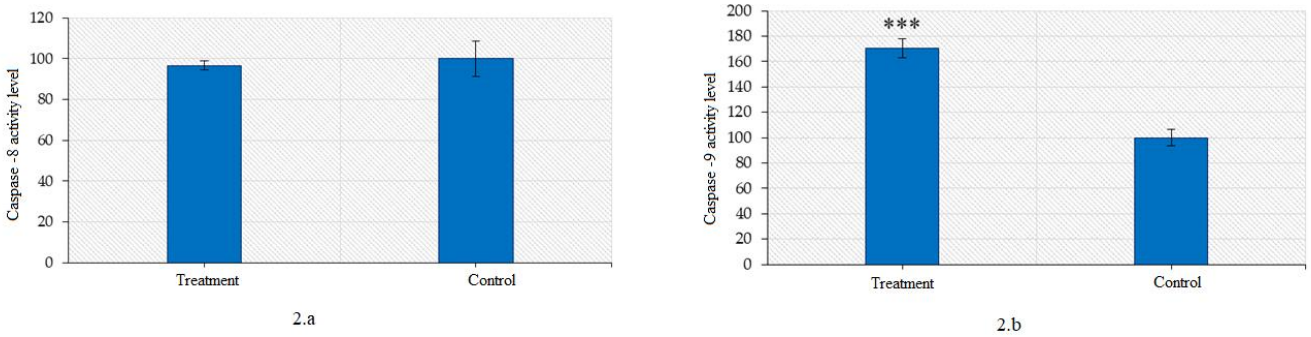

Figure 2. The effect of aspirin on caspase-8 (2.a) and caspase-9 (2.b) activity level in response to IC50 concentration of aspirin. * indicates significant difference (***: p<0.001).

In the present study we have shown that aspirin has cytotoxic effects on cervical cancer cells *in vitro* which is partly mediated by increased caspase-9 activity level. In consistent with our finding, it has been shown that aspirin has anticancer effects on certain types of cancer including ovarian cancer (Baandrup et al., 2015). A large body of evidence supports the role of aspirin as an anti-cancer agent, and a number of randomized trials are currently underway aiming to assess the potential benefit of aspirin in the treatment of cancer. The evidence are growing to show that aspirin has a pivotal role in prevention of the development and recurrence of gynaecological cancers including ovarian, endometrial and cervical cancer (Joharatnam-Hogan et al., 2020). Prolonged treatment with aspirin has been reported to induce apoptosis by altering expressions of Bcl2, Bax and Cytochrome Cin cervical cancer cells (Das et al., 2022). Aspirin inhibits ovarian tumor progression which might be mediated by p53 acetylation and subsequent activation of p53 target genes (Guo et al., 2021). Indeed, the studies build support for the hypothesis that frequent, low-dose aspirin reduces ovarian cancer risk among younger postmenopausal women (Hurwitz et al., 2020). It has been shown that use of non-aspirin NSAIDs was not associated with reduced endometrial cancer. Rather, it has been reported that high-intensity and large cumulative amount of non-aspirin NSAID use may be associated with increased endometrial cancer mortality (Sperling et al., 2021). The use of aspirin was associated with a reduced risk of endometrial cancer, and the reduced risk was closely related to the high-frequency of use (Wang et al., 2020; Matsuo et al., 2019). Recent findings show that aspirin induces apoptosis in cervical cancer cells (Lee et al., 2022). The present study shows that aspirin increases caspase-9 activity level and does not change significantly the caspase-8 activity level and since caspase-8 mediates extrinsic, and caspase-9 initiates the intrinsic pathway of apoptosis, we can assume that aspirin initiates intrinsic pathway of apoptosis leading to cervical cancer cell death.

4 Conclusion

The results of this study indicate that aspirin has cytotoxic effects in cervical cancer cells *in vitro*, however, it does not kill the normal cells. Aspirin cytotoxic effects on cervical cancer cells is partly mediated by increased activity level of the caspase-9. Further research are required to reveal the exact mechanism behind aspirin action on cervical cancer cells.

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Conflict of interests

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

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