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Changes in P53 and Bcl2 Gene Expression in Ags Cell Line Due to Ascorbic Acid Treatment

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ABSTRACT

Background: Antioxidants have a great influence on the control of oxidative stress and free radicals, and they cause the expression of apoptosis genes. Therefore, they have an important role in preventing cancer. The aim of this study was to Mechanism of ascorbic acid apoptosis on p53 and Bcl2 gene expression in AGS cell line.
Method: To investigate the cytotoxicity influences of the treatments, the MTT test was applied to test the impact of various concentrations of ascorbic acid on the gene expression of p53 and Bcl2 using Real-time PCR.
Results: The findings revealed that the mortality of the cancer cells enhanced significantly by treatment, compared with the untreated cells. The treatment, compared to untreated group, significantly affected the gene expression of p53 and BCL2.
Conclusion: the results indicated that might be effective in inhibiting the growth of cancer cells.

Keywords: Ags Cell Line, Ascorbic Acid, Cancer, P53, Bcl2.

Introduction

Cancer is a main health problem resulting in death in spite of the discovery of many novel anticancer medicines. One of the most important strategies for prevention of gastric cancer is the use of antioxidant rich diet. Gastric cancer risk is lower in people with healthy lifestyles who are on a diet rich in fruits and vegetables, but smoking and inadequate nutrition are associated with an increased risk of gastric cancer(1). Free radicals cause damage to vital cell macromolecules, such as DNA and genetic damage, which results in cell death through apoptosis and cancer cell growth. On the other hand, antioxidants by trapping free radicals can eliminate and reduce the lethal effect of these radicals in the cell(2). Reactive Oxygen Species (ROS) and Reactive Nitrogen Species can be produced in different organelles in response to different stimuli. The main sources of ROS production include mitochondria, endoplasmic reticulum, plasma membrane and cytosol(3). If oxidative stress persists, oxidative damage occurs to vital biomolecules (such as the genome), and accumulation of these damages results in some biological effects such as alterations in message transmission, alterations in gene expression, mitogenesis, transformation, mutations and cell death. They are important in controlling oxidative stress(4). Ascorbic acid (vitamin C) is a water soluble vitamin and is well-known for its antioxidant capacity. Therefore, it is of considerable interest in the present field to prepare water-soluble stable magnetic nanofluids with the use of ascorbic acid. Ascorbic acid, as an antioxidant, scavenges reactive oxygen species and free radicals. It has been identified to be toxic to cancer cells and is also a well-known reducing agent both in vitro and in vivo(3). It has been found that ascorbic acid could target molecules that control cancer progression such as p53 and BCL2. Activation of p53 in enucleated cytoplasts is adequate to directly or indirectly initiate apoptosis by inducing proapoptotic Bcl-2 family members. Tumor suppressor p53 gene is located on chromosome 17 (17p13.1) in human. The significance of p53 in preserving genome stability is demonstrated by the finding that about half of all human cancers carry mutant p53(5). Tumor suppressor B cell lymphoma 2 (Bcl-2) gene is located on chromosome18 (18q21.33) in human. Key managers of the intrinsic apoptotic pathway are the family of B cell lymphoma 2 (Bcl-2) proteins. The activity of the prototypical Bcl-2 protein is typically considered antiapoptotic(5). In this study, we evaluated the distribution of ascorbic acid in gastric cancer cell line. Furthermore, the current study aimed to give insight into the possible mechanism of application of ascorbic acid in cancer treatment by examining the expression of tumor suppressor genes, p53 and Bcl-2, in in gastric cancer cell line.

Materials and Methods

Treatment and MTT test

Cell counts were assessed by a haemocytometer, and 8000 cells were applied for all experiments. To calculate LD50, cells treated with ascorbic acid were added to each well (60 wells of 96 wells) and the plate returned to the incubator for either 24, 48 or 72 hours.Each treatment was performed in 6 replicates and each replicate in 9 different

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concentrations. The first row in each pellet was treated as control as 6 replicates. Anti-tumor activities on the AGS cell line, was determined by 3-4, 5-dimethylthiazol-2-yl 2,5-iphenyltetrazolium bromide (MTT) colorimetric assay. After 24, 48 or 72 h of Treatment, MTT (Sigma) was added and then incubated for a further 4 h at 37°C. Then, 20µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated for an additional 4 h followed by adding 200µl of MTT solvent and shacked for 10 minutes. Then, spectrophotometric absorbance was measured at 570 nm and read by ELISA reader and Optical density (OD) for each well was determined.

RNA extraction and complementary DNA synthesis

RNA was extracted from two high doses and two low doses of LD50 for ascorbic acid, as well as from negative control samples. Then, 6 × 108 cells were centrifuged for 2 minutes / 12000 g, and the supernatant was removed. RNA extraction was done using RNX- Plus Solution (EX6101, sinaclon, iran). After centrifugation, the sediment was stored at -80° C until analyses. The complement DNA (cDNA) was synthesized with the use of a cDNA synthesis kits (Sinaclon, Iran). Materials used to conduct Reverse transcription polymerase chain reaction(RT-PCR) included.

Real-time polymerase chain reaction

Real-time PCR was done using a Corbett Rotor-Gene 3000 (Corbett Robotics, Australia), with the use of eva Green Premix Ex Taq II (BioFact, Daejeon, Korea). The specific primers used in the present study are reported in Table 1. PCR mixtures included1µL of first strand cDNA ,10 µL Eva Premix Ex Taq II (2×), 8 µL of DEPC-treated water, and 1 µL of each specific primers (10 pmol in a final volume of 20 µL.

Table 1: Primer sequence

Gene	Sense 5´- 3´	Antisense 5´- 3´	Product size (bp)
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG	496
P53	CAGTGCTCGCTTAGTGCTCC	GTGTTTGTGCCTGTCCTGGG	107
Bcl2	GAGGCAGGCAGTAGTATGGTG	AGGATAACGGAGGCTGGACA	100

Temperature conditions consisted initial denaturation at 95°C for 10 minutes, followed by 40cycles of denaturation at 95°C for 15 seconds , annealing at 60°C for 40 seconds, and extension at 72°C for 30 seconds.The relative level expressions of all investigated genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as the endogenous house-keeping gene.The relative value of Ct was compared to that of control cells as a reference for estimating the fold change of mRNA expression among the samples.Triplicates were conducted for each pairs of primers.

Statistic Analysis

SPSS software version 23 was used for statistical analysis and $p < 0.05$ was considered significant.

Results and Discussion

The morphological characteristics of AGS cancer cells treated with different concentrations of ascorbic acid were examined by invert microscopy (Figure1,A, B and C). The results showed that at mentioned concentrations a number of morphological changes such as chromatin condensation and nucleus fragmentation and apoptotic bodies formation occur in AGS cancer cells However, no change was observed in control cells and normal cellular structure was observed. These changes were in fact an introduction to the formation of apoptotic bodies and the initiation of the apoptotic process. In addition to morphological changes, the number of apoptotic cells was also significantly increased after treatment with the mentioned concentrations compared to the control group.

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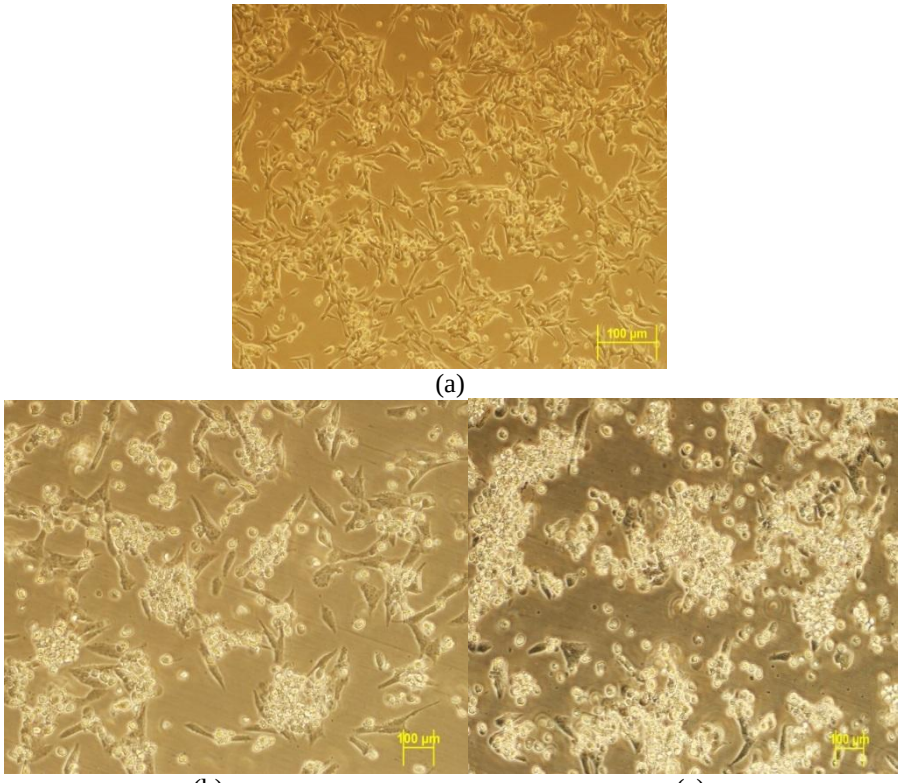

(a)

(b) (c)

Figure 1: (a) Without treatment, (b) Low-dose ascorbic acid treatment (c) High-dose ascorbic acid treatment.

MTT analyze

Result of this investigation find that, after 48 h, there was a significant difference in the cell viability ascorbic acid in monothearpy. Moreover, cell viability was lowest in ascorbic acid group (16). There was a dose-dependent reduction in cell viability so that with the increasing the doses of ascorbic acid, the cell viability also decreased (shown in Figure 2). The concentrations of each agent giving 50% inhibition of cell viability (IC50) were estimated from ic50 calculation software . The ic50 value was calculated for ascorbic acid 29 µg/ml.

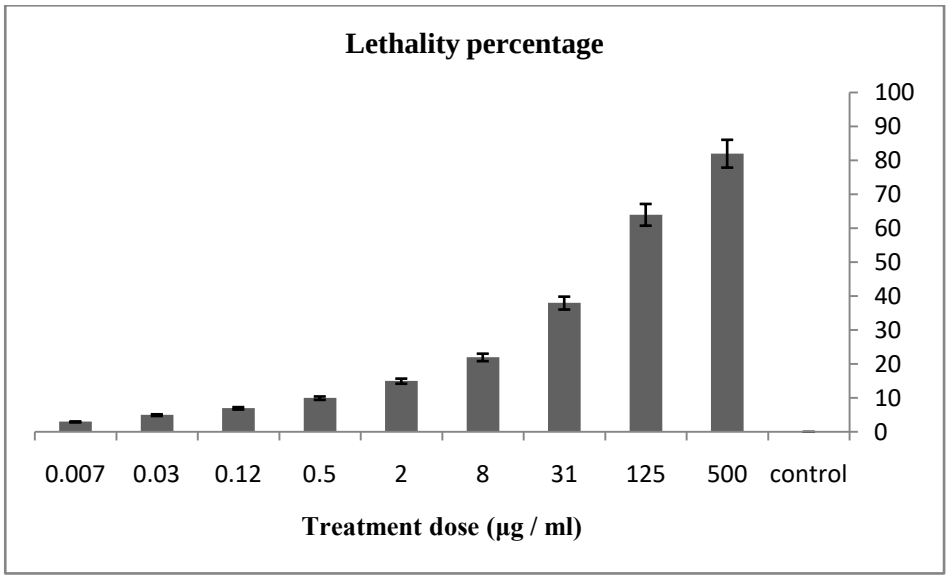

Figure 2. Effect of different concentrations of ascorbic acid on Ags cell line mortality using MTT assay($P<0.05$).

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p53 and Bcl2 genes expression

Figure 3 and 4 presents changes in the gene expression by ascorbic acid. We studied the influence of various concentrations of ascorbic acid on the expression of p53 and Bcl2 with use of Real-time PCR. The results revealed that p53 and Bcl2 expression were significantly (p, 0.05) increased and decreased in treated ascorbic acid groups, respectively, compared to control group. Furthermore, the gene expression of p53 and bcl2, increased and decreased significantly, respectively under the treatment with high doses compared to the control group. In Ascorbic acid group, the rate of p53 gene expression in high doses was 9.8 fold and in the low doset was 2.2 fold. The rate of expression of Bcl2 gene in high doses reduced by 81% and in the low dose decreased by 19%.

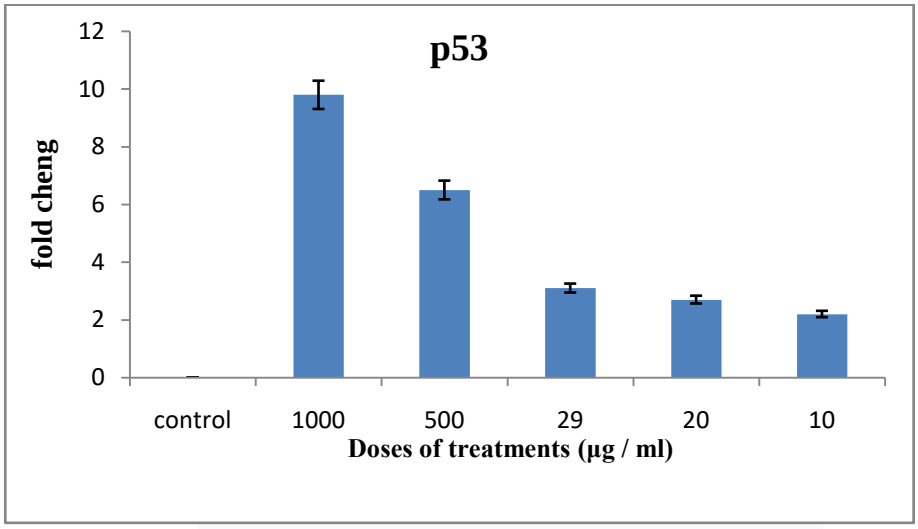

Figure 3. Expression of p53 gene by different doses of ascorbic acid

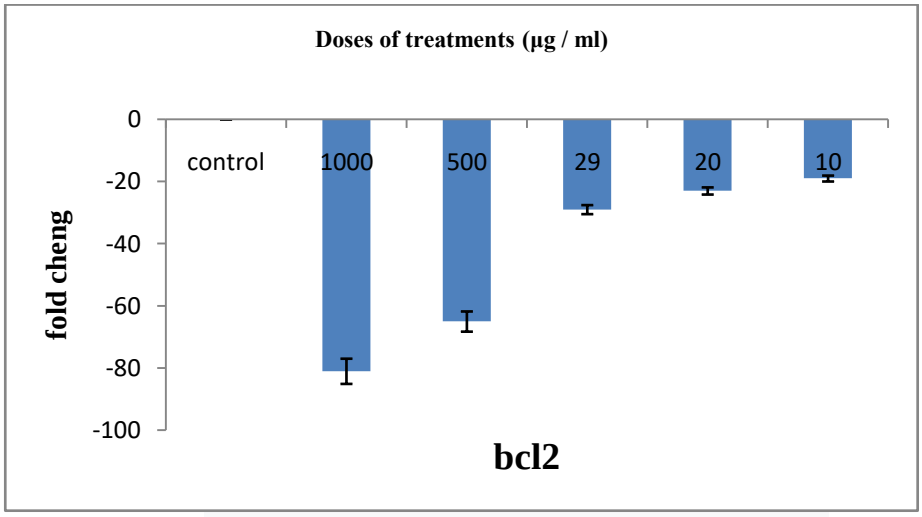

Figure 4. Expression of p53 gene by different doses of ascorbic acid

Discussion

One of the major causes of death in the world is cancer. Gastric cancer is the most common cancer and the most common cause of death among men and women. Lifestyle plays an important role in the development of cancer, including poor nutrition and stressful life. The role of antioxidants in the prevention and treatment of cancers has been reported by many studies(6). The antioxidant impact of ascorbic acid has been reported by researchers including Anarkooli et al, Raghavan et al, and Anbara et al(7-9). Nevertheless, studies have identified that these nutrients play an important role in the prevention and treatment of cancer, because of antioxidant activity, induce

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death of cancer cells by reducing free radicals and Ros(10). studies have found that ROS generetion is a normal physiological process, its existence vital for safety and coordination in the transmission signaling pathways, yet the excessive and random production of ROS is recognized as a chief reason of disease and cancer. The regulation of gene expression through antioxidants is a promising treatment(11, 12). This study was done to investigate the mechanism of ascorbic acid apoptosis on the expression of p53 and Bcl2 genes in gastric cancer cells. Based on the findings of the MTT test, it was found that when cancer cells are exposed to ascorbic acid for 48 hour, these treatments result in the death of cancer cells. With increasing the dose of ascorbic acid, the mortality rate of the cancer cells also enhanced, and the highest dose had the highest effect on cancer cell death. One of the suggested mechanisms for the selective cytotoxic action of ascorbic acid in cancer cells is antioxidant effect(10).

After approving the reduction in the proliferation and cell survival in the studied cell line, it is essential to know that these findings are because of factors such as this one that one of the mechanisms suggested for the selective cytotoxic activity of ascorbic acid in cancer cells is oxidative stress(13, 14). In addition to cellular toxicity, a high level of ascorbic acid also shows a remarkable decrease in peroxide levels, shownig that it becomes more harmful species of the cell, as radical hydroxyl or nitrite peroxide. The findings of this study are in line with the study by Salomé Pires et al., which showed that active species cause damage to cells, but vitamin C reduces free radicals and reactive oxygen species. The researchers cited that ascorbic acid is involved in gathering reactive oxygen species, acting as an antioxidant to preserve the balance of intrinsic cellular and minimize the oxidative damage produced by these free radicals, which matched the results of our research(15). An imbalance between antioxidant defense and oxidative stress can results in cancer, thus, ascorbic acid play an important role in preventing cancer and through the upregulation of apoptosis genes.

The findings (Figure3 and 4) indicated that ascorbic acid significantly increase the p53 expressionand reduce the expression of BCL2 gene in cancer cells, compared to the control group. And doses also had a greater effect on the expression of genes, so that in p53 and BCL2, higher doses had respectively, higher expression and decreased expression than the lower doses. our findings are in agreement with the study by Bassiony et al., which reported that nanoparticles hinder the tumor through the upregulation of apoptosis genes(1). Oxidative stress happens when excessive production of active species occurs due to an external (eg, ultraviolet radiation) or an internal source (at the cellular level involved in mitochondria)(11, 12, 15). The most important mechanism justifying the impact of ascorbic acid on the expression of p53 and BCL2 is its antioxidant property, which enhances the expression of genes involving in the apoptosis, decreases free radicals, regulates cellular signaling, regulates cell cycle, regulates the reaction of oxidation and restores and prevents mutation(11, 12, 15, 16, 17). In vivo and in vitro experiments reveal that antioxidants could impede the growth of Neoplastic cells, induces apoptosis, stimulates cell differentiation and inhibits sprotein kinase C and adenylyl cyclase. Antibacterial proof also confirms that high dose therapy could be beneficial in improving prognosis and therapeutic efficacy(18). These reports confirm our findings.

Ascorbic acid is shown to increases the expression of pro-apoptotic proteins, such as Bax and P53 , paralleled with the deceased of the anti-apoptotic Bcl-2 protein expression, thus resulting in cell death(19). Signaling between P53 and Bcl-2 has been demonstrated to be of high significance for cancer screening. P53 gen is activated in several cellular stresses and acts as an intermediary to stop the cell cycle. The p53 gene increases the Bax gene expression(20). Besides, activaation of Bax by P53 might overcome the antiapoptotic influences of Bcl-2. For instance, in the absence of Bax, the expression of the concentration of oncogenesis induced by p53-dependent apoptosis is reduced. Thus, the regulation of the P53 mediator of the Bax ratio to the Bcl-2 level could affect cell function during stress response(20 , 21). The cyclin-dependant kinase decrease the p21 protein and cause the cell to stop at the preG1 stage. This reaction can be observed in response to DNA damage and oxidative stress, which depends on the attendance of active p53 in the nucleus. Furthermore, cancer cells often display excessive expression of the Bcl-2 protein, which prevents cytokromic release of mitochondria, and provides the cell with chemotherapy resistance and avoiding apoptosis(19). Since the expression has increased P53 is associated with an increase in the Bax / Bcl-2 ratio in ascorbic acid induced apoptosis. In addition, more evidence proposes that the key role of p53 in apoptosis is mainly reliant on transcriptional activity.

Conclusion

In conclusion, the current study revealed that ascorbic acid are effective in inhibiting the growth of cancer cells and the gene expression of p53 and BCL2, which led to cells trigger programmed cell death via apoptosis in tumor cells. Thus, applying these compounds is might be effective in the treatment of cancers.

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