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METFORMIN IS CYTOTOXIC AGAINST CERVICAL CANCER HELA VIA MODULATION (P53, CASPASE-3, BCL2, COX-1, CYCLIN-D1) GENES EXPRESSION

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ABSTRACT

Cervical cancer is the second female cancer worldwide. Could it be treated safely and efficiently? Would these women not losing their fertility for treatment? We aimed to estimate whether metformin treatment against cervical cancer HeLa cell line could affect cells viabilityor not and to evaluate the genes expression of p53, caspase-3, Bcl2, COX-1 and cyclin-D1 as an apoptotic and proliferation markers genes.HeLa cell line was cultured and treated with different concentrations of metformin. Cells viability was investigated by MTT proliferation assay. Cells was treated by metformin for 48 h and 72 h and harvested for RNA extraction. RNA was transformed to cDNA and p53, caspase-3, Bcl2, COX-1 and cyclin-D1 genes' expression were assessed by qPCR.Metformin was cytotoxic by 66% death at1000 µg/mLfor HeLa cervix cancer cells and reduced the survival fraction in a dose response relationship. Metformin affects many genes' expression that reflects life and death. P53 and caspase-3 genes' expression were raised by the drug effect while Bcl-2, COX-1 and cyclin-D1 genes' expression levels were reduced.Metformin showed potent apoptotic activities against cervical cancerHeLa cell line through the elevation of p53 and caspase-3 and the elevation of Bcl-2, COX-1 and cyclin-D1 genes' expression. Metformin could be the new, safe and effective treatment against cervical cancer.

KEYWORDS: HeLa, Cervix, p53, caspase-3, Bcl2,COX-1, cyclin-D1

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INTRODUCTION

The second female cancer worldwide is the cancer of cervix as the most common malignancy in both incidence andmortality[1]. Unfortunately, more than 80% of cervical cancer cases are found in developing countries [2].Many lines treatments were developed and usedfor cervical cancer, but each of them has apparent drawbacks.Surgical treatment line is restricted only for patientswith early stage and the young patients who have lost fertility [3]. Radiotherapy and chemotherapy are randomized, not specific to only cancer cells and often bring severe adverse effect including bone marrow suppression, nerve injury, gastrointestinal adverse reactions, renal impairment and second cancer occurrence [3]. In spite of the advanced technology and methods, up to one third of patients will still develop persistent/ recurrence metastatic disease when the treatment results are poor. New therapeutic strategies must be evaluated to improve survival. Thus, finding a safer and more efficient treatmentremains an arduous tasksuch as the anticancer activities of metformin.Metformin belongs to a class of compounds called biguanines that were first isolated from the plant *Galegaofficinalis* (French lilac or goat's rue) known for its medicinal value [4]. It is the most widely prescribed antidiabetic drug for diabetes type 2 in the world [5]. Metformin's beneficial effects in diabetic patients have been shown to be largely through repression of hepatic gluconeogenesis, which reduces the glucose levels. In addition, it also increases insulin sensitivity and glucose uptake [6].In the past decade, metformin has gained wide attention for its anticancer properties. Studies have shown that in vitro treatment with metformin inhibited the growth of myriad cancer cell lines [7].An early study done in breast cancer mouse model showed that metformin treatment significantly decreased the tumor accumulation of mammary adenocarcinomas accompanied by increase in the life span of mice [8]. Metform in is also being tested as an adjuvant cancer therapy[9], studies on both laboratory animals and humans, showed that metformin not only exerts a major protective effect against the development of a wide range of cancers but also improves prognosis in those found to have these cancers[10]. It was suggested that it inhibits cancer cell proliferation, tumor growthand provokes cell-cycle arrest or apoptosis in G0–G1[11].Many genes can be considered as an apoptosis markers such as p53 and caspase-3. Not only p53 can be considered a marker gene for apoptosis, but also it controls cellular apoptosis and proliferation[12]. P53 plays a major role in many cancer cells death specially cervix cancer[13]. In the same way, caspase-3 is considered a death cascade promoter for the cancerous cells. The High levels of this gene may demonstrate the degree of HeLa cell death[14].On Contrarily, The anti-apoptotic Bcl2 gene expression reflects the cancer cells viability and vitality[15]. Additionally, the novel biomarker in many types of cancers isCOX-1. Recently, COX-1 gene expression has a vital role as biomarker for the detection of cervical cancer development and progression[16-17].Moreover, Cyclin-D1 gene was observed to be over expressed in cervical carcinoma[18].The emergence of metformin as a potential anticancer and cancer-preventive therapeutic tool is exciting. With the added benefits of being readily available, economical, and easily tolerated with good safety profile, it can be effortlessly transitioned from bench to bedside for cancer therapy[4]. We aimed to evaluate whether there is any effect of metformin treatment on the viability of cervical cancer HeLa cell line or not and to assess the gene expression of p53, caspase-3, Bcl2, COX-1 and cycin-D1 as an apoptotic and proliferation markers genes.

MATERIALS AND METHODS

Dulbecco's modified Eagle medium (DMEM), fetal calf serum, and 3-(4, 5-dimethylthiazol-2-yl)- 2, 5-diphenyltetrazoliunbromide (MTT) were purchased from GIBCO BRL (Grand Island, NY, USA). Trypsin 2.5%, penicillin, streptomycin, metformin and all other chemicals employed in this study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, USA).

Cell culture and cell proliferation assay

Metformin was dissolved in complete DMEM, the pH value adjusted to 7.2 and sterilized through a 0.2 µm filter to the desired working solutions (equivalent to 15.6–1000 µg/mL, w/v) [19]. Human cervical carcinoma cell line (HeLa) was provided by American Type Culture Collection (ATCC, Minisota, U.S.A.). Cells were cultured in DMEM medium supplemented with 5% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 units/mL penicillin at 37 °C in a humidified incubator in an atmosphere of 5% CO_2. HeLa cells were seeded in 96-well plates (10^3- 10^4 cells/well) for 24 h incubation, cell viability was evaluated using MTT assay as described previously [20]. In brief, cells were treated with metformin at a various concentration 0, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 µg/ml for 48 h and untreated cells served as a control. Prior to determination, 10 µL MTT (2.5 g/L) was added to each well. The wells were incubated for 4h, and then the culture media were discarded. 100 µL of detergent reagent was added to each well which was vibrated for 10 min. After colour appearance the absorbance (A) was measured at 570 nm by a microplate reader (ELISA reader) for the tested wells and was compared to the control (without test compound). Viable cells percentage was calculated as follows: (A of experimental group/A of control group) × 100%. After that, the IC_{50} (cytotoxic concentration for 50% cell death) was estimated from the dose-response curve.

Total RNA isolation

After cells detachment by trypsin (2.5%), the total RNA was isolated with RNA easy Mini Kit (Qiagen), then analyzed for quantity and quality with Beckman dual spectrophotometer (USA). The integrity of RNA and the (house keeping gene) GAPDH- RNA ratio were used as the quality control.

Real Time PCR (qRT-PCR) for quantitative expression of p53, Caspase-3, Bcl2, COX-1 and CyclinD1

The mRNA expression level was quantified by qRT–PCR (Real time PCR).1000 ng of the total RNA from each sample by High capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA).

Subsequently, the cDNA was amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95^0C for enzyme activation followed by 40 cycles of 15 seconds at 95^0C, 20 seconds at 55^0C and 30 second at 72^0C for the amplification step. The expression changes in of each target gene were calculated relative to the mean critical threshold (CT) values of GAPDH housekeeping gene by the ΔΔCt method. 1μM was needed from both primers that was specific for each target gene. Primers sequence and annealing temperature specific for each gene demonstrated in table (1).

Table 1
Primers sequence and annealing temperature specific for each gene

Target gene	Primer sequence: 5`- 3`	Gene bank accession number
P53	Forward: AGAGTCTATAGGCCCACCCC Reverse: GCTCGACGCTAGGATCTGAC	KJ897694.1
Caspase-3	Forward: GCTATTGTGAGGCGGTTGT Reverse: TGTTTCCCTGAGGTTTGC	KJ890827.1
Bcl2	Forward: CCGCTCGAGCCCTGGCCTGAAGAAGAGAC Reverse: TAAGAATGCGGCCGCAGGGACGAGGAAACCTTCAA	NG_009361.1
COX-1	Forward: TTCGCCGACCGTTGACTATTCTCT Reverse: AAGATTATTACAAATGCATGGGC	KM220890.1
Cyclin- D1	Forward: CCTCCTCTCCGGAGCATTT Reverse: CTGTAGCACAACCCTCCTCC	NG_007375.1
GAPDH	Forward: CCTCTACTGGCGCTGCCAAGGCT Reverse: GTCCACCACTGACACGTTGG	NT_009759.16

Primers were obtained according their genes bank accession numbers

STATISTICAL ANALYSIS

Results were disclosed as means ± standard deviations. One-way ANOVA and Tukey's multiple comparison post hoc tests were performed. *P*< 0.01 was considered significant. Non parametric correlations between various genes expressions were done by Spearman correlation coefficient.

RESULTS

Metformincytotoxocityand IC_{50}against HeLa cell line

Metformin significantly reduced HeLa cell line viability in a dose response relationship after 24 h incubation period (**Figure 1**). Beginning with the lowest metformin concentration 15.6 μg/mL, it decreased the cancer cell line viability significantly to 95.25%±2.19%; P< 0.001 against the untreated control. IC50; the cytotoxocity concentration value that fifty percent of cells were dead; was calculated and examined to equal 720.5μg/mL. The maximum cytotoxicity effect against HeLa cell line was reached 33.75%±4.40%; P < 0.001 survival percent against the untreated control and 66.25% death percent after the exposure to 1000 μg/mL.

P53 and caspase-3 elevated genes expression after metformin treatment

Both death marker genes were elevated for HeLa cell line 48 h after the metformin treatment as shown in figure 2. Cells p53 expression was increased significantly (0.177±0.075; P< 0.001 compared to untreated control group 0.034±0.012) and remain unchanged after 72 h treatment. Similarly, caspase-3 expression was nearly doubled after 48 h drug treatment (0.77±0.26;P< 0.001) and remained constant after 72 h when compared to control value (0.44±0.11).

Bcl-2, COX-1 and Cyclin-D1 reduced genes expression after metformin treatment

Complementary to the previous results, Bcl-2; a marker gene for cells viability or life; expression was significantly reduced in a time dependent to almost one half each time after 48 h and 72 h metformin treatment against the control (0.74±0.27 for 48 h and 0.43±0.21 for 72 h; P < 0.001 for both compared to 0.122±0.17). In the same direction, COX-1 gene expression value was reduced in a time dependent manner after 48 h and 72 h that reflect the diminishing of cancer conditions (1.23 ±1.04 for 48 h and 0.95±0.64 for 72 h; P < 0.001 for both compared to control3.8±1.3). Parallel to these results, Cyclin-D1 gene expression was markedly decreased after 48 h and 72 h metformin treatment when compared to control (5.62±4.4 for 48 h and 2.3±1 for 72 h; P < 0.001 for both compared to 9.68±4.7).

Nonparametric Correlations between various genes expressions

Even though the gene expression for both P53 and caspase-3 genes were positively correlated with each other at48 hrs metformin treatment times periods, these two genes were negatively correlated with Bcl-2, COX-1 and cyclin-D1 genes expressions at most different treatment periods.On the other hand, Bcl-2, COX-1 and cyclin-D1 genes expressions were correlated positively with each other for most different groups and inversely negatively correlated with P53 and caspase-3 genes expression.

Table 2

Nonparametric Correlations between various genes expressions; p53, caspase-3, Bcl-2, COX-1 and Cyclin-D1; for different groups; control, 48 hrs and 72 hrs metformin treated groups

CorrelationR/Group	Gene expression	P53	Caspase-3	Bcl-2	COX-1	Cyclin-D1
Control	P53	1.000	**-0.007**	0.158	**-0.350**	0.0910
48 hrs		1.000	0.119	**-0.343**	0.664	**-0.217**
72 hrs		1.000	**-0.463**	0.225	0.095	**-0.154**
Control	Caspase-3	**-0.007**	1.000	0.093	**-0.263**	**-0.214**
48 hrs		0.119	1.000	**-0.273**	0.168	**-0.273**
72 hrs		**-0.463**	1.000	0.035	**-0.497**	**-0.021**
Control	Bcl-2	0.158	0.093	1.000	0.452	**-0.168**
48 hrs		**-0.343**	**-0.273**	1.000	**-0.329**	0.112
72 hrs		0.225	0.035	1.000	0.252	0.322
Control	COX-1	**-0.350**	**-0.263**	0.452	1.000	**-0.028**
48 hrs		0.664	0.168	**-0.329**	1.000	0.301
72 hrs		0.095	**-0.497**	0.252	1.000	**-0.126**
Control	Cyclin-D1	0.091	**-0.214**	**-0.168**	**-0.028**	1.000
48 hrs		**-0.217**	**-0.273**	0.112	0.301	1.000
72 hrs		**-0.154**	**-0.021**	0.322	**-0.126**	1.000

Correlations done by Spearman correlation coefficient

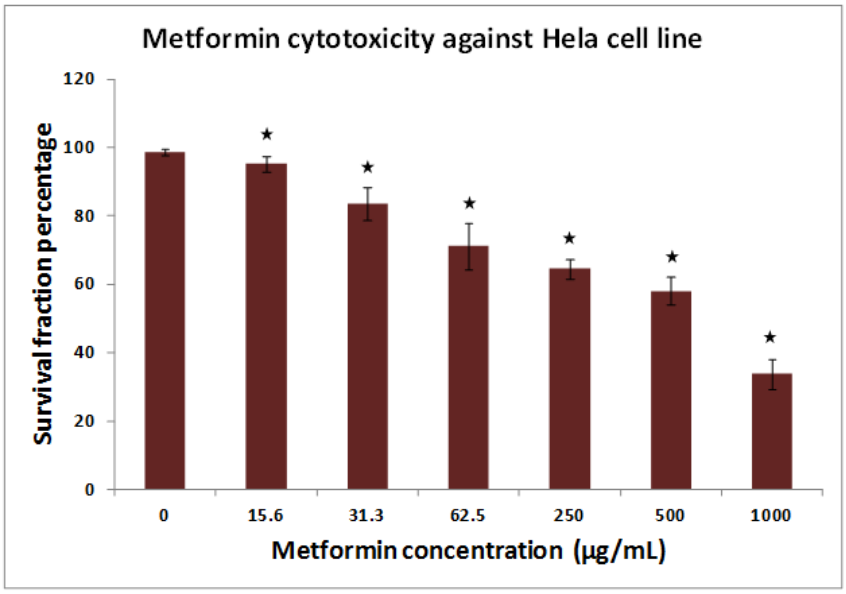

Figure 1

Metformin cytotoxicity against HeLacell line, survival fraction percentage of HeLa cells were reduced after treatment of different metformin concentrations. Data are means ± SD,(★) p value value≤ 0.001versus untreated control group.

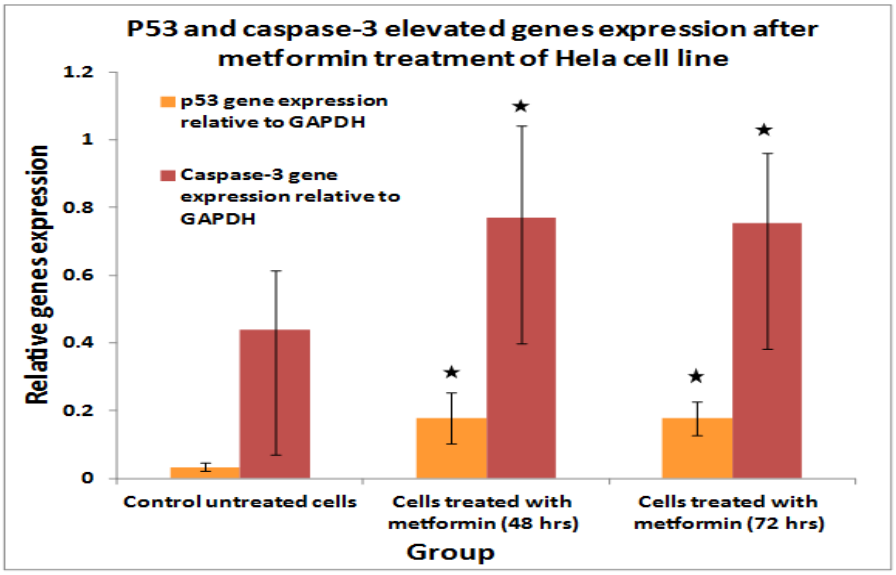

Figure 2

P53 and caspase-3 elevated genes expression after metformin treatment of HeLa cell line.Data are means ± SD, (★) p value value≤ 0.001versus untreated control group.

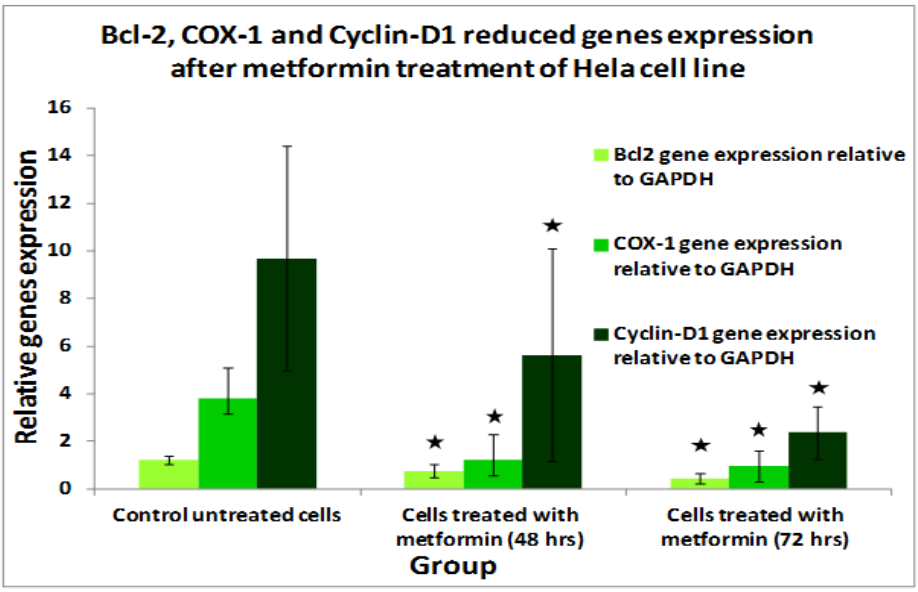

Figure 3

Bcl-2, COX-1 and Cyclin-D1 reduced genes expression after metformin treatment of HeLa cell line.Data are means ± SD, (★) p value value≤ 0.001versus untreated control group.

DISCUSSION

For the first time, Metformin was significantly cytotoxic for HeLa cervix cancer cell line in a dose dependent manner as shown in (Figure 1). This apoptotic effect conceded with previous anticancer, prophylactic and adjuvant activities against other types of cancers[7-11]. Recently, metformin was effective against pancreatic[21], ovarian[22], breast[23], melanoma[24] andneuroblastoma[25]. Metformin was not so far from its anti-diabetic group in cancer treatment[26].The anticancer effect of metformin was explained throughits relationship and regulation of the transcriptional certain genes (CHOP, CAV-1, HO-1, SGK-1 and Par-4) on MCF-7 cell line[27]. Build and complementary for this informationtranscriptional, regulator and apoptotic genes' expression were examined for p53, caspase-3, Bcl-2, COX-1 and cyclin-D1 genes. Moreover, each mentioned gene affects cell proliferation or apoptosis of cancer cells specially HeLa cervix cancer cells[12-18]. Not only p53 gene expression was participated for cervix cancer cells death[13], but also metformin affect the cancer cells susceptibility for p53 to be dead[28]. Even though the low concentration of metformin induces a p53-dependent senescence in hepatoma cells via activation of the AMPK pathway[29]. In the same direction, caspase-3 was induced in cancer by

metformin[30]. Moreover, both p53 and caspase-3 genes were induced by the effect of metformin in prostate cancer[31].On the other hand, Metformin reduces growth of cutaneous squamous cell carcinomaby targeting mTOR signaling pathway and the reduction of Bcl-2 gene expression[32].Futhermore, metformin and aspirin reduced COX-1 gene expression by targeting AMPK-mTOR and inflammation for pancreatic cancer prevention and treatment[33].Confirmed with our cyclin-D1 results, metformin suppresses hepatocellular carcinoma cell growth through induction of cell cycle G1/G0 phase arrest and p21CIP and p27KIP expression and downregulation of cyclin D1 in vitro and in vivo[34].The direct relationship between p53 and caspase-3 genes expressions that positively correlated reflect the degree of death for cancer cells by the effect of metformin treatment. While there indirect relationship with Bcl-2, COX-1 and cyclin-D1negatively correlations were in the same direction, hence these genes reflect the viability, inflammation and progression of breast cancer disease.Contrarily, Bcl-2, COX-1 and cyclin-D1 correlated positively together as mentioned to express the worse survival state of cancer cells and inversely with p53 and caspase-3 genes expressions that marked directly to cells death.Precisely, metformin impairs growth of endometrial cancer cells that neighbouring cervical cancer via cell cycle arrest and concomitant autophagy and apoptosis[30].Accordingly, Metformin could be the new, safe and effective treatment against cervical cancer.

CONCLUSION

Cervical cancer was the new type of cancer treated by metformin. Metformin has an apoptotic potent cytotoxic activities were shown by the dose response relationshipagainst human cervical cancer HeLa cells. Even if metformin HeLa treatment raised p53 and caspase-3 genes' expression as apoptotic markers, it reduced Bcl-2, COX-1 and cyclin-D1 genes' expressions as proliferation markers.

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CONFLICT OF INTEREST

The authors declared no any conflict of interest.

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