

Induction of Apoptosis by a Combination of 2-Deoxyglucose and Metformin in Esophageal Squamous Cell Carcinoma by Targeting Cancer Cell Metabolism

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What's Known

- The effect of 2-deoxyglucose (2DG) has not yet been tested on esophageal cancer cells. It is shown that metformin decreases cancer cell viability and induces cell autophagy in esophageal cancer cells.
- The effect of combined 2DG and metformin on cancer cell metabolism and growth is presently unknown.

What's New

- A treatment with combined 2DG and metformin causes more damage to cancer cells than the individual use of each drug. It leads to the inhibition of cell viability in esophageal cancer cells and induces p53-dependent apoptosis.
- The findings highlight the potential use of such combination in anti-cancer therapy.

Abstract

Background: Both mitochondrial dysfunction and aerobic glycolysis are signs of growing aggressive cancer. If altered metabolism of cancer cell is intended, using the glycolysis inhibitor (2-deoxyglucose (2DG)) would be a viable therapeutic method. The AMP-activated protein kinase (AMPK), as a metabolic sensor, could be activated with metformin and it can also launch a p53-dependent metabolic checkpoint and might inhibit cancer cell growth.

Methods: After treatment with 5 mM metformin and/or 500 μ M 2DG, the TE1, TE8, and TE11 cellular viability and apoptosis were assessed by MTT, TUNEL, and ELISA methods. The changes in p53 and Bcl-2 genes expression levels were examined using real-time PCR method. Data were analyzed by Kruskal-Wallis test using the SPSS 17.0 software.

Results: Metformin and 2DG, alone and in combination, induced apoptosis in the cell lines. Real-time PCR revealed that metformin induced apoptosis in TE8 and TE11 cells by activating p53, down-regulating Bcl-2 expression. The induced apoptosis by 2DG raised by metformin and the combination modulated the expression of Bcl-2 protein in all cell lines and it was more effective in TE11 cell line.

Conclusion: Metformin induced apoptosis in ESCC by down-regulating Bcl-2 expression, and up-regulating p53 and induced apoptosis increased by 2-deoxy-d-glucose. Thus, the combination therapy is an effective therapeutic strategy for esophageal squamous cell carcinoma.

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Introduction

The third most common cancer in the digestive tract is esophageal squamous cell carcinoma (ESCC) and it is the world's sixth deadliest cancer.¹⁻⁴ Most cases of ESCC are diagnosed at an advanced stage and mainly metastasis to the regional lymph nodes occurs.⁵ Currently, no effective therapeutic methods and chemopreventive agents are available for this fatal illness. One of the primary metabolic changes associated with proliferating tumor cells is the induction of aerobic glycolysis.⁶ Therefore, most cancer cells use an elevated amount of glucose for anabolic

reactions and are more dependent on aerobic glycolytic metabolism to generate ATP than on mitochondrial metabolism. These biological alterations present a major challenge in cancer treatment, as exemplified by the resistance of cancer cells to chemotherapeutic agents and radiation therapy in hypoxic environment.⁷ Also, the enhanced dependency of cancer cells to glycolysis for energy production could be used to preferentially kill these cells by inhibition of glycolysis.

2-deoxyglucose (2DG) acts as an inhibitor of glucose metabolism since it inhibits hexokinase, which is the first limiting factor enzyme of glycolysis.⁸ The result is intracellular ATP depletion⁹ and autophagy induction. Also, the process of cell survival, as a reaction to nutrient deprivation, is influenced.¹⁰ Since a tumor is dependent on glycolysis, 2DG has been considered as a possible anticancer factor and aggregation of chemotherapeutic factors. 2DG has been applied successfully in mice.¹¹

Metformin is a commonly prescribed drug for the treatment of type 2 diabetes and is used by more than 120 million people. It inhibits hepatic glucose production by reducing hyperglycaemia.¹² Many recent studies have revealed that metformin reduces tumor growth and cancer cell viability in xenograft models.¹³⁻¹⁶ Also, retrospective epidemiologic research disclosed a reduction in the occurrence of cancer in patients who were treated with metformin.^{17, 18} In a similar way to 2DG, metformin affects cell metabolism and barricades the signaling pathways of mTOR that are sensitive to energy.¹⁹ Mammalian target of rapamycin (mTOR) is a central regulator of translation, transcription, differentiation, and metabolism; thereby controlling cell growth, survival, and stress. Metformin prevents the respiratory chain complex 1 in hepatocytes²⁰ and it destroys the consumption of oxygen in colon cancer cells,¹⁴ which is incompatible with the prevention of oxidative phosphorylation. We started by combining 2DG and metformin, two different drugs that target the two different sources of cell energy, which may have a major advantage over common chemotherapies. Note that the result of using this combination on cancer cell metabolism and growth is currently unknown.

The expansion of ESCC is related to the accumulation of multifold genetic/epigenetic changes, like oncogenes activation and/or the tumor suppressor genes inactivation. Changes in the p53 and p16 pathways^{21, 22} and mutations of p53²³⁻²⁵ are involved in the outreach of ESCC. Bcl-2 family members are very important regulators for apoptosis and their changed expression is

mostly imported in oncogenesis (these proteins are often expressed in esophageal carcinomas) and particularly in primitive lesions.^{26, 27} We have sufficient information about the important role of apoptosis in the aggressiveness, pathogenesis, and the effectiveness of cancer treatment. The proteins of Bcl-2 have the same importance with p53 as the regulators of apoptosis. Herein, we showed that metformin and 2DG act synergistically to induce apoptosis ESCC. It led to a stronger inhibitory effect on cell viability than the drugs alone. Finally, we showed that 2DG and metformin operate synergistically for the induction of a massive amount of ATP evacuation in esophagus cancer cells.

Materials and Methods

Cell Lines

The ESCC cell lines TE1 (RBRC-RCB1894), TE8 (RBRC-RCB2098), and TE11 (RBRC-RCB2100) were purchased from a cell bank (RIKEN, Tsukuba, Ibaraki, Japan) and cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, US) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, US), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco Inc., UK) under the condition of 5% CO₂ in a humidified incubator at 37 °C. Media were changed every 24 hours and cells were in 80-90% confluence.

Chemo Treatments

Initially, the stock solution of 2DG, ≥98% GC (Sigma) in phosphate-buffered saline (PBS), was provided and attenuated in culture medium to 500 µM. Then, the cultured cells were treated for 2 days.²⁸ Metformin (1, 1-bimethylbiguanide hydrochloride) was purchased from Sigma-Aldrich (St. Louis, MO, US) and finally attenuated in culture medium to 5 mM concentration.

MTT Assay

The cytotoxic effect of 2DG and metformin on TE1, TE8, and TE11 cells was studied by MTT assay during 48 hours of treatment. Briefly, ESCC cells were seeded in a concentration of 8×10³ cells in each well of the 96-well plate and the treatment commenced with the 2DG and metformin. During the subsequent two days, the rate of cellular proliferation was measured continuously.²⁹ In summary, 20 µl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltrazolium bromide) (Sigma-Aldrich, St. Louis, MO, US) was added to each well and the plates were incubated at 37 °C and 5% CO₂ for 4 hours. Then, the cells were treated with 200 µl of dimethyl sulfoxide to solubilize the colored

formazan product and were solubilized by 25 μ l Sorenson buffer. Eventually, the absorbance of each well was measured by ELISA plate reader (BioTeck, Bad Friedrichshall, Germany) at 570 nm wavelength, and the computed data were compared with the untreated and normalized cells. To study the effect of 2DG, metformin, and their combination on p53 and Bcl-2 gene expression, 1×10^6 cells/well were treated in a 6-well plate with 5 mM metformin and/or 500 μ M 2DG for 48 hours.

RNA Extraction

Using RNX-Plus reagent, the total RNA was extracted (Cinagen Co., Tehran, Iran). In summary, the cells (1×10^6 cells) were treated using 1 mL of RNX solution at 25 °C for 5 minutes. Then, 200 μ L chloroform was added and centrifuged at 12,000 rpm at 4 °C for 15 minutes. Then, the upper phase was combined with an equal volume of isopropanol in the new tube and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The supernatant was thrown away and the RNA-pellet was washed using 1 mL of 75% ethanol. Finally, the RNA-pellet was dissolved in diethyl phosphorocyanide treated water. Optical densities at the wavelengths of 260 and 280 nm were used to determine the density of the purified RNA.

Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis Kit (MBI, Fermentas, Lithuania). To prevent DNA contamination, the RNA was treated by DNase I (Invitrogen, Carlsbad, CA, USA) before the cDNA synthesis. The cDNA synthesis reaction was performed with 20 μ l reaction solution that comprised 5 μ g total RNA, reaction buffer, RNase inhibitor (20 units); dNTP mix (20 nM), random hexamer primer, oligo (dt) primer, and 200 units of M-MuLV reverse transcriptase. Reverse transcription (RT) was performed at 42 °C during one hour and terminated by heating at 70 °C for 5 minutes.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

For the qRT-PCR, specific primer sequences were designed for Bcl-2, p53, and β -actin by Primer3 software (a free online program) as: Bcl-2 primers: 5'-GAGACAGCCAGGAGA AATCA-3' (forward primer) and 5'-CCTGTGGATGACTGAGTACC-3' (reverse primer), p53 primers: 5'-TGGGCGTGAGCGCTTCGAGA-3' (forward primer) and 5'-GGTGGCTGGAGTGAGCCCTGC-3' (reverse primer), β -actin primers: 5'-TCCCTGGAGAAGAGCTACG-3'

(forward primer) and 5'-GTAGTTTCGTGGA TGCCACA-3' (reverse primer).

β -actin housekeeping gene was used as an internal control and the relative gene expression was measured by the $2^{-\Delta\Delta C_t}$ method. We executed whole real-time PCR reactions in 72-well reaction tubes comprising 2 \times SYBER Green PCR Master Mix reagent (ABI, Vernon, CA, USA), 190 nM primer, and 1 μ g cDNA in 20 μ L reaction volume. Quantitative RT-PCR was performed with Corbett Rotor-Gene 6000 thermal cycler (Corbett Life Science, Sydney, Australia). The thermal cycle condition was performed as follows: one cycle at 95 °C for 5 minutes, followed by 42 cycles at 95 °C for 20 seconds (denaturation), at 60 °C for 20 seconds (annealing), and at 72 °C for 20 seconds (extension). After the completion of each test, the accuracy of the reaction was corroborated by the melting curve analysis using Corbett Rotor-Gene 6000 software (Corbett Life Science, Sydney, Australia).²⁹

TUNEL Assay

TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling) staining was performed to confirm the induction of apoptosis by detecting fragmented DNA with an in Situ Cell Death Detection Kit (Roche Diagnostics). The cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton-100, and 0.1% sodium citrate. Then, the cells were incubated with TUNEL reaction mixture using alkaline phosphatase-conjugated anti-fluorescein antibody, stained with nitro blue tetrazolium-5/bromo-4-chloro-3-Indolyl phosphate, and counterstained with methyl green. For negative controls, cells were incubated in TUNEL reaction mixture without the addition of terminal transferase. For positive controls, the fixed and permeabilized cells were treated with DNase I (3 U/ml in 50 mM Tris-HCL, pH 7.5, 1 mg/ml BSA) for 10 minutes at room temperature before incubation with TUNEL reaction mixture. Subpopulations were analyzed by flow cytometry to detect FITC-labeled cells.

Cell Death Detection ELISA

For the determination of cytoplasmic histone-associated DNA fragments, the photometric enzyme-immunoassay was used for the quantitative and qualitative in vitro (mono- and oligonucleosomes) after induced cell death. The assay is based on the quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. It provides a specific designation of mono- and oligonucleosomes in the

cytoplasmatic fraction of cell lysates. Histone-associated DNA fragmentation during apoptosis was analyzed using an ELISA kit (Roche Applied Science, Germany). Approximately 6.0×10^3 cells were plated in each well of a 96-well plate and left overnight for attachment. After being treated with metformin (5 mmol/L) and 2DG (0.5 mmol/L) alone and in combination for 48 h on TE1, TE8, and TE11 cells, the cells were processed according to the manufacturer's instructions. The absorbance was measured using an ELISA plate reader at 405 nm and the percentage of apoptosis and necrosis obtained from the ratio of absorbance in the treated samples to that of the untreated controls.

Statistical Analyses

The data were obtained in triplicate and analyzed using the SPSS software version 17.0. The results were expressed as mean \pm SD. The significance of the difference between the control and each experimental test condition was analyzed by ANOVA and Kruskal-Wallis test. $P < 0.05$ was considered statistically significant.

Results

Cell Viability

The viability of cells was assessed using the MTT assay. The cells were treated by the previously mentioned agents and cell viability was assessed after 48 hours. The results of the MTT assay for cell viability are shown in figure 1.

Expression of p53 and Bcl-2 in TE1, TE8, and TE11 Cell Lines

The mRNA expression profiles of p53 and Bcl-2 of the cells were analyzed using the real-time PCR assay. The goal was to confirm that p53, which plays a key role in apoptosis,³⁰ is essential for metformin/2DG-induced apoptosis in the three cell lines. 2DG alone influenced the p53 protein level while metformin somewhat enhanced the expression of p53. Noticeably, their combination forcefully induced p53 expression. The cell lines treated for 48 hours with metformin (5 mmol/L) and 2DG (0.5 mmol/L), an inhibitor of glycolysis, alone and in combination, were measured by the quantitative RT-PCR. As shown in figure 2, the relative levels of Bcl-2 mRNA were decreased. After treatment by the agents, all cell groups influenced the expression of Bcl-2 mRNA ($P < 0.05$). However, the b-actin mRNA expression was not influenced in each group.

To identify the relationship between Bcl-2 and p53, we examined p53 gene expression in TE1, TE8, and TE11 cells treated with 2DG-metformin

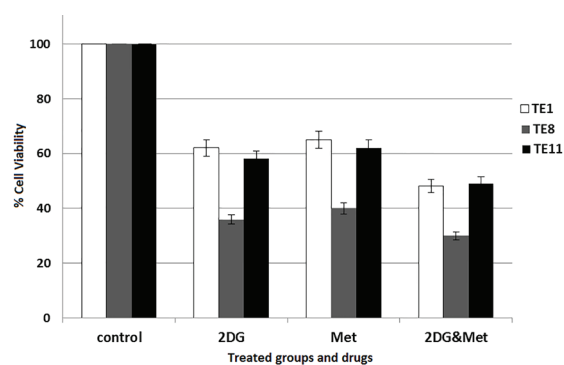


Figure 1: TE1, TE8, and TE11 cells were treated with metformin (5 mmol/L) and 2DG (0.5 mmol/L) alone and in combination for 48 hours. The cell viability is expressed as OD values. A significant decrease in cell viability was observed in 2DG-metformin combination group. Data are expressed as the mean \pm SD; $n=3$ ($P < 0.05$).

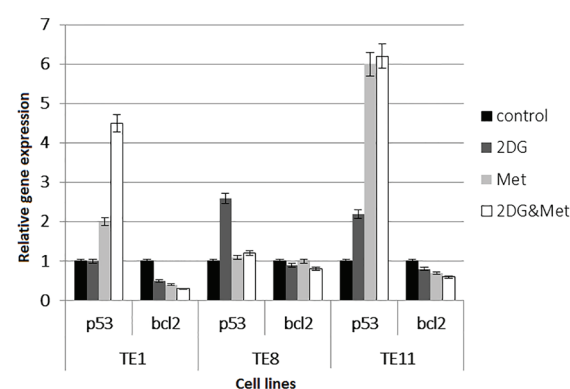


Figure 2: Relative p53 and Bcl-2 gene expression in TE1, TE8, and TE11 cells and the control group (untreated). Data are expressed as mean \pm SD ($P < 0.05$).

combination for 48 hours. The results showed a significant increase in p53 mRNA level in TE1, TE8, and TE11 cells while Bcl-2 gene expression decreased simultaneously.

Cell Apoptosis

The combination of 2DG and metformin increased the repetition of apoptosis in TE1, TE8, and TE11 cells. Terminal deoxytransferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay was applied to monitor the measure of DNA fragmentation due to apoptosis (figure 3).

Cell Death Detection ELISA

The measurement of apoptotic and necrotic cell death in three cell lines was assessed by the cell death ELISA detection kit. The cell lines were treated for 48 hours using metformin (5 mmol/L) and 2DG (0.5 mmol/L) alone and in combination (figure 4).

Two different forms of eucaryotic cell death could be described by morphological and biochemical criteria: necrosis and apoptosis. Necrosis occurs at the same time with increasing

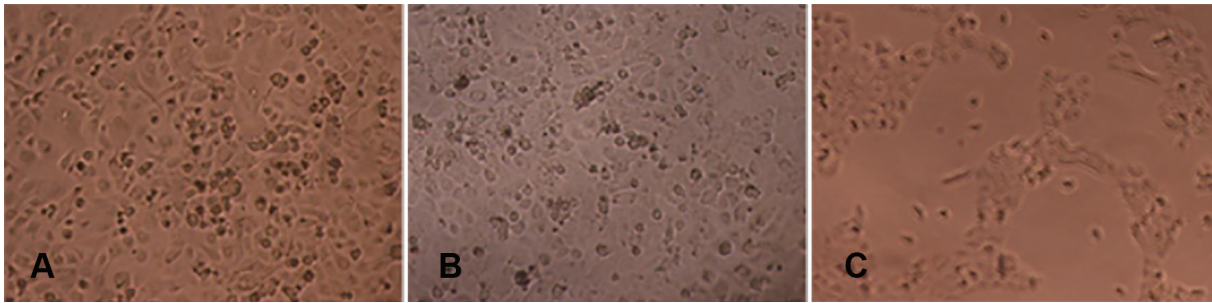


Figure 3: Metformin and 2DG combination induces apoptosis in TE1 (A), TE8 (B), and TE11 (C) cell lines. TUNEL assay was carried out at 48 hours after treatment by 2DG 0.5 mM and metformin 5 mM to detect apoptotic cells.

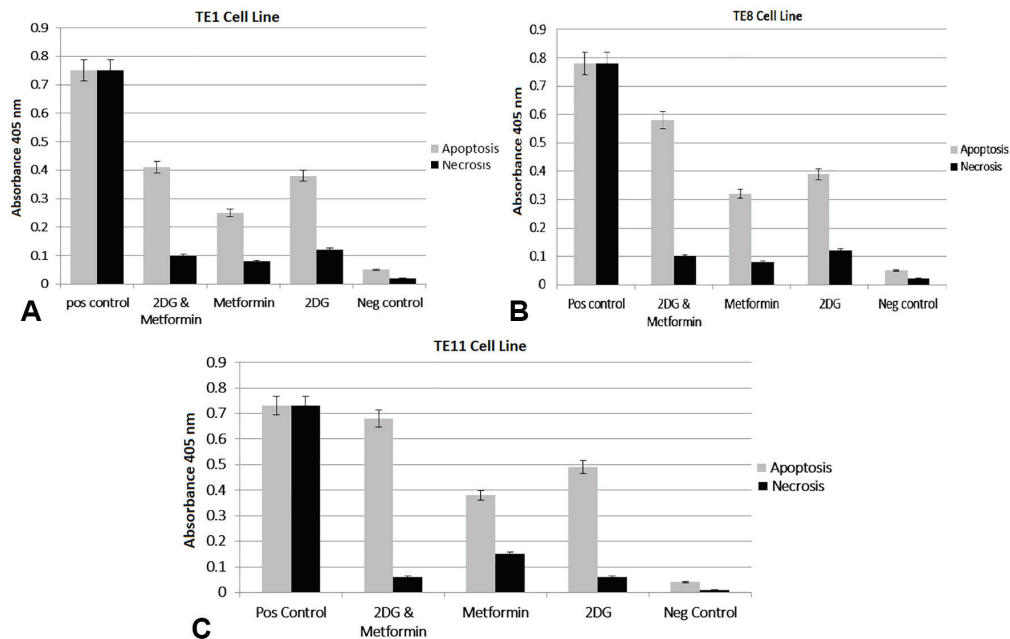


Figure 4: Effects of 2DG 0.5 mM and metformin 5 mM on cytotoxicity and proliferation of TE1 (A), TE8 (B), and TE11 (C) cells. Squamous cell carcinomas were treated with agents and appraised after 48 hours using the cell death ELISA. The dosage of the drugs significantly decreased cell proliferation and increased apoptosis in all cell lines. Left bar: Apoptosis, $P < 0.05$, $n = 3$; Right bar: Necrosis, $P < 0.05$, $n = 3$.

ion permeability of the plasma membrane; the cell swells and the plasma membrane tears in just a few minutes (osmotic lysis). Apoptosis was distinguished using membrane blebbing (zeiosis), the condensation of cytoplasm, and an endogenous endonuclease activation as well as specific proteases.

Discussion

Metformin alone represents a powerful anti-proliferative function in multiple cancer cell lines,²⁸⁻³⁴ whereas 2DG alone sensitizes cancer cells in response to the radiation or chemotherapeutic agents.^{32, 35-37} We have illustrated that these drugs in a combined form have a stronger damaging effect than individually. The combination of 2DG and metformin leads to a drastic decrease of intracellular ATP via the inhibition of the mitochondrial complex 1 and

glycolysis. Sahra et al. demonstrated that the combination of 2DG and metformin induced p53-dependent apoptosis in prostate cancer cells.³⁸ In their study, apoptosis was AMPK-mediated and p53 was required.

Consistent with the ability of the combined metformin and 2DG to induce cell death in vitro, such combination significantly suppressed tumor growth in two xenograft models in vivo.³⁹ These outcomes indicated that the tumor cell bioenergetics can be targeted and the combined metformin and 2DG warrants further clinical evaluations. Recently, metformin has gained much attention due to its anti-tumor activity in some cancer types in preclinical assays¹⁴ with AMPK activation being suggested as a major mechanism of action. AMP-activated protein kinase (AMPK) is a major cellular regulator of lipid and glucose metabolism. It is reported that metformin activates AMPK in hepatocytes. As a

result, acetyl-CoA carboxylase (ACC) activity is reduced, fatty acid oxidation is induced, and the expression of lipogenic enzymes is suppressed. Briefly, evaluating the clinical advantage of the blockade of the Warburg effect requires the concomitant inhibition of numerous ingredients of the energy pathways of cells. In oncology, the Warburg effect is the observation that most cancer cells predominantly produce energy by a high-rate of glycolysis followed by lactic acid fermentation in the cytosol rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells. Any preemptive blockade of the Warburg effect and the mechanisms of compensatory can illustrate to be predominant on the growth and survival promoting influences of growth factors or activated oncogenes. Since 2DG and metformin are already used in PET scan or seizure disorders and in type II diabetes, respectively, the expedited assessment of the clinical effect of deprivation of cancer bioenergetics could be easily tested in cancer patients.

Apoptosis induction is a huge challenge since it can reclaim the effects of classic anticancer factors. Currently, the dependence of chemotherapy and metformin in breast cancer cases is continuously tested in clinical examinations,¹⁹ while other researchers have already concluded the useful effect of 2DG alongside radiation in glioma.³⁷ Considering the findings of the present study that the combination of 2DG and metformin induces apoptosis and increases the antiproliferative effect, more influence on cancer is anticipated. The 2DG and metformin drugs have the clinical benefits of inducing no direct harmful side effects. However, metformin may slightly lead to lactic acidosis.⁴⁰ Past experiences have emphasized the positive antitumoral effect of combined 2DG and Metformin in vitro.

The results of the present study have shown that apoptosis induction is associated with a drop in ATP. In fact, 2DG or metformin causes a moderate reduction of ATP, less AMPK activation, and a modest reduction in mTOR activity compared to the potent effect of their combination that induces apoptosis. In all of the three cell lines, where the combined drugs mildly reduced intracellular ATP concentration, cell viability was slightly influenced. Hence, in line with other researchers, it is advocated that ATP level regulates the initiation of apoptosis.^{41, 42}

Conclusion

Our observations highlight the importance

of confirming the antitumoral effect of the combined metformin and 2DG in vitro. Indeed, the remarkable efficiency of their combination to affect cell viability by inducing apoptosis may have important implications in the treatment of ESCC. One of the effective therapeutic strategies for esophageal squamous cell carcinoma is the combination therapy.

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Conflict of Interest: None declared.

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