RESEARCH ARTICLE

Effects of Metformin on Cell Kinetic Parameters of MCF-7 Breast Cancer Cells *in Vitro*

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Abstract

In this study, the antiproliferative effects of the metformin was evaluated on MCF-7 Cells (human breast adenocarcinoma cell line). For this purpose cell kinetic parameters including cell proliferation assay, mitotic index and labelling index analysis were used. 30 μM, 65 μM and 130 μM Metformin doses were applied to cells for 24, 48 and 72 hours. The results showed that there was a significant decrease in cell proliferation, mitotic index and labelling index for all experimental groups ($p<0.05$) for all applications.

Keywords: Breast cancer - MCF-7 cells - cell proliferation - mitotic index labelling index

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Introduction

Breast cancer that effects many women in the world is complex and heterogeneous disease and is divided into many subtypes (Cetin and Topcul, 2014). Epidemiological studies show that patients with diabetes mellitus (DM) have an increased risk of breast carcinoma and that metformin treatment is associated with decreases the incidence of cancer and cancer-related mortality in diabetic patients (Larsson et al., 2007; Goodwin et al., 2011; Evans et al., 2005; Bowker et al., 2006).

Insulin can promote tumorigenesis via a direct effect on epithelial tissues, or indirectly by affecting the levels of other modulators, such as insulin-like growth factors, sex hormones, and adipokines (Calle and Kaaks, 2004; Pollak et al., 2004; Wolf et al., 2005; Renehan et al., 2006; Yee, 2006).

The anticancer effects of metformin are associated with both direct (insulin- independent) and indirect (insulindependent) actions of the drug (Dowling et al., 2011). The indirect, insulin-dependent effects of metformin are mediated by the ability of AMPK to inhibit the transcription of key gluconeogenesis genes in the liver and stimulate glucose uptake in muscle, thus reducing fasting blood glucose and insulin (Cusi et al., 1996; Witters, 2001). The direct, insulin-independent effects of metformin originate from LKB1-mediated activation of AMPK and a reduction in mTOR signaling and protein synthesis in cancer cells (Dowling et al., 2007).

Although the metformin mechanism of action is still under investigation, preclinical studies suggest a direct antineoplastic activity (Cazzaniga et al., 2009), Activation of LKB1/AMPK pathway, induction of cell cycle arrest and/or apoptosis, inhibition of protein synthesis, reduction in circulating insulin levels, inhibition of the unfolded protein response (UPR), activation of the immune system, and eradication of cancer stem cells are have been suggested as several potential for the ability of metformin to suppress cancer growth *in vitro* and *in vivo* (Kourelis and Siegel, 2011).

The aim of the present study was to investigate the effect of metformin on MCF-7 cell line using cell kinetic parameters including cell proliferation assay, mitotic index and apoptotic index.

Materials and Methods

Cell line

The MCF-7 cell line used in this study was obtained from European Cell Culture Collection (CCL). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco Lab) containing 10% fetal bovine serum (FBS, Gibco Lab), 100 µg/ml streptomycin (Streptomycin sulphate, I. E. Ulugay), 100 IU/ml penicilin (Pronapen, Pfizer), amphotericin B (Sigma, USA) and 2 mM glutamine at 37°C in humidified atmosphere of 5% CO_2. The pH of the medium was adjusted to 7.4 with $NaHCO_3$.

Drug doses

Metformin concentrations that were used in the present study were determined based on previous *in vitro* and clinical studies. At first 1 mg/ml stock solution was prepared with methanol. Three different doses were prepared by dilution of stock solution. These doses were determined as D1: 30 μM, D2: 65 μM and D3: 130 μM. Cells were treated with these doses for 24, 48 and 72 hours.

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Cell proliferation assay

The effects of Metformin on cell proliferation were measured by MTT assay. This method is based on the ability of live cells to convert tetrazolium salt into purple formazan. For this application MCF-7 cells were seeded into 96-well plates at a density of $2x10^4$ cells per well and incubated overnight. Then the cells were treated with 30 μM, 65 μM and 130 μM Metformin concentrations. At the end of the experimental period, the medium in each well was removed and 40 µl fresh MTT solution (5 mg/ml in PBS) were added into each well and cells were incubated at 37 °C for 4 h. Then, 160 µl DMSO (Dimethyl Sulfoxide, Sigma) was added into each well and cells were shaked thoroughly for 1 h on a shaker. Then, the absorbance of the samples was measured against a background control as a blank using an Elisa reader (µQuant, Bio-Tek Instruments Inc) at 450-690 nm.

Mitotic index analysis

For mitotic index analysis MCF-7 cells were seeded into round coverslips which were in 24-well plates at a density of 2x104 cells per well and incubated overnight. Then the cells were treated with 30 μM, 65 μM and 130 μM Metformin concentrations. At the end of the experimental period cells were fixed with Carnoy's fixative (3:1 methanol-acetic acid). Mitotic index were studied by the methods of Feulgen. Before the cells were treated with Feulgen, they were prepared with 1 N HCl at room temperature for 1 minute and then hydrolized with 1 N HCl for 10.5 minutes at 60°C. After coverslips were treated with Feulgen, they were rinsed for few minutes in distilled water and stained with 10% Giemsa stain solution pH 6.8, for 3 minutes and washed twice in phosphate buffer. After staining, the coverslips were rinsed in distilled water. And then the coverslips were air dried. At last mitotic index were calculated by counting metaphases, anaphases and telophases for each tested drug concentration and control. At least three thousands cells were examined from each coverslip for mitotic index.

^{3}H-thymidine labelling index analysis

For ^{3}H-thymidine labelling index analysis which determine cells in the synthesis phase, MCF-7 cells were seeded into round coverslips which were in 24-well plates at a density of $2x10^4$ cells per well and incubated overnight. Then the cells were treated with 30 μM, 65 μM and 130 μM Metformin concentrations. At the end of the experimental period, cells were treated with medium containing 1 µCi/mL 3H-thymidine for 20 min to evaluate the labelling index.

Autoradiography

After labelling, the cells were fixed with Carnoy's fixative (3:1 methanol-acetic acid) and the remaining radioactive material was washed twice with 2% perchloric acid at 4°C for 30 min. Coverslips were prepared and were coated with K.2 gel emulsion (Ilford) prepared with distilled water at 40°C to determine thymidine labelling index. After 3 days exposure at 4°C, autoradiograms were washed with D-19 b developer (Kodak) and fixed with Fixaj B (Kodak). The coverslips were evaluated after being stained with Giemsa for 3 min. The labelled cells were counted on each coverslip. At least three thousands cells were examined from each coverslip.

Statistics

Values of proliferation rate, MI and AI were evaluated relative to controls and to each other. For this reason, values obtained from all experimental groups were analyzed using one-way ANOVA test. The significance between control and experimental groups was determined by DUNNETT's test and the significance between experimental groups was determined by Student's t-test.

Results

Cell proliferation

After administration of 30 μM, 65 μM and 130 μM Metformin concentrations for 0-72 h, the cell proliferation values of MCF-7 cells decreased significantly. The differences between the control and all experimental groups were significant ($p<0.05$). As seen in Figure 1, cell proliferation values decreased from 879x10-^{3} to 743x10-^{3}, 582 x10-^{3} and 458 x10-^{3} at 24 h; from 1197 x10-^{3} to 1095 x10-^{3}, 747 x10-^{3} and 409 x10-^{3} at 48 h and from

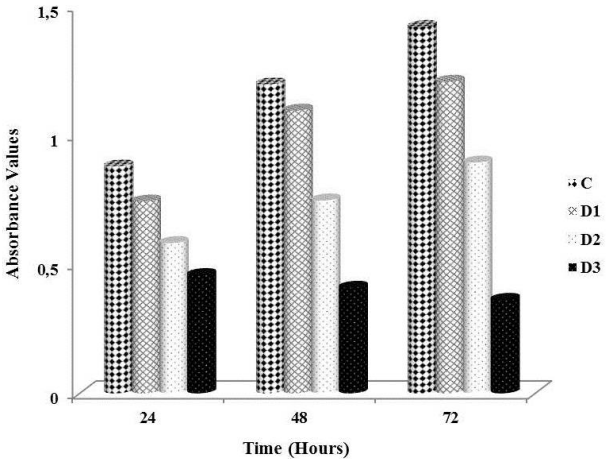

Figure 1. Absorbance Values of Mitochondrial Dehydrogenase Activity of MCF-7cells Treated with D1, D2 and D3 dose of Metformin (D1: 30 μM, D2: 65 μM and D3: 130 μM) for 0-72 h (450-690 nm) ($p<0.05$)

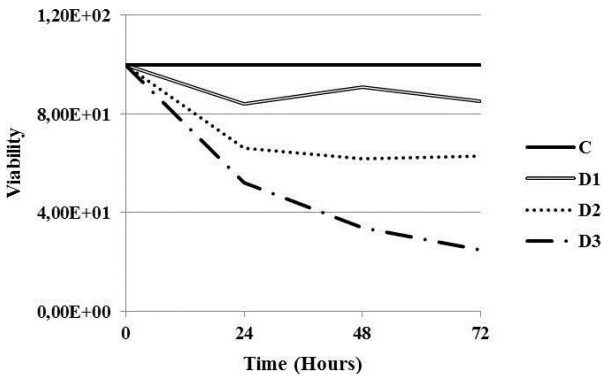

Figure 2. Percent Viability Values of MCF-7 Cells Treated with D1, D2 and D3 dose of Metformin (D1: 30 μM, D2: 65 μM and D3: 130 μM) for 0-72 h (450-690 nm) ($p<0.05$)

1419 x10-^{3} to 1207 x10-^{3}, 894 x10-^{3} and 362 x10-^{3} at 72 h respectively for 30 μM, 65 μM and 130 μM. As seen in Figure 2, cell viability values 84%, 66% and 52% at 24 h; 91%, 62% and 34% at 48 h; 85%, 63% and 25% at 72 h respectively for 30 μM, 65 μM and 130 μM according to control group which was taken as 100%.

Mitotic index

After administration of 30 μM, 65 μM and 130 μM Metformin concentrations for 0-72 h, the mitotic index values of MCF-7 cells decreased significantly. The differences between the control and all experimental groups were significant ($p<0.05$). As seen in Figure 3, mitotic index values decreased from 5,79to 4,34; 2and 2,02at 24 h; from 4,93to 2,94; 2,21and 2,261at 48 h and from 3,43to 2,54; 1,53 and 1,29 at 72 h respectively for30 μM, 65 μM and 130 μM.

Labelling index

After administration of 30 μM, 65 μM and 130 μM Metformin concentrations for 0-72 h, the labelling index values of MCF-7 cells decreased significantly. The differences between the control and all experimental groups were significant ($p<0.05$). As seen in Figure 4, labelling index values decreased from 8,42 to 7,67; 4,87 and 2,81 at 24 h; from 8,87 to 7,06; 4,32 and 1,86 at 48 h and from 9,68 to 6,59; 1,93 and 0,68 at 72 h respectively for30 μM, 65 μM and 130 μM.

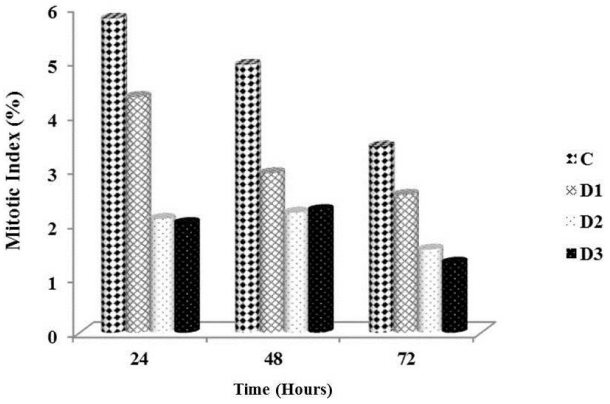

Figure 3. Mitotic Index (%) Values of MCF-7 Cells Treated with D1, D2 and D3 dose of Metformin (D1: 30 μM, D2: 65 μM and D3: 130 μM) for 0-72 h ($p<0.05$)

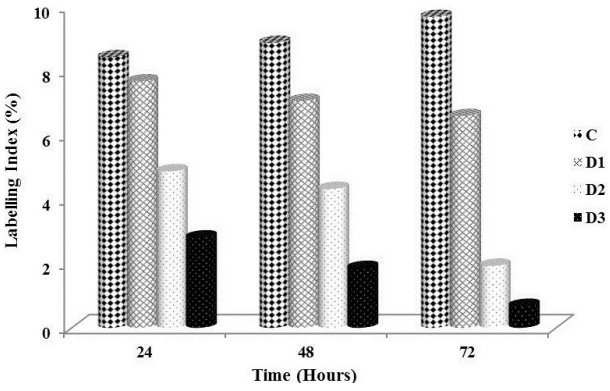

Figure 4. Labelling Index (%) Values of MCF-7 Cells Treated with D1, D2 and D3 dose of Metformin (D1: 30 μM, D2: 65 μM and D3: 130 μM) for 0-72 h ($p<0.05$)

Discussion

Investigation of potential indications of metformin in oncology is appealing because the drug is inexpensive, relatively safe, and seems to involve, at least in part, modulation of energy metabolism, which is a cancer research theme that is attracting increasing interest (DeBerardinis et al., 2008; Vander Heiden et al., 2009; Cairns et al., 2011; Koppenol et al., 2011; Vander Heiden, 2011; Ward and Thompson, 2012).

Effects of metformin on cell proliferation were evaluated by many investigators using different cell lines. Bao et al. conducted MTT and clonogenic assays using gemcitabine-resistant AsPC-1 and MiaPaCa-2 cells (AsPC-1-GTR and MiaPaCa-2-GTR cells). They found that metformin significantly inhibited cell survival by a dose-dependent manner in AsPC-1 and MiaPaCa-2 cells as well as gemcitabine- and Tarceva-resistant cells (AsPC-1-GTR and MiaPaCa-2-GTR cells). They also found that metformin significantly decreased the clonogenicity in both drug-sensitive and drug-resistant pancreatic cancer cells compared with those cells without metformin treatment (Bao et al., 2012). Costa et al. evaluated antiproliferative effect of metformin on in human SKNBE2 and SH-SY5Y neuroblastoma cell lines and on SKNBE2 cells in which differentiation is induced by retinoic acid treatment or stable overexpression of NDM29 non-coding RNA using xCELLigence RTCA DP system. SKNBE2 growth curves showed a time-dependent decrease in the proliferation rate of metformin-treated cells, resulting in a statistically significant difference after 48 h and lasting up to the end of the experimental observation (72 h) (Costa et al., 2014).

In a study which was evaluate the anti-estrogenic effect of metformin on endometrial histology in comparison with progesterone, researchers showed that metformin could induce endometrial atrophy in 21 out of 22 patients (95.5%) while this positive response was achieved in only 13 out of 21 patients (61.9%) in the megstrol group. In addition two low grade endometrial carcinomas in the metformin group responded very well (Tabrizi et al., 2014).

In a study which aimed to investigate the effects of cisplatin combined with metformin on the proliferation, invasion and migration of HNE1/DDP human nasopharyngeal carcinoma (NPC) cells, and to provide a new target for treating metastasis, it was shown that cisplatin combined with metformin has effects on proliferation, invasion, and migration of human NPC cells (Sun et al., 2014).

In a study Yu Xiong et al. (2012) reported effects of metformin on hepatocellular carcinoma (HCC) Hep-G2 cells and details of molecular mechanisms of metformin activity. Their research indicated that metformin displays anticancer activity against HCC through inhibition of the mTOR translational pathway in an AMPK-independent manner, leading to G1 arrest in the cell-cycle and subsequent cell apoptosis through the mitochondrion-dependent pathway (Xiong et al., 2012).

In recent years treatment of breast cancer have gained a new direction with the discovery of cancer stem cells

(Topcul et al., 2013). If stem cell differentiation potential becomes impaired and their proliferative capacity becomes uncontrolled, these mutated, potentially tumorigenic, self-renewable stem cells have the potential to cause cancer and are called cancer stem cells (CSCs). CSCs are responsible for tumor relapse and metastasis (Cetin and Topcul, 2012). In a study it was showed that low doses of metformin, a standard drug for diabetes, inhibits cellular transformation and selectively kills cancer stem cells in four genetically different types of breast cancer cell lines (MCF-7, SKBR3, MDA-MB-486 and MCF10A ER-Src) (Hirsch et al., 2009).

In the present study, treating MCF-7 cells with various concentrations of metformin for 24, 48 and 72 hours decreased cell viability, mitosis and S-phase cells. The results showed that metformin decreased cell viability, mitotic index and labelling index of MCF-7 cells, depending upon time and applied concentrations. When compared to the control, this decrease was found statistically significant in each group ($p<0.05$).

Acknowledgements

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