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The Effect of Metformin on the Expression of Caspase 3, 8, 9 and PARP-1 in Human Breast Cancer Cell Line T47D

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Abstract

Background: *Metformin is used to lower blood sugar in patients with type II diabetes. Recent research showed that metformin has effects on cancer cell growth. Studies show that metformin can induce apoptosis in certain cancer cell lines. In this study, we examined the effect of metformin on apoptosis in the T47D breast cancer cell line.*

Methods*: The T47D breast cancer cell line was selected and purchased from the Pasteur Institute (Tehran, Iran). Cells were treated with doses of 5, 10, and 50 μM of metformin at 24, 48, and 72 hours. The transcription levels of genes involved in apoptosis, including caspase-3, -8, -9, and PARP-1, were evaluated by real-time PCR.*

Results*: The results of this study showed that at all three doses (5, 10, and 50 μM) of metformin and at three times (24, 48, and 72 h), the expression of caspase-8 and caspase-9 were increased. Also, at all doses metformin increased the expression of PARP-1 at 48 and 72 hours, but at 24 hours the expression of PARP-1 was not affected.*

Conclusions*: These results indicate that metformin does not affect expression of caspase-3 at any dose or time point. This study showed that metformin, by increasing the transcription of caspase-8 and caspase-9, causes cell death through apoptosis.*

Keywords: Breast cancer, Metformin, Apoptosis.
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Introduction

Breast cancer is the most common cancer in women and the leading cause of cancer death in women 40–44 years old. This malignancy is responsible for 33% of female cancers and 19% of cancer-related deaths. Evidence shows a steady increase in the prevalence of breast cancer in the mid-1940s[1,2]. Three methods are used for breast cancer treatment: surgery, chemotherapy, and radiotherapy.[3] Recent studies have focused on drugs that can induce apoptosis in cancer cells. These drugs may be known drugs with different indications for the treatment of cancer. Metformin is a drug used to lower blood glucose in patients with type II diabetes. Anti-diabetic activity occurs through activation of AMPK (AMP-activated protein kinase) and the subsequent reduction in liver glucose and increase glucose uptake in skeletal muscle. The effects of biguanides, such as metformin, on the metabolic effects in non-diabetic cancer patients have been examined.[4,5] Reports suggest that patients with type 2 diabetes who were prescribed metformin compared with patients who did not use metformin are less likely to get breast cancer.[5] Treatment with metformin reduces the size and distribution of breast cancer in female transgenic HER-2/neu mice[6] and reduces the effects of carcinogens that cause pancreatic cancer in rats. Evidence suggests that metformin inhibits growth in cultured breast, colon, prostate, and glioma cancer cells.[7-10] Apoptosis is a programmed cell death that plays a critical role in normal and pathological development of a wide range of tissue and results in shrinkage of cytoplasm and fragmentation of cell nucleus. In most cancer cells, apoptosis is blocked, which allows malignant cells to survive despite their genetic and morphological variation. Therefore, the search for substances that can initiate apoptosis in tumor cells a new strategy in anti-cancer drug discovery.[1,2,11-14] Most drugs that induce apoptosis are often targeted to mitochondria and increase caspase activation. PARP is a nuclear protein implicated in DNA repair and is the earliest protein cleaved to a specific 89 kDa fragment (cleaved PARP) during apoptosis.[15] In some cells, such as placental cells, it was revealed that cleavage of PARP by caspase-3 prevents PARP from repairing damaged DNA; this lack of repair can lead to apoptosis.[15] Therefore, in this study, the effect of metformin on genes involved apoptosis in the breast cancer cell line T47D was studied.

Materials and Methods

The human breast cancer cell line T47D (Cell Bank Code: NCBI: C203) was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 units of penicillin/ml and 10 mg/ml streptomycin) in an incubator at 37°C in a humidified atmosphere containing 5% CO2. Twenty-four hours before the test, a million cells in volume of 5 ml were added to each flask and were incubated for 24 h. After the incubation period, three flasks for each dose of metformin (5, 10, 50 μM) and control conditions were evaluated at 24, 48, and 72 h of treatment. At these time points, cells were counted and collected. After cell counting, cells were separated from the culture medium by centrifugation. The pellet was kept for one hour at −20 °C. Then, RNA was extracted and kept at −70°C.

In this study, total RNA from control and treated T47D cells was extracted using RNeasy Plus Mini Kit purchased from Qiagen (Germany) according to the instructions that came in the kit. The concentration and purity of RNA were determined by measuring the absorbance at 260 nm of the extracted samples using Picodrop. Integrity and size distribution of total RNA was investigated by agarose gel electrophoresis with gels stained with SYBR Green.

In this study, cDNA was synthesized by a kit (QuantiTect Reverse Transcription 50 reaction) purchased from Qiagen (Germany) according to instructions that came in the kit.

Primers corresponding to GAPDH (internal control), caspase-3, caspase-8, caspase-9, and PARP-1 were designed using Gene Runner software. The suitability of the primers confirmed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to find amplified fragment length and confirm that it has no non-specific binding sites on the same gene or positions of similar sequences in other species. The primers used were appropriate, and protected areas were selected in order to avoid errors caused by amplification of genomic DNA binding region of exon–exon.

Table 1: Profile primers

Official name	Primer sequences(5′-3′)	Tm(°C)	GCcontent
Caspase 8	Forward:	57.6	50%
	GAAAAGCAAACCTCGGGGATAC	55.5	50%
	Reverse:		
	CCAAGTGTGTTCCATTCCTGTC		
Caspase 9	Forward:	59.5	54.6%
	CCAGAGATTCGCAAACCAGAGG	60	54.6%
	Reverse:		
	GAGCACCGACATCACCAAATCC		
GAPDH	Forward:	55.9	55%
	TGCACCACCAACTGCTTAGC	57.3	57.1%
	Reverse:		
	GGCATGGACTGTGGTCATGAG		
Caspase 3	Forward:	58.55	55%
	CTGGACTGTGGCATTGAGAC	58.48	50%
	Reverse:		
	ACAAAGCGACTGGATGAACC		
PARP-1	Forward:	60.5	55%
	GGAAAGGGATCTACTTTGCCG	61.2	60%
	Reverse:		
	TCGGGTCTCCCTGAGATGTG		

To investigate the quantitative expression of caspase-3, caspase-8, caspase-9, and PARP-1, quantitative real-time PCR reactions with the same conditions for all genes were done by real-time PCR (ABI-7300) using a SYBR Green PCR kit (ABI, USA) based on the kit instructions. Study of real-time PCR for the six genes listed in different groups was performed using the $2^{-\Delta\Delta Ct}$ method.

Data was entered into the statistical software SPSS16 and was analyzed using ANOVA, and Tukey's test.

Results

In the present study, we observed that metformin increased the expression of caspase-8 and caspase-9 genes at 24, 48, and 72 h, and the maximum effect was observed at 72 h. No effect was observed on caspase-3 at any time point. Also, the results showed that all three doses, 5, 10, and 50 µM, of metformin increased expression of caspase-8 and caspase-9, and the maximum effect was observed at a dose of 50 µM. None of the three dose of metformin affected caspase-3. In simultaneous survey of metformin dose and the incubation time, the most effective dose of metformin on gene expression of caspase-8 and caspase-9 were recorded in 50 µM and 72 h. (Figure 1, Figure 2, and Figure 3)

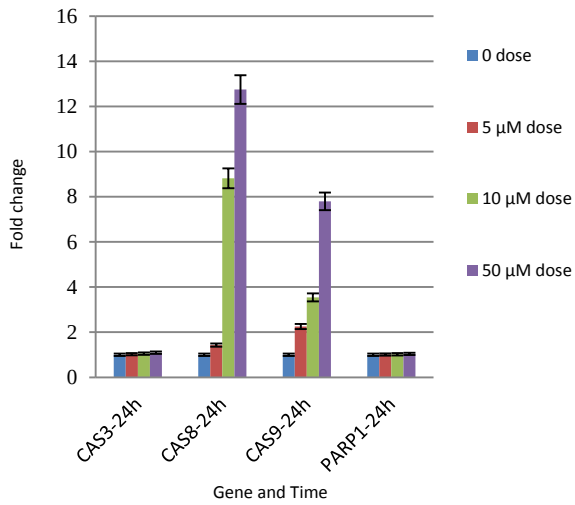

Figure 1: Caspase-3, caspase-8, caspase-9, and PARP-1 gene transcription in three doses at the 24 h time point. Statistically significant differences of caspase-8 and -9 gene expression were observed between metformin (5, 10, and 50 µM) and control groups at the 24 h time point ($p \leq 0.001$).

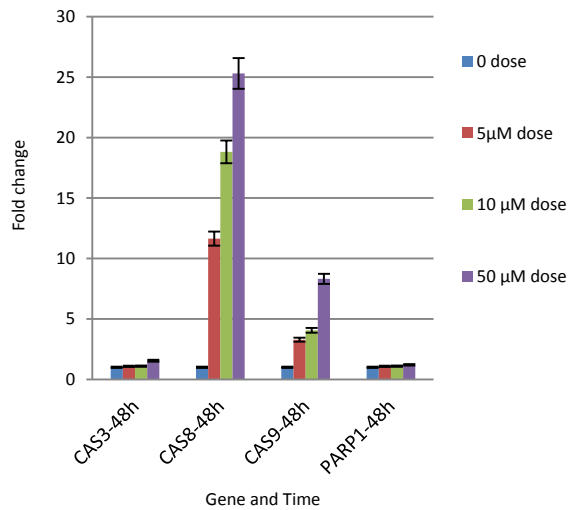

Figure 2: Caspase-3, caspase-8, caspase-9, and PARP-1 gene transcription in three doses at the 48 h time point. Statistically significant differences of caspase-8 and -9 and PARP-1 gene expression were observed between metformin (5, 10, and 50 µM) and control groups at the 48 h time point ($p \leq 0.001$).

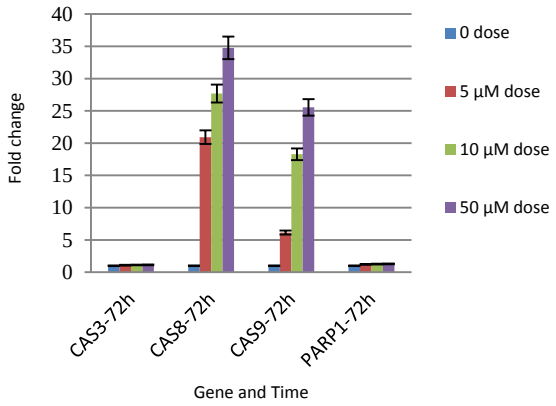

Figure 3: Caspase-3, caspase-8, caspase-9, and PARP-1 gene transcription in three doses at the 72 h time point. Statistically significant differences of caspase-8 and -9 and PARP-1 gene expression were observed between metformin (5, 10, and 50 µM) and control groups at the 24 h time point ($p \leq 0.001$).

The results of PARP-1 gene transcription rates in effect of metformin at the different time and dose: In this study it was observed that metformin on the PARP-1 gene expression is unaffected at 24 hours, but at 48 and 72 h increased the expression of PARP-1 at three doses 5, 10, 50 µM. In simultaneous survey of metformin dose and the incubation time, the maximum effect were recorded in 50 µM and 72 h (Figure 1, 2, 3).

Discussion

In this study, we showed that metformin increases gene expression of caspase-8 and caspase-9 in breast cancer cells but did not affect expression of caspase-3. In other studies, metformin activated or elevated expression of caspase-3, -8, and -9. This data suggests that metformin affects caspase-3 activity and the expression of caspase-3 at the protein level but does not affect the expression of caspase-3 on the mRNA level. Caspase-3 plays a key role in both the death receptor pathway, initiated by caspase-8, and the mitochondrial pathway, involving caspase 9. In addition, several studies have shown that caspase-3 activation is required for apoptosis induction in response to chemotherapeutic drugs, e.g., taxanes, 5-fluorouracil, and doxorubicin.[16-18]

In our study we found that metformin has no effect on PARP-1 at 24h, but it elevated the expression of PARP-1 at 48 and 72h. In the other studies (zhaung y 2011, liu b 2009), metformin effected PARP-1.While PARP-1 is constitutively expressed, its characteristic ability of being activated by DNA strand breaks makes poly (ADP-ribosylation) an immediate and drastic cellular response to DNA damage as induced by ionizing radiation, alkylating agents, and oxidants.[19] Hyperactivation of PARP also may cause cell death by depleting cellular NAD+ levels. Because NAD+ is required for both glycolysis and oxidative metabolism in mitochondria, depletion could lead to cellular energy collapse and subsequent cell death.[19-21] PARP-dependent cell death of metformin-treated cells appears to be delayed relative to apoptotic cell death and is associated with changes in mitochondrial morphology.[22] This study showed that metformin, by increasing the transcription of caspase-8 and caspase-9, causes cell death through apoptosis. These results indicate that metformin does not affect the expression of caspase-3 at any dose.

Conflict to Interest

The authors declared that they have no conflict of interest.

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