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A Study of the Effect of Aspirin and Atorvastatin on the Phenotypes of Liver Cancer Cells in a Cell Culture Model

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ABSTRACT

BACKGROUND AND OBJECTIVE: In patients with type 2 diabetes, liver diseases are the major causes of liver cancer. The invention of new methods and medicinal compounds has led to a significant increase in our ability to treat cancers. This study aims to evaluate the effect of two known compounds, atorvastatin and aspirin, on the phenotypes of liver cancer cell lines.

METHODS: In this experimental study, after preparing HepG2 cell line from National Cell Bank of Iran and culturing it, the cytotoxic, apoptotic and metastatic effects of both atorvastatin and aspirin were investigated at concentrations of 50 – 100 – 200 µM in 7 treatment groups and one control group by MTT assay, flow cytometry and zymography tests.

FINDINGS: The results of these tests indicated the cytotoxic effects of atorvastatin at all concentrations of 50 – 100 – 200 µM (48%, 99.96% and 100%), and the low cytotoxic effects of aspirin at all concentrations except for 200 µM, mainly observed as necrosis ($p<0.05$). In both compounds, apoptosis induction was initiated at a specific concentration and the simultaneous use of these two compounds increased the apoptosis from 6.8 and 3.22, respectively for atorvastatin and aspirin, to 20.22 ($p < 0.05$). Investigating the activity of the MMP-2 enzyme as a key enzyme in metastases indicated a decrease in this phenotype.

CONCLUSION: The results of this study showed that co-administration of both atorvastatin and aspirin compounds is capable of inducing programmed cell death at low concentrations.

KEY WORDS: *Atorvastatin, Aspirin, Hepatocellular carcinoma, Metastasis, Matrix metalloproteinase*.

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Introduction

One of the causes of death in patients with type 2 diabetes mellitus is chronic liver disease, such as nonalcoholic fatty liver disease. The severe form of the disease causes liver failure and ultimately hepatocellular carcinoma (HCC), which causes mortality in people (1). Findings from several studies in developed countries indicate the incidence of hepatocellular carcinoma, even before the onset of cirrhosis, in about fifty percent of patients with nonalcoholic fatty liver disease, which creates a new challenge in the process of faster diagnosis (2).

Despite remarkable advances in molecular biology, molecular mechanisms effective at the stages of tumor formation and metastasis in this cancer are not clearly known yet (3 – 5). Studies have shown that proteolytic activity of a specific group of endopeptidase (matrix metalloproteinase –2) by cancer cells plays an important role in hepatocellular carcinoma and metastasis (6, 7). Matrix metalloproteinases (MMPs) are proteases that degrade the extracellular matrix and are controlled by endogenous inhibitors. In pathologic processes, the expression and activity of these proteolytic enzymes is increases by secretion of proinflammatory cytokine, which will result in exacerbation of inflammation. In recent years, various studies have been carried out on the important role of metalloproteinases in processes such as angiogenesis and cancer cell proliferation, and the production of compounds that inhibit these structures plays a special role in studies. Several treatments including chemotherapy and radiotherapy have been proposed for the treatment of this disease (8, 9).

In a clinical trial, statin and its derivatives have shown their effects on controlling blood lipids as competitive inhibitors of HMG-CoA reductase. These compounds are prescribed in clinical medicine in order to prevent or reduce the risk of cardiovascular disease despite increasing liver transaminases (10). There are contradictory reports about the effect of statins on malignancy. Some have considered them to be ineffective and some have shown positive effects on different types of cancer, such as lymphoma and melanoma (11–17).

Another known drug as a non – steroidal anti – inflammatory drug (NSAID) is aspirin, which is prescribed as an analgesic and antipyretic. Due to its anticoagulant effects, it is used at low concentrations to prevent heart attacks and stroke. This compound inhibits cyclooxygenase enzymes non-selectively. Aspirin is currently the most widely used drug in the world, and some studies indicate a reduction in the risk of colorectal cancer and a lack of effect on other cancers, such as lung cancer (18,19).

Li et al. also showed that aspirin has beneficial effects in patients with HCC (20). Due to the variety of factors that affect the pathogenesis of HCC and the increased prevalence and mortality resulting from it, research to find drugs that solve this problem by various mechanisms is highly sought after. Most of the existing drug compounds, especially at concentrations used in treatments, have severe and non-specific cytotoxic effects, and there are contradictory reports on some of them, such as statins. One of the potential solutions would be co-administration at lower concentrations. The aim of this study was to investigate the effect of atorvastatin and aspirin on phenotypes of cancer cells and their inhibitory effect on the proliferation of cancer cells and metalloproteinases.

Methods

Cell culture: This experimental study was carried out in the ethics committee of Tehran University of Medical Sciences (code 7718) on HepG2 cell line in a cell lab. Cells of the liver cancer model were purchased from the Iranian Cell Bank in the Tehran Pasteur Institute. The study variables, that is cytotoxic, apoptotic and gelatinolytic effects, were respectively analyzed using MTT, flow – cytometry and zymography tests at concentrations of 50 – 100 – 200 µM aspirin and atorvastatin, alone and together, in 7 treatment groups and one control group. At least three to five wells were used according to the standard for control groups and evaluated materials. The pure compounds of atorvastatin and aspirin were purchased from Daroo Pakhsh Co. in Iran and were diluted in solvent (Dimethyl Sulfoxide, Merck, USA).

Cellular viability using MTT assay: The liver cancer cell model (HepG2 cell line) was stored in Cell culture laboratory of Tehran University of Medical Sciences and stored in RPMI 1640 medium (Gibco, USA) containing 5% fetal calf serum (Gibco, USA). Penicillin and streptomycin antibiotics (Gibco, USA) were cultured in 5% CO2 incubator at 37 °C, and saturated moisture content. The cytotoxicity test was performed according to the study of Khoramizadeh et al. (21). Ten thousand cells were cultured in 96 – well plates and treated with atorvastatin and aspirin at

concentrations of 50 – 100 – 200 µM for one night in incubator in the seven treatment groups. The control group had no medication. The cell culture medium was then replaced with a fresh medium containing tetrazolium bromide solution at a concentration of 5 mg / mL. The cells were placed in a new medium for three hours, and then the supernatant was discarded and 100 µl of isopropanol was poured into plate cavities instead. Finally, the ambient color was measured by the ELISA reader at a wavelength of 570nm.

Evaluation of gelatinase activity by zymography test: Zymography test was performed to evaluate the semi-quantitative activity of gelatinase A enzyme (MMP-2). Samples from supernatant test cells were electrophoresed with 1% gelatin in 7% polyacrylamide gel. The gel was then placed overnight in a solution containing calcium and zinc, which activates the gelatinase enzyme. After 24 hours, the gel was stained with Coomassie Blue and then bleached with a mixture of ethanol solvent and water. The gels were then photographed using the Gel Documentation (UVP) device and were quantitatively analyzed using the UViTech (UK) Density Software (21). The results are shown in columnar graphs.

Flow – cytometric study of programmed cell death with Annexin-v: Apoptosis assay was done using Annexin-V kit (ROCHE, Germany) based on flow – cytometry method. The cells were fixed after treatment and then, using the above-mentioned solution, which was conjugated with FITC, and were evaluated through spectrophotometric analysis by flow – cytometer (Partech, USA) and using propidium iodide solution, which is used to detect necrotic cells. The results read by the device software were used for subsequent comparative analyses. The results of the differences in growth of treated cells and activity of gelatinase from three independent tests were investigated using SPSS 16 software using T-test, while $p<0.05$ was considered significant.

Results

The results of cellular viability using MTT assay: To evaluate the cytotoxic effects of atorvastatin and aspirin, Hep-G2 cells were treated with different concentrations of these compounds and MTT assay was performed (Fig 1). By increasing the atorvastatin concentration, the percentage of live cells decreased to 50, 0.04 and 0. Based on the results of 50IC, atorvastatin is 50 µM. In addition, after cell treatment with aspirin at various concentrations of 50, 100 and 200 µM, the percentage of live cells was 100, 94.7 and 73.7, respectively, indicating that the difference between concentrations in these two compounds is statistically significant ($p<0.05$).

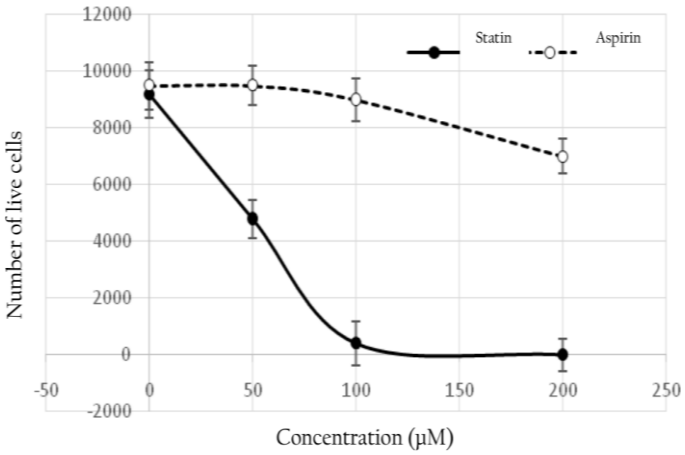

Figure 1. Evaluation of cytotoxic effects of atorvastatin and aspirin in different concentrations on Hep-G2 cells. As shown in the chart, with increasing atorvastatin concentration, the number of live cells is reduced in a dose-dependent manner, while up to 100 µM aspirin has no significant effect on Hep-G2 cells.

Evaluation of the activity of matrix metalloproteinase-2 (MMP-2) after hep-G2 cell incubation using zymography: To study the effects of aspirin and atorvastatin drug combinations on MMP-2 activity, different amounts of these compounds were tested on HepG2 cell line. Following the chemical treatment, the level of activity of matrix metalloproteinase-2 decreased and these changes were calculated by analyzing the density of zymogram gel and based on the mean area under the curve. This decrease was significant at 200 µM for atorvastatin (Fig 2) ($p<0.05$).

Results of programmed Hep-G2 cell death at various concentrations of drug: Flow –cytometric results of HepG2 cell treatment with statin at different concentrations (0 – 50 – 100 – 200 µM) showed that after the chemical treatment, the percentage of necrotic cells increased with increasing drug concentration; at 200 µM atorvastatin, all cells were destroyed. This decrease in all concentrations of 50 – 100 – 200 µM was 33.2, 23.15 and 0, respectively, which was statistically significant compared to zero concentration ($p<0.05$). It was also found that this compound was not effective in the induction of programmed cell death,

and with increasing drug concentration, the percentage of apoptotic cells reached 6.80, 2.77 and 0, respectively (Fig 3).

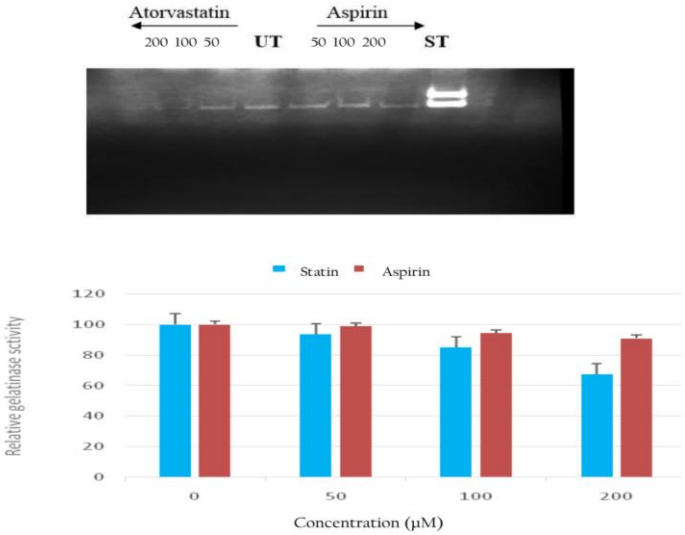

Figure 2. Effect of aspirin and atorvastatin on the activity of matrix metalloproteinase-2. Panel A: Electrophoretic analysis of gelatinase A (MMP-2) activity from atorvastatin and aspirin treatment with concentrations of 50–100–200 µM. UT (untreated) and ST (standard control). Panel B. Effects of chemical treatment of cells with aspirin and atorvastatin on the activity of matrix metalloproteinase-2 (MMP-2) using zymography technique. Reduction of gelatinase A (MMP-2) activity at 200 µm atorvastatin concentration was significant ($p \leq 0.05$)

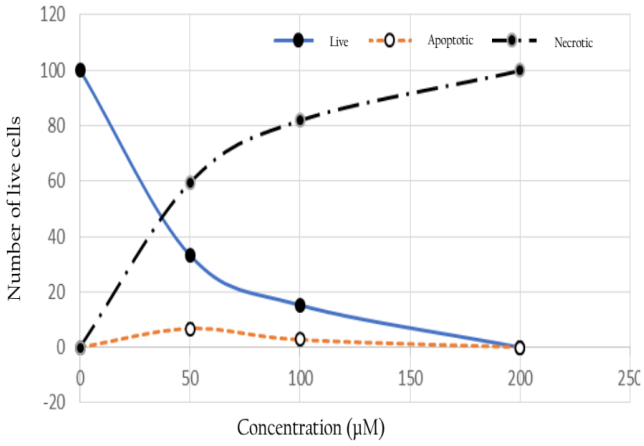

Figure 3. The percentage of apoptosis and necrosis of cells treated with different concentrations of atorvastatin. Apoptosis decreases with increasing statin concentration. Significant differences were observed in necrosis and apoptosis of Hep-G2 cells in all atorvastatin concentrations ($p<0.05$).

HepG2 cell treatment with aspirin at various concentrations (0–50–100–200 µM) has a small effect on processes such as necrosis and apoptosis and only causes cell death at high concentrations (Fig 4). Based on the results of HepG2 cancer cell line treatment with different concentrations of atorvastatin and aspirin, the combination of these two was used up to 50 µM of each. The combination of these two compounds strongly induces programmed cell death, which increases by up to three times compared to statin. This increase is statistically significant (Fig 5) ($p<0.05$).

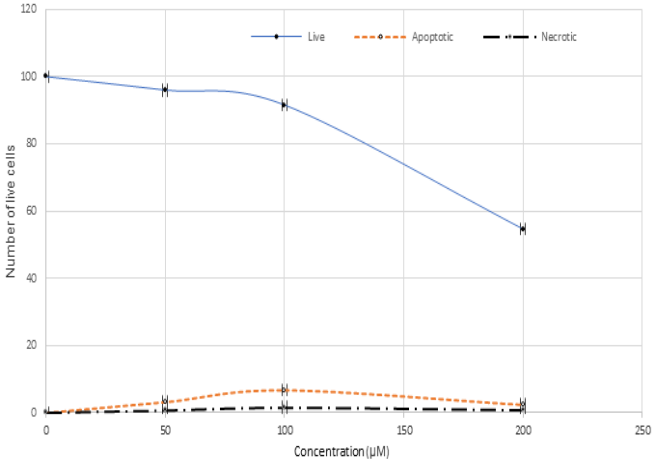

Figure 4. The percentage of cells with apoptosis and necrosis after treatment with different concentrations of aspirin. With increasing aspirin concentration, the apoptosis changed in a wave form, although there was no significant difference between necrosis and apoptosis of in Hep-G2 cells in all aspirin concentrations.

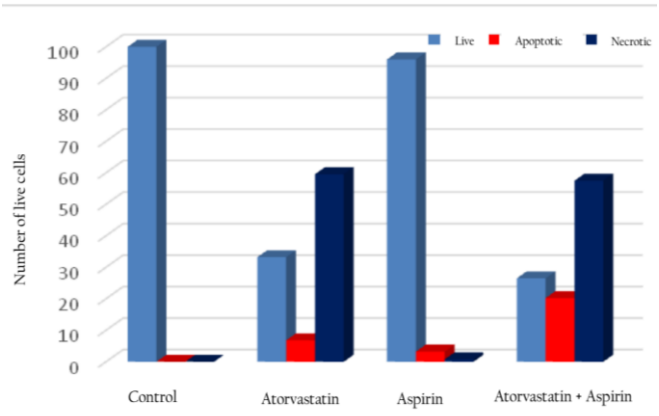

Figure 5. Results of analysis of programmed cell death at a concentration of 50 µM atorvastatin and aspirin. Significant differences were observed in Hep-G2 cell apoptosis in co-administration of statin and aspirin compared to each of the two compounds separately ($p \leq 0.05$).

Discussion

The results of this study indicate the ability of inducing programmed cell death by combining atorvastatin with aspirin at low concentrations. The co-administration of these two compounds increases the programmed cell death by up to three times compared to statin. Due to the unlimited proliferation of cancer cells and the high levels of production of nucleic acids and proteins, these cells, while escaping from the apoptosis process, have potential to invade the adjacent tissues and ultimately locate in secondary sites. Different statins have different effects on HCC (22). Results of a study by Simon et al. indicate that hepatocellular carcinoma (HCC) is 50% lower in patients with hepatitis C (23), while Lai et al. estimate it to be around 28% (24).

The present study examined the effects of both compounds of atorvastatin and aspirin on one of the most well-known cellular models alone and in a comparative way, in order to better understand the anticancer role of these compounds. The results of cytotoxicity tests revealed severe cytotoxic effects of atorvastatin compared with aspirin. Our findings in the HepG2 cell model confirmed the cytotoxic effects of atorvastatin even at low concentrations, as confirmed by Leszczynska et al. (25).

On the other hand, this finding is consistent with previous results regarding the effects of cytotoxic drugs (NSAIDs) (26). What was found about the effects of these compounds on the metastasis phenotype using the gelatinase A enzyme activity assay showed the highest inhibitory effects on atorvastatin (at a concentration of 200 μM) and the lowest inhibitory effects on aspirin. This finding is consistent with the results of other researchers; statin does not affect MMP-9 and prevents increased MMP-2 secretion (27 and 28). The results of apoptosis–necrosis test using flow – cytometry showed that the effect of atorvastatin on induction of cell death in all concentrations has a significant difference with untreated samples, although this effect is mainly due to non-apoptotic cell death. Of course, at a concentration of 50 μM, apoptosis was higher than that of 100 μM. This finding is consistent with some of the findings that suggest suppression of Ras / ERK and Ras / mTOR pathways is effective in the induction of statin-induced apoptosis (14).

In the case of aspirin, the cell death phenomenon, including necrosis or apoptosis, is very low and gradually increases with concentration, which is consistent with the observations of other researchers (29 and 30). Unlike atorvastatin treatment, apoptotic cells in aspirin treatment increase and then decrease as a wave at a concentration, compared with necrotic cells. This decrease is related to dose-dependent statin, but high-dose aspirin seems to cause cell death in another ways such as autophagy (31).

On the other hand, the co-administration of these two compounds, with far less concentrations, increases the programmed cell death by up to three times. According to the results of this study, the ability to induce programmed cell death increased significantly by co-administration of statin and aspirin at a concentration of 50 μM. Compared to aspirin and statin, this increase is seven and three times as much, respectively. The main feature of cancer disease is that it has multiple branches. Thus, it is better to design new generations of drugs that can affect multiple targets simultaneously in cancer cells without causing toxicity in the host's healthy cells.

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