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In vitro anticancer effects of ibuprofen on HeLa cell line

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Abstract: Several studies have reported the anticancer effects of ibuprofen on cervical cancer cells, but the molecular pathway is still unclear in many aspects. This study aimed to investigate the effects of cytotoxic dose of ibuprofen on BAX, BCL-2, caspase-3, MMP-9, KAI-1 and NM23 gene expression levels and evaluation of caspase-3, -8 and -9 activity level in cervical cancer (HeLa) cells. Cervical cancer cells were divided into untreated (control) group and ibuprofen treated groups. Expression levels of BAX, BCL-2, caspase-3, MMP-9, KAI-1 and NM23 genes were evaluated by Real-time PCR and caspase-3, -8 and -9 activity levels were determined using colorimetric method. Hoechst staining was used to confirm apoptosis. The data were statistically analyzed between groups using ANOVA and independent t-test. Significant increase in expression levels of caspases-3, and BAX/BCL-2 ratio, caspase-3, -8 and -9 activity level and significant decrease in NM23, KAI-1 genes expression level were observed in HeLa cells treated with ibuprofen cytotoxic concentration. The apoptosis observed in HeLa cells was confirmed by Hoechst 333285 staining and flow cytometry analysis. Ibuprofen increased nuclear condensation and induced apoptosis in HeLa cells by both intrinsic and extrinsic apoptosis pathways.

Keywords: Ibuprofen; HeLa; Caspase activity; Apoptosis.

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1 Introduction

Currently cervical cancer is the second most common cancer among women worldwide (Schiffman et al., 2007). Exponential growth of this fatal disease and the struggle against cancer has led to development of current therapeutic agents and investigation of the most potent and novel therapeutic approaches. It has become a major area of interest to explore new agents with no or less unpleasant side-effects that could be used against the aggressive tumor. Using and producing novel antitumor agents including natural or synthetic non-cytotoxic nutrients have gained significant attention over the last decades (Hussain et al., 2011). Non-steroidal anti-inflammatory drugs (NSAIDs) presents a good case in this respect. Several epidemiological studies showed that NSAIDs have been associated with reduced risks of cancers (Zhou et al., 2014). Consistent evidence has demonstrated both selective and non-selective NSAIDs have protective effects and remarkably prevent chemically induced carcinogenesis of epithelial tumors (Collet et al., 1999; Harris et al., 2005). Anti-inflammatory, antipyretic, and analgesic properties of NSAIDs have made them a potential option for clinical use (Kim et al., 2003). Carcinogenesis pathway in many cancers is involving overexpression of the inducible cyclooxygenase-2 (COX-2) gene and deregulation of prostaglandin (PG) biosynthesis (Harris et al., 2005). NSAIDs suppress the production of prostaglandins by blocking cyclooxygenase (COX), an essential enzyme in PG production (Zhou et al., 2014). NSAIDs by involving and stimulating the production of arachidonic acid and ceramide play an essential role in apoptosis commencement (Kim et al., 2003). NSAIDs show their anticancer effects either on both the tumors or the tumor microenvironment and inhibit proliferation, decrease migration, prevent angiogenesis, and overcome apoptosis resistance in a COX-2 dependent and independent manner (De Groot et al., 2007; Rayburn et al., 2009). Apoptosis occurs resulted from a subset of enzyme activity including caspase proteases, in particular, caspase-3, -6, and -7 (executioner caspases) that can be activated by caspase- 8, -9, and -10 (initiator caspases), cleaving specific substrates (proteins) and lead to biochemical and morphological changes in the cell and finally cell death (Green and Kroemer, 2005; Große et al., 2016). These events can trigger apoptosis via two main ways: the extrinsic pathway (death receptor-mediated) involving the cell surface death receptor, and the intrinsic pathway (mitochondrial-mediated) (Hu and Kavanagh, 2003; Green and Kroemer, 2004). BCL-2 family (a negative regulator of apoptosis proteins) plays a critical role in controlling mitochondrial pathway (Hu and Kavanagh, 2003; Willis et al., 2003). BCL-2 protein has also a key role in drug resistance in cancer patients and prevents cell death induced by anticancer agents (Oltersdorf et al., 2005). BAX and BAK (BCL-2 family proteins) can switch apoptosis on or off by assembling at mitochondria and releasing cytochrome C (Große et al., 2016; Salvador-Gallego et al., 2016). Metalloproteinase-9 (MMP-9) is produced mainly by the tumor cells and has an essential role in metastasis triggering and cellular invasion. This protease breaks down extracellular matrix components and exposes cryptic peptide epitopes in the extracellular matrix that leads to cell death (Mehner et al., 2014; Guo et al., 2016). The NM23 gene is a metastatic suppressor gene and involves metastasis by affecting the MAPK and the cytoskeleton-organizing pathways (Yoshida et al., 2002; Prabhu et al., 2012). As reported, it exerts its metastasis-suppressing impacts at early stages of metastatic propagation, hence inhibiting invasive migration (Boissan and Lacombe, 2012). KAI-1/CD82 is a member of TM4SF family and has a central role in cell fusion, adhesion, migration, signaling, fertilization, differentiation, and invasion (Yu et al., 2014). It appears that decreased CD82/KAI-1 expression contributes to metastasis which makes it an ideal marker. The mechanism and how KAI-1/CD82 exhibit their inhibitory effects on metastasis of cancers is not clear, however, it has been suggested that KAI-1/CD82 inhibits cell cycle progression (Wu et al., 2012; Zhang et al., 2013).

Although ibuprofen is widely used in patients with cancer as anti-inflammatory and analgesic drug, few studies have been carried out to investigate the apoptotic effects of ibuprofen on cancer cells in vitro and in vivo. The present study aimed to reveal the cytotoxic effects of ibuprofen on cervical (Hela) cancer cells and expression level of BAX, BCL-2, caspase3, MMP9, NM23, KAI-1/CD82 genes.

2 Materials and Methods

2.1. Ibuprofen:

Ibuprofen was purchased from Abureyhan Pharmaceutical Company (Tehran, Iran). Stock solution was prepared in DMSO at 200 mM concentration and aliquots were kept at -20 ^{o}C.

2.2. Cell culture:

HeLa cell line was obtained from National Cell bank of Iran (NCBI) affiliated to Pasteur Institute of Iran and maintained at 37 °C in a humidified atmosphere (90 %) containing 5 % CO2. The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium;SIGMA, USA) supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % antibiotic (gentamicin). Cells were then incubated in a humidified atmosphere with 5 % CO2 at 37 °C incubator. PBS and trypsin-EDTA were used to wash the cultured cells at 70-80 % confluency and to detach the cells from the flask, respectively. Culture medium containing 10 % FBS was added to neutralize the excess trypsin-EDTA activity. The cell suspension was centrifuged, and the cell pellet was re-suspended in fresh culture medium and ready for the use of subsequent experiments (Sun et al., 2020).

2.3. Cell viability assay:

Cell viability was analyzed using the MTT method. The viability of HeLa cells treated by ibuprofen was determined. Cells were seeded into 96-well plates at a density of 5×0^4 cells in a well, after 24 hours incubation, the culture medium discarded and changed with DMEM (supplemented with 10 % FBS. Then, the cells treated with ibuprofen (0.0001, 0.001, 0.01, 0.1, 1 and 10) mg/mL were incubated for 24 hours. After treatments, the culture medium was discarded and washed with PBS. Finally, the MTT solution was added into the culture (25 µL) and incubated for another 3 hours. The culture medium discarded and 125 µL DMSO was added to dissolve the MTT formazan crystals and absorbance was measured at 570 nm by plate reader (BioTek, USA). Cells cultured in DMEM were used as negative control (NC). Cell viability was calculated as percentage of viable cells in total population were and inhibitory concentration (IC50) of ibuprofen was calculated from curve constructed by plotting cell survival (%) versus drug concentration. Each experiment was performed in eight replicates (Marks et al., 1992).

2.4. Morphological studies of HeLa cells before and after treatment with ibuprofen:

Morphological changes in HeLa cells elicited by ibuprofen were studied using a normal inverted microscope (Labomed). The untreated cells and cells treated with IC50 concentration of ibuprofen for 24 hours were used for the morphological studies.

2.5. RNA extraction and quantitative RT-PCR:

The total RNAs were extracted with the high purity RNA extraction kit (Takara, Japan) according to the manufacturer's instructions and reverse-transcribed into cDNAs. Then, real-time quantitative PCR (RT-PCR) was conducted to analyze BAX, BCL-2, caspase-3, KAI-1, NM23, MMP9 and GAPDH expression levels. The primer sequences are shown in Table 1.

Table 1. Primer Sequences used in RT-PCR.

Genes	Directions	Primers
BAX	F	5'CGGCAACTTCAACTGGGG-3'
=	R	5'TCCAGCCCAACAGCCG-3'
BCL-2	F	5'GGTGCCGGTTCAGGTACTCA-3'
=	R	5'TTGTGGCCCTTCTTTGAGTTCG-3'
Caspase-3	F	5'GCCTGCCGTGGTACAGAACT-3'
=	R	5'GCACAAAGCGACTGGATGAAC-3'
MMP9	F	5'GGCGTCGTGGTTCCAACT-3'
=	R	5'CGGTCGTCGGTGTCGTAGT-3'
NM23	F	5'GTTGACCTGAAGGACCGTCC-3'
=	R	5'GATGGTCCCAGGCTTGGAGT-3'
KAI-1	F	5'ACAACTGGACAGACAACGCTGA-3'
=	R	5'GCCACGCCTGCACCTTC-3'
GAPDH	F	5'CCCACTCCTCCACCTTTGAC-3'
=	R	5'CATACCAGGAAATGAGCTTGACAA-3'

The expression level of genes was calculated based on $2^{\Delta\Delta Ct}$ method and was normalized to the loading control, GAPDH (Luo et al., 2017).

2.6. Caspase activity assay:

Cells were treated with IC_{50} concentration of ibuprofen for 24 hours. A caspase colorimetric protease assay kit (Abnova, Taiwan) was used to measure enzymatic activity of caspases. In brief, harvested cell pellets were lysed in the lysis buffer and supernatants were collected. Equal amounts of protein were incubated with reaction buffer and colorimetric substrate, for instance, Acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETD-pNA) for caspase-8, acetyl-Leu-Glu-His-Asp-p-nitroaniline (Ac-LEHD-pNA) for caspase-9, and Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) for caspase-3, at 37 °C for 2 hours in the dark. Caspase activity was calculated following the manufacturer's instructions (Li et al., 2017).

2.7. Measurement of apoptotic morphology:

The morphological analysis of (HeLa) cell apoptosis was confirmed by nuclear staining with Hoechst 33258 dye. The cells were seeded at a concentration of 5×0^4 in a 25 mL culture flask for 24 hours to allow cell attachment. The cells were then treated with IC_{50} concentration of ibuprofen and incubated for 24 hours. After removal of the culture medium, the cells were fixed with ethanol acetone 80 % for 30 minutes. The cells were then washed twice with PBS and stained

with Hoechst 33258 dye (5 µg/mL) for 5 min in an incubator with 5 % CO2. The cells were identified by fluorescence microscopy (Nikon Corporation, Chiyoda-ku, Tokyo, Japan). Apoptotic cells were identified according to the characteristic nuclear morphological changes in cells, this includes reduction in volume and chromatin condensation (Li et al., 2017).

2.8. Flow Cytometry Analysis:

The Annexin-V-FITC/PI double staining assay was used to detect cellular apoptosis. The HeLa cells were treated with ibuprofen at a concentration of IC_{50}, for 24 hours. The cells collected after washing twice with cold PBS buffer, were suspended at 1×0^4 cells/mL in Annexin-V binding buffer. The cells were then incubated with 5 µL Annexin-V-FITC and 5 µL PI solution at room temperature for 15 min. Finally, the cells were washed with Annexin buffer and binding buffer (400 µL) was then added into each tube. Apoptotic cells were quantified by flow cytometer within 1 hour of staining and analyzed by the Cell Quest software. Data were obtained from three independent experiments (Li et al., 2017).

2.9. Statistical analysis:

Data were analyzed by SPSS and Excel software. The Kolmogorov-Smirnov test was used to normalize the distribution of data. Then, the one-way ANOVA and student's t-test was used to analyze the data followed by Tukey's post hoc multiple comparison test. All data were expressed as the mean±standard deviation (S.D.) and $P<0.05$ was considered significant.

3 Results and Discussions

3.1. IC_{50} of Ibuprofen:

The data obtained from the curve constructed by plotting HeLa cell viability (%) versus ibuprofen concentration. The IC50 value obtained for ibuprofen was at 3.22 mg/mL (Figure 1).

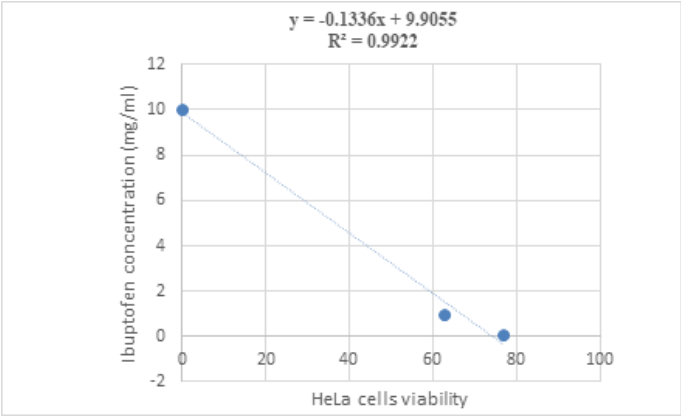

Figure 1: IC_{50} of ibuprofen calculated from curve constructed by plotting HeLa cell viability (%) versus ibuprofen concentration.

3.2. Effects of Ibuprofen on BAX, BCL-2, Caspase-3, KAI-1, MMP9 and NM23 genes expression level:

Relative quantification (RQ) of apoptotic BAX, anti-apoptotic BCL-2, and caspase-3 genes

expression levels significantly increased in ibuprofen (IC_{50} at 3.22 mg/mL) treated cells compared to control group ($P<0.001$). However, there was significantly higher BAX than BCL-2 expression level ($P<0.05$). Compared to control group, RQ of KAI-1 and NM23 significantly decreased in ibuprofen (IC_{50}) treated cells ($P<0.001$). MMP9 gene expression level did not change significantly in ibuprofen (IC_{50} at 3.22 mg/mL) treated cells compared to untreated cell (Figure 2).

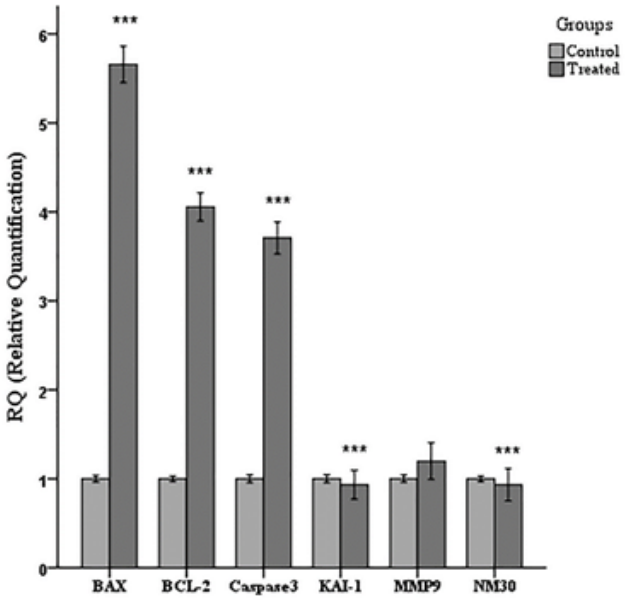

Figure 2: Relative gene expression level of BAX, BCL-2, caspase3, KAI-1, MMP9 and NM23 genes in cervical cancer cells treated with IC_{50} at 3.22 mg/mL of ibuprofen compared with control group. *** represents significant difference compared to control group (***: $P<0.001$).

3.3. Effects of Ibuprofen on the caspase-3, caspase-8 and caspase-9 activity levels:

Compared to control group, the caspase-3, -8 and -9 activity levels significantly increased in HeLa cells treated with IC_{50} at 3.22 mg/mL of ibuprofen ($P<0.001$, $P0.001$ and $P<0.01$, respectively) (Figure 3).

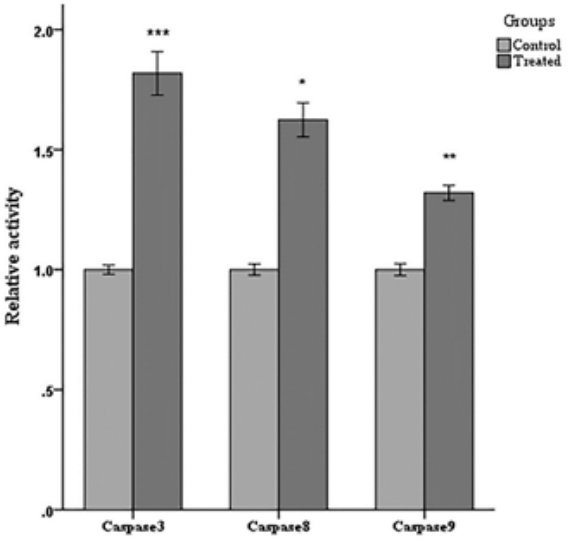

Figure 3: Activity level of caspase-3, -8 and -9 in HeLa cells treated with IC_{50} at 3.22 mg/mL of ibuprofen compared with control group.*. *, ** and *** represent significant difference compared to control group (*: $P<0.05$, **: $P<0.01$ and ***: $P<0.001$).

3.4. Morphological characteristics of HeLa cells nuclei with Hoechst staining:

Hoechst staining method was used to further confirm apoptosis and to determine nuclear morphology characteristics of HeLa cells. Nuclear morphology was evaluated with membrane-permeable blue Hoechst 33258. Figure 3 shows representative Hoechst 33258 fluorescence photomicrographs of cultured HeLa cells with and without treatment of ibuprofen at IC_{50} of 3.22 mg/mL. In the untreated HeLa cells, nuclei of the cells appeared with regular contours and were round and large. Rarely HeLa cells with smaller nuclei and condensed chromatin were seen. In contrast, most nuclei of ibuprofen-treated HeLa cells appeared hyper-condensed (brightly fluorescent), this is due to chromatin being dense caused by apoptosis (Figure 4).

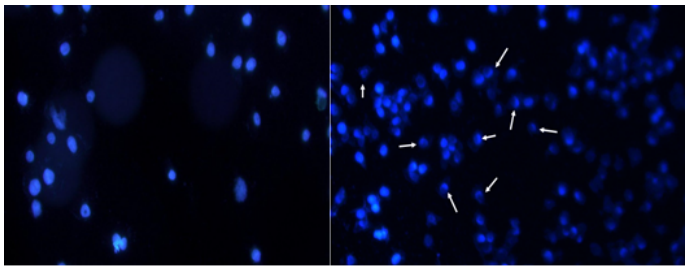

Figure 4: Hoechst staining left image is control cells and the right image is ibuprofen -treated cells. The right image demonstrates that IC_{50} at 3.22 mg/mL of ibuprofen on HeLa cells causes morphological changes. Arrows indicate nuclear condensation and apoptosis body in HeLa cells treated with ibuprofen.

3.5. Detecting HeLa cells apoptosis by flow cytometry:

The apoptosis of HeLa cells was detected using Annexin V-FITC/PI-FCM. The results indicated a significant increase in early and late apoptotic cells (from 2.0 % to 83.7 %) and a significant decrease in normal live cells (from 92.4 % to 4.98 %) which confirmed the apoptosis-induced after treatment of HeLa cells with IC_{50} at 3.22 mg/mL of ibuprofen (Figures: 5 and 6).

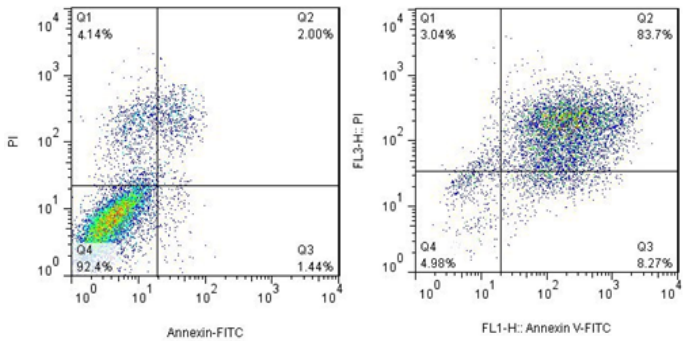

Figure 5: Flow cytometry analysis of HeLa cells after treatment with IC_{50} at 3.22 mg/mL of ibuprofen. Numbers in the respective quadrant profiles indicate the percentage of the cells present in this area. Q1: Necrosis; Q2: Late apoptosis; Q3: Early apoptosis; Q4: Viable cells.

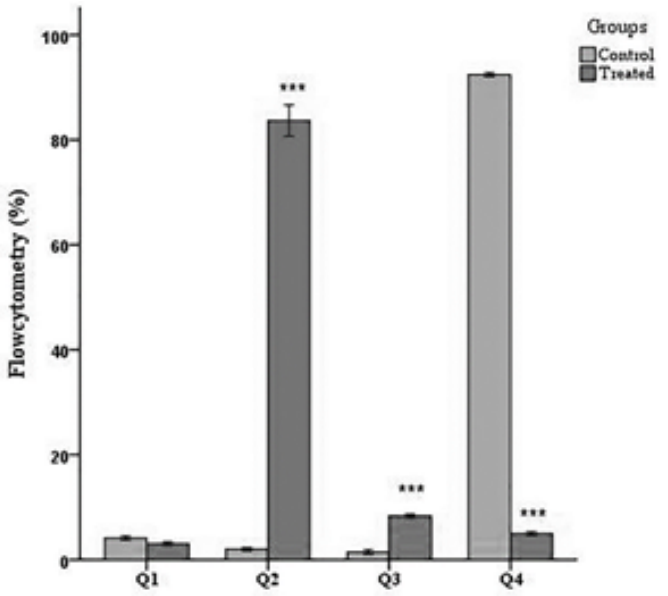

Figure 6: Percentage of apoptosis in HeLa cells induced by IC_{50} at 3.22 mg/mL of ibuprofen compared to control groups. Q1: Necrosis; Q2: Late apoptosis; Q3: Early apoptosis; Q4: Viable cells. (***) represents significant difference compared to control group (***: $P<0.001$).

3.6. Ibuprofen effects on BAX and BCL-2 gene expression level in HeLa cells:

This research demonstrated that the IC_{50} at 3.22 mg/mL of ibuprofen significantly increased the BAX/BCL-2 ratio, indicating that ibuprofen exerts its cytotoxic effect on HeLa cells through BAX dependent apoptotic pathway. The ratio of BAX to BCL-2 is one of the important factors in apoptosis investigations and its increase has a significant effect on caspase-3 activity and apoptosis induction in cancer cells (Fridman and Lowe, 2003). It has been reported that ibuprofen can induce BAX dependent apoptosis in cancer cells. In a study, the findings demonstrated that NSAIDs inhibited cell proliferation and induced apoptosis in both gastric cancer (AGS) cell lines (wild-type p53) and MKN-28 (mutant p53) with an increase in caspase-3 activity level. Also, there was a significant increase in BAX pro-apoptotic protein levels after treatment with the NSAIDs (Zhang et al., 2001). Another study proved that BCL-2 plays an important role in melanoma pathogenesis (Iervolino et al., 2002). However, it has been shown that change in BCL-2 expression level may have no impact on apoptosis induction (Tiwari, 2012).

3.7. Ibuprofen effects on KAI-1 gene expression level in HeLa cells:

We found that IC_{50} at 3.22 mg/mL of ibuprofen led to a remarkable decrease of KAI-1/CD82 gene expression level in HeLa cells. It has been shown that there is a close association between the positive expression of KAI-1 gene and the development of carcinoma cells (Huang et al., 2016). KAI-1 gene, as a tumor suppressor gene, inhibits the migration and invasion of gastric carcinoma cells (Guo et al., 2015). Recent studies showed that the expression of KAI-1 is associated with tumor grade, invasive depth, lymph node metastasis, and prognosis of cervical squamous cell carcinoma (CSCC). Also demonstrated a clear relationship between the increased expression of KAI-1 and higher survival rate among patients (Gong et al., 2015). It has been shown that KAI-1 is a metastasis-related factor in cervical cancer and low expression of this gene promotes cervical cancer progress and metastasis, such that KAI-1 expression was related negatively to the clinical stage of cervical cancer and positively to differentiation degree and lymph node metastasis (Zhou and Wang, 2015). In this study, IC50 at 3.22 mg/mL of ibuprofen resulted in a significant decrease in KAI-1/CD8 gene expression and showed that although ibuprofen has apoptotic effects, it does

not have KAI-1/CD8 dependent anti-metastatic effects on HeLa cell.

3.8. Ibuprofen effects on NM23 gene expression level in HeLa cells:

This study showed that the IC_{50} at 3.22 mg/mL of ibuprofen caused a considerable reduction in NM23 gene expression in HeLa cells. NM23 was the first identified tumor metastasis suppressor, which has been reported to relate to genomic stability maintenance and cancer (Xue et al., 2019). So, we can conclude that IC_{50} at 3.22 mg/mL of ibuprofen does not have an NM23 dependent anti-metastatic impact on cervical cancer cells in vitro; however, in vivo research is needed to clarify the anti-metastatic effects of ibuprofen on cervical tumors. On the contrary, some studies have shown the opposite results, for example, an investigation by Liu and other researchers in 2002 showed that increased expression level of NM23 gene is associated with cell differentiation in thyroid follicular carcinomas (FTC) and metastasis in papillary carcinomas (PTC)(Liu et al., 2002).

3.9. Ibuprofen effects on MMP9 gene expression level in HeLa cells:

MMPs play a central role in many biological processes, such as angiogenesis and metastasis in cancers (Visse and Nagase, 2003). There is no doubt that MMPs have a major role in metastasis and are involved in the facilitation of the physical barrier's breakdown in this process (Visse and Nagase, 2003). It has been proven that there is a positive correlation between matrix metalloproteinase (MMPs) overexpression and an invasive phenotype in numerous cancer types and in many cancer cell lines (Martinella-Catusse et al., 2001). We found that the cytotoxic concentration (IC_{50} at 3.22 mg/mL) of ibuprofen does not have any significant effects on MMP9 gene expression in HeLa cells. As MMP9 protein has an important role in extracellular matrix proteins digestion and metastasis induction, we concluded that the cytotoxic concentration (IC_{50} at 3.22 mg/mL) of ibuprofen does not have significant impact on MMP9 dependent metastasis in cervical cancer cells.

3.10. Ibuprofen effects on caspase-3 expression level in HeLa cells:

Our findings revealed a significant increase in caspase-3 expression level in ibuprofen (IC_{50} at 3.22 mg/mL) treated cervical cancer cells, triggering the apoptosis pathway in HeLa cells. Caspase-3 gene encodes a protein that belongs to a highly conserved family of proteases. Caspase-3 is also considered as an essential regulator of programmed cell death and leads to proteolytic cleavage of apoptotic target proteins. It is involved in tissue differentiation, regeneration, and neural development as well (Shalini et al., 2015). Caspase-3 is one of the most important enzymes in apoptosis and it is involved in both intrinsic and extrinsic apoptosis pathways.

3.11. Ibuprofen effects on caspase-8, -9 activity levels in HeLa cells:

Different investigations have proven the inhibitory effects of non-steroidal anti-inflammatory drugs on caspases activity, including a recent study, in which, inhibitory effects of diclofenac on different caspases were evaluated and it was revealed that although diclofenac does not have any impacts on caspase-8 activity, it significantly affects caspase-9, so the mitochondrial pathway is the only pathway involved in diclofenac-induced apoptosis (Gómez-Lechón et al., 2003). Diclofenac also influenced caspase-9 activity in cervical cancer cells after treatment but did not have any impacts on caspase-8 activity (Robertson et al., 1998). In our study, there was a significant increase in

caspase-8, -9 gene activities in HeLa cells after treatment with IC50 at 3.22 mg/mL of ibuprofen. Because caspase-9 is a part of the intrinsic apoptosis pathway and caspase-8 acts in the extrinsic pathway, we suggest that 3.22 mg/mL of ibuprofen induces both intrinsic and extrinsic apoptotic pathways in HeLa cells.

4 Conclusion

In conclusion, the results of the present study suggest that ibuprofen induce cytotoxic effects on HeLa cells and the effects are mediated by apoptotic pathways. In this study, ibuprofen triggers extrinsic and intrinsic apoptotic pathways by increasing the BAX and caspase-3 genes expression and increasing the caspase -3, -8 and -9 activities in HeLa cells. In a broader context, this study may provide fundamental knowledge to further find out the association between ibuprofen treatment and cervical cancer recurrences among patients, thereby laying the groundwork for further clinical investigations and improving patients' conditions.

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6 Conflict of interests

The authors declare that there are no competing interests.

References

Boissan, M., & Lacombe, M.L. (2012). NM23, an example of a metastasis suppressor gene. Bull Cancer. 99(4):431–440. French. doi: 10.1684/bdc.2012.1550.

Collet, J.P., Sharpe, C., Belzile, E., Boivin, J.F., Hanley, J., & Abenhaim, L. (1999). Colorectal cancer prevention by non-steroidal anti-inflammatory drugs: effects of dosage and timing. British Journal of Cancer, 81(1): 62–68. https://doi.org/10.1038/sj.bjc.6690651.

De Groot, D.J.A., De Vries, E.G.E., Groen, H.J.M., & De Jong, S. (2007). Non-steroidal anti-inflammatory drugs to potentiate chemotherapy effects: from lab to clinic. Critical reviews in oncology/hematology, 61(1): 52–69. doi: 10.1016/j.critrevonc.2006.07.001.

Fridman, J.S., & Lowe, S.W. (2003). Control of apoptosis by p53. Oncogene; 22(56): 9030–9040. https://doi.org/10.1038/sj.onc.1207116.

Gómez-Lechón, M.J., Ponsoda, X., O'connor, E., Donato, T., Castell, J.V., & Jover, R. (2003). Diclofenac induces apoptosis in hepatocytes by alteration of mitochondrial function and generation of ROS. Biochemical pharmacology, 66(11): 2155–2167.

Gong, X., Tao, Y., Zhou, L., Yu, L., Wu, S., Song, W., Wang, D., & Cheng, Z. (2015). Expressions of Snail, Slug and KAI1 proteins in cervical carcinoma and their clinicopathological significance.

Nan fang yi ke da xue xue bao=Journal of Southern Medical University, 35(12): 1733–1738. Chinese. PMID: 26714907.

Green, D.R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. Science, 305(5684): 626–629. doi: 10.1126/science.

Green, D.R., & Kroemer, G. (2005). Pharmacological manipulation of cell death: clinical applications in sight? The Journal of clinical investigation, 115(10): 2610–2617.

Große, L., Wurm, C.A., Brüser, C., Neumann, D., Jans, D.C., & Jakobs, S. (2016). BAX assembles into large ring- like structures remodeling the mitochondrial outer membrane in apoptosis. The EMBO journal, 35(4): 402—413. doi: 10.15252/embj.201592789.

Guo, J., Fan, K.X., Xie, L.I., Xiao, J.J., Chen, K., Hui, L.N., & Xu, Z.F. (2015). Effect and prognostic significance of the KAI1 gene in human gastric carcinoma. Oncology letters, 10(4): 2035–2042. doi: 10.3892/ol.2015.3604.

Guo, Y., Zhang, Y., Yang, X., Lu, P., Yan, X., Xiao, F., Zhou, H., Wen, C., Shia, M., Lua, J., & Meng, Q.H. (2016). Effects of methylglyoxal and glyoxalase I inhibition on breast cancer cells proliferation, invasion, and apoptosis through modulation of MAPKs, MMP9, and Bcl-2. Cancer biology & therapy, 17(2): 169–180.

Harris, R.E., Beebe-Donk, J., Doss, H., & Burr Doss, D. (2005). Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade. Oncology reports, 13(4): 559–83.

Hu, W., & Kavanagh, J.J. (2003). Anticancer therapy targeting the apoptotic pathway. The lancet oncology, 4(12): 721–729. doi: 10.1016/s1470-2045(03)01277-4.

Huang, W., Yang, J., Ren, J., & Tang, J. (2016). Expression of PTEN and KAI1 tumor suppressor genes in pancreatic carcinoma and its association with different pathological factors. Oncology letters, 11(1): 559–562. doi: 10.3892/ol.2015.3932.

Hussain, A., Brahmbhatt, K., Priyani, A., Ahmed, M., Rizvi, T.A., & Sharma, C. (2011). Eugenol enhances the chemotherapeutic potential of gemcitabine and induces anticarcinogenic and anti-inflammatory activity in human cervical cancer cells. Cancer Biotherapy & Radiopharmaceuticals, 26(5): 519–527. doi: 10.1089/cbr.2010.0925.

Iervolino, A., Trisciuoglio, D., Ribatti, D., Candiloro, A., Biroccio, A., Zupi, G., & Del Bufalo, D. (2002). Bcl-2 overexpression in human melanoma cells increases angiogenesis through VEGF mRNA stabilization and HIF-1-mediated transcriptional activity. The FASEB Journal, 16(11): 1453–1455. 16(11):1453-1455. doi: 10.1096/fj.02-0122fje.

Kim, K.Y., Seol, J.Y., Jeon, G.A., & Nam, M.J. (2003). The combined treatment of aspirin and radiation induces apoptosis by the regulation of bcl-2 and caspase-3 in human cervical cancer cell. Cancer Letters, 189(2): 157–166. doi: 10.1016/s0304-3835(02)00519-0.

Li, J., Li, Q., Huang, H., Li, Y., Li, L., Hou, W., & You, Z. (2017). Overexpression of miRNA-221 promotes cell proliferation by targeting the apoptotic protease activating factor-1 and indicates a poor prognosis in ovarian cancer. International journal of oncology, 50(4):1087–1096. doi: 10.3892/ijo.2017.3898.

Liu, Y., Jiang, C., & Tan, Y. (2002). Pathological study on the expression of cell adhesion molecules and metastasis suppressor gene in thyroid follicular carcinoma and papillary. Carcinoma, 31(4): 322–326..

Luo, S.W., Kang, H., Kong, J.R., Xie, R.C., Liu, Y., Wang, W.N., Xie, F.X., Wang, C., & Sun, Z.M. (2017). Molecular cloning, characterization and expression analysis of (B-cell lymphoma-2) Bcl-2 in the orange-spotted grouper (Epinephelus coioides). Developmental & Comparative Immunology, 76: 150–162. doi: 10.1016/j.dci.2017.06.003.

Marks, D.C., Belov, L., Davey, M.W., Davey, R.A., & Kidman, A.D. (1992). The MTT cell viability assay for cytotoxicity testing in multidrug-resistant human leukemic cells. Leukemia research, 16(12):1165-73. doi: 10.1016/0145-2126(92)90114-m.

Martinella-Catusse, C., Polette, M., Noel, A., Gilles, C., Dehan, P., Munaut, C., Colige, A., Volders, L., Monboisse, J.C., Foidart, J.M., & Birembaut, P. (2001). Down-regulation of MT1-MMP expression by the 3 chain of type IV collagen inhibits bronchial tumor cell line invasion. Laboratory Investigation, 81(2): 167–175. doi: 10.1038/labinvest.3780224..

Mehner, C., Hockla, A., Miller, E., Ran, S., Radisky, D.C., & Radisky, E.S. (2014). Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. Oncotarget, 5(9): 2736–2749. doi: 10.18632/oncotarget.1932.

Oltersdorf, T., Elmore, S.W., Shoemaker, A.R., Armstrong, R.C., Augeri, D.J., Belli, B.A., Bruncko, M., Deckwerth, T.L., Dinges, J., Hajduk, P.J., Joseph, M.K., Kitada, S., Korsmeyer, S.J., Kunzer, A.R., Letai, A., Li, C., Mitten, M.J., Nettesheim, D.G., Ng, S.C., Nimmer, P.M., O'Connor, J.M., Oleksijew, A., Petros, A.M., Reed, J.C., Shen, W., Tahir, S.K., Thompson, C.B., Tomaselli, K.J., Wang, B., Wendt, M.D., Zhang, H., Fesik, S.W., & Rosenberg, S.H. (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature, 435(7042): 677–681.

Prabhu, V.V., Siddikuzzaman, S., Grace, V.M., & Guruvayoorappan, C. (2012). Targeting tumor metastasis by regulating Nm23 gene expression. Asian Pacific Journal of Cancer Prevention, 13(8): 3539–3548. https://doi.org/10.7314/APJCP.2012.13.8.3539.

Rayburn, E.R., Ezell, S.J., & Zhang, R. (2009). Anti-inflammatory agents for cancer therapy. Molecular & cellular pharmacology, 1(1):29–43. doi: 10.4255/mcpharmacol.09.05.

Robertson, F.M., Parrett, M.L., Joarder, F.S., Ross, M., Abou-Issa, H.M., Alshafie, G., & Harris, R.E. (1998). Ibuprofen-induced inhibition of cyclooxygenase isoform gene expression and regression of rat mammary carcinomas. Cancer letters, 122(1-2): 165–175.

Salvador- Gallego, R., Mund, M., Cosentino, K., Schneider, J., Unsay, J., Schraermeyer, U., Engelhardt, J., Ries, J., & García-Sáez, A.J. (2016). Bax assembly into to membrane pores. The EMBO journal, 35(4): 389–401. doi: 10.15252/embj.201593384.

Schiffman, M., Castle, P.E., Jeronimo, J., Rodriguez, A.C., & Wacholder, S. (2007). Human papillomavirus and cervical cancer. The Lancet, 370(9590): 890–907. doi: 10.1016/S0140-6736(07)61416-0.

Shalini, S., Dorstyn, L., Dawar, S., & Kumar, S. (2015). Old, new and emerging functions of caspases. Cell death and differentiation, 22(4), 526 pp.

Sun, Y.S., Thakur, K., Hu, F., Cespedes-Acuña, C.L., Zhang, J.G., & Wei, Z.J. (2020). Icariside II suppresses cervical cancer cell migration through JNK modulated matrix metalloproteinase-2/9 inhibition in vitro and in vivo. Biomedicine & Pharmacotherapy, 125:110013. doi: 10.1016/j.biopha.2020.110013.

Tiwari, M. (2012). Apoptosis, angiogenesis and cancer therapies. Journal of cancer therapeutics & Research; 1(1): 3.

Visse, R., & Nagase, H. (2003). Metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circulation research, 92(8): 827–839.

Willis, S., Day, C.L., Hinds, M.G., & Huang, D.C. (2003). The Bcl-2-regulated apoptotic pathway. Journal of cell science, 116(Pt 20):4053–4056. doi: 10.1242/jcs.00754.

Wu, S., Lan, Y., Wenqing, S., Lei, Z., & Yisheng, T. (2012). Expression and clinical significance of CD82/KAI-1 and E-cadherin in non-small cell lung cancer. Journal of Archives of Iranian medicine, 15(11): 707-712.

Xue, R, Peng, Y., Han, B., Li, X., Chen, Y., & Pei, H. (2019). Metastasis suppressor NME1 promotes non-homologous end joining of DNA double-strand breaks. DNA Repair (Amst), 77:27–35. doi: 10.1016/j.dnarep.2019.03.003.

Yoshida, B.A., Dubauskas, Z., Sokoloff, M.H., Welch, D.R., & Rinker-Schaeffer, C.W. (2002). Metastasis-Suppressor Genes: A Review and Perspective on an Emerging Field. Journal of the National Cancer Institute, 92(21): 1717–1730. doi: 10.1093/jnci/92.21.1717.

Yu, L., Zhou, L., Wu, S., Gong, X., Feng, Z., Ma, L., Zhu, B., Yao, N., Wang, D., & Dong, H. (2014). Clinicopathological significance of cancer stem cells marked by CD133 and KAI-1/CD82 expression in laryngeal squamous cell carcinoma. World journal of surgical oncology, 12(1): 118. https://doi.org/10.1186/1477-7819-12-118.

Zhang, B., Liu, W., Li, L., Lu, J., Liu, M., Sun, Y., & Jin, D. (2013). KAI-1/CD82 and CyclinD1 as biomarkers of invasion, metastasis and prognosis of laryngeal squamous cell carcinoma. International journal of clinical & experimental pathology, 6(6): 1060–1067.

Zhou, X.L., & Wang, M. (2015). Expression levels of survivin, Bcl-2, and KAI1 proteins in cervical cancer and their correlation with metastasis. Genetics & Molecular Research, 14(4): 17059–17067. doi: 10.4238/2015.

Zhou, X.L., & Wang, M. (2015). Expression levels of survivin, Bcl-2, and KAI1 proteins in cervical cancer and their correlation with metastasis. Genet Mol Res, 14(4): 17059–67.

Zhou, Y., Boudreau, D.M., & Freedman, A.N. (2014). Trends in the use of aspirin and drugs in the general US population. Pharmacoepidemiology & Drug safety, 23(1): 43–50.