Research Article

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Metformin suppresses the proliferation and invasion through NF-kB and MMPs in MCF-7 cell line

Metformin MCF-7 hücre hattında NF-kB ve MMP'ler yoluyla proliferasyonu ve invazyonu baskılar

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Abstract

Objective: MCF-7 cells, a breast cancer cell line, are used for experiments of estrogen receptor (ER)-positive breast cancer and many sub-clones representing different classes of ER-positive tumors. We aimed to determine the efficacy of metformin, a potential anti-cancer agent, on the cell proliferation, and the expressions of NF-kB (p65), MMP-2 and MMP-9 in MCF-7 cell line.

Materials and methods: MCF-7 cells (human breast adenocarcinoma) were treated with elevating doses of metformin (0–50 mM) for 24 h. The anti-proliferative effect of metformin was studied by BrdU proliferation assay, and the expression levels of NF-kB (p65), MMP-2 and MMP-9 were analyzed by immunocytochemical staining.

Results: The percentage of cell proliferation was reduced significantly by 10 and 50 mM doses of metformin ($p < 0.001$). The expression levels of nuclear NF-kB (p65), MMP-9 and MMP-2 were considerably reduced in 50 mM metformin treated cells while the expression of cytoplasmic NF-kB (p65) elevated compared to control group ($p < 0.05$). Ten millimolar metformin also reduced expression of MMP-9 significantly ($p < 0.05$).

Conclusion: Metformin may act on the proliferation, and the processes of invasion and metastasis of MCF-7 cells through blocking NF-kB, which is intensely expressed in breast cancer cells, and through diminishing the expression of MMP-2 and MMP-9 significantly.

Keywords: Breast cancer; Matrix metalloproteinase; MCF-7; Metformin; NF-kB.

Öz

Amaç: Bir meme kanseri hücre soyu olan MCF-7, östrojen reseptörü (ER) pozitif meme kanseri ve ER-pozitif tümörlerin farklı sınıflarını temsil eden birçok alt klonun deneyleri için kullanılır. Potansiyel bir anti-kanser ajanı olan metforminin, MCF-7 hücre soyunda hücre çoğalması ve NF-kB (p65), MMP-2 ve MMP-9 ekspresyonları üzerindeki etkisini belirlemeyi amaçladık.

Gereç ve Yöntem: MCF-7 hücreleri (insan meme adenokarsinomu) 24 saat boyunca metformin artan dozları (0–50 mM) ile muamele edildi. Metforminin anti-proliferatif etkisi, BrdU proliferasyon deneyi ile incelendi ve NF-kB (p65), MMP-2 ve MMP-9 ekspresyon seviyeleri ise immünositokimyasal boyama ile analiz edildi.

Bulgular: Hücre proliferasyonunun yüzdesi, 10 ve 50 mM'lik metformin dozları ile önemli ölçüde azaldı ($p < 0.001$). Nükleer NF-kB (p65), MMP-9 ve MMP-2'nin ekspresyon seviyeleri, 50 mM metformin ile muamele edilmiş hücrelerde önemli ölçüde azalırken, sitoplazmik NF-kB'nin (p65) ekspresyonu, kontrol grubuna göre ($p < 0.05$) arttı. 10 mM metformin de MMP-9 ekspresyonunu anlamlı şekilde azalttı ($p < 0.05$).

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Sonuç: Metformin, meme kanseri hücrelerinde yüksek oranda eksprese edilen NF-kB(p65) ve MMP-2 ve MMP-9 ekspresyon seviyelerini önemli ölçüde azaltarak, MCF-7 hücrelerinin proliferasyonu, invazyonu ve metastazı üzerinde etkili olabilir.

Anahtar Kelimeler: Meme kanseri; Matrix Metalloproteinaz; MCF-7; Metformin; NF-kB.

Introduction

Breast cancer, predominantly common in female population worldwide [1], is expressed as a heterogeneous disease with clinical course and its outcome, since it is related to many biological factors that are the nature of the cancer. The available evidence seems to suggest that understanding this heterogeneity is important in terms of targeted prevention of breast cancer and the biological process that is an obstacle to treatment [2]. Epidemiological studies indicate that women with Type 2 diabetes mellitus (T2DM) may have a tendency to develop breast cancer. Along similar lines, a meta-analysis study argues that especially postmenopausal women with T2DM are related to a higher rate in the risk of breast cancer by 23% [3].

A biguanide drug, namely metformin (1-(diaminomethylidene)-3,3-dimethylguanidine), reducing circulating insulin levels as an insulin sensitizer which enhances insulin sensitivity, is not only considered as a gold standard for the treatment of T2DM but also an agent that diminishes carcinogenic risk and inhibits cancer cells' proliferation [4]. The type 2 diabetics using metformin in long-term periods have been found to have a lower risk of cancers, especially of the breast cancer [5]. On the basis of the epidemic evidence currently available, it seems fair to suggest that the diminished probability of getting cancer in Type 2 diabetic people through metformin uptake becomes widespread in the field of oncology.

Being at the center of many complex biochemical processes, invasion and metastasis in cancer are mostly accountable for cancer-associated deaths. Interactions between tumor cells and extracellular matrix (ECM), degradation of intercellular adhesion molecules and ECM invasion via circulatory vessels are crucial processes for both tumor invasion and metastasis [6]. Several proteolytic enzymes act a part and assume critical roles in cancer microenvironment degradation such as the ECM and the basement membrane [7]. Underlining the engagement of matrix metalloproteinases (MMPs) in tumor invasion & metastasis is remarkable as they block tumor cells' spreading [8]. MMPs are structural and functional zinc-dependent endopeptidases participating in the several ECM components' proteolysis process [8]. The activity of these proteins is accurately regulated to block tissue degradation under physiological conditions. The balance of physiological conditions may likely to be broken down in cancer producing tumor cells which are able to invade the other tissues [9]. Until now, most of the researchers have focused on the expression patterns of MMP-9 and -2 which are able to degrade type IV collagen [10]. Tumor invasion begins with an obligatory step of epithelial cells-stroma separation, namely the basal membrane degradation, proceed with both enhanced activity and expression of MMP-9 and MMP-2 [11]. During the transcription process, activator proteins such as NF-kB (nuclear factor kappa B) and AP-1 (activator protein 1) collaborate for the expression of MMP-2 and -9 in the transcription level.

NF-kB has more than 100 target genes to be regulated in many diseases and acts a crucial role in inflammation and numerous stages of cancer initiation and progression [12]. The NF-kB transcription factor has five distinct homo and heterodimer complex proteins and one of these, p65 subunit, forms the p50/p65 heterodimer with p50 subunit. The p50 subunit binds to the DNA, while the p65 subunit carries out transcriptional activation [13]. NF-kB is inhibited by cytoplasmic Inhibitor of kappa B (IkB), bound to the p50/p65 heterodimer. After the IkB phosphorylation by IkB kinase, NF-kB activates the transcription of its target genes on DNA, following the translocation of the released p50/p65 and p50/p65 to the nucleus [14].

Cell lines are becoming important tools used for the molecular diagnosis in breast cancer as in vitro models in cancer research. As for breast cancer, MCF-7 cell line is a potent candidate that can be used in studies to mimic the estrogen receptor (ER)-positive breast cancer and many sub-clones, which have been established for different classes of ER-positive tumors [15].

The preventive efficacy of metformin against cancer cell has been supported by several investigations, but there are many unanswered questions to illuminate the underlying anti-cancer mechanisms of metformin on MCF-7 cell line. Therefore, the target of this study was to evaluate the anti-proliferative effect of metformin in MCF-7 cell populations and to figure out the role of NF-kB, MMP-9 and MMP-2 expressions, if any, in regulating invasiveness and metastasis of breast cancer cells.

Materials and methods

Cell culture

The MCF-7 cell line (human breast adenocarcinoma, estrogen receptor–and progesterone receptor positive) acquired from the American Type Culture Collection was exerted in our study. The cells were cultured through incubation at 37°C in 5% CO_2 incubator using the recommended media (Dulbecco's Modified Eagle Medium)(DMEM/F-12, Sigma-Aldrich) with 5.5 mM glucose, 2 mM glutamine and 15 mM HEPES, supplemented with 100 IU/mL penicillin (Pronapen, Pfizer), 50 mg/mL streptomycin, 10% fetal bovine serum (FBS, Gibco Lab). The pH of the medium was adjusted to 7.4 using $NaHCO_3$. The proliferation and immunocytochemistry (ICC) assays were performed by seeding 0.5×10^5 cells in 24-well plates and culturing the cells in media for 24 h.

BrdU cell proliferation assay

Metformin (Metformin HCL-Wanbury, H10000691) concentrations were identified with respect to the former implementation of clinical and in-vitro studies. Three different experimental doses (2 mM, 10 mM and 50 mM) diluted from a 100 mM stock metformin were obtained and adjusted in 24-well plates.

The anti-proliferative efficacy of metformin on cell population was evaluated by testing 5-bromo-2′-deoxyuridine (BrdU) integration into cellular DNA during the replication. Shortly, 0.5×10^5 cells were seeded in a 24-well plate using DMEM. After seeding, the cells are treated with a set of metformin (0–50 mM) for 24 h and incubated at 37°C and under condition of 5% CO_2. Following the incubation, 50 µL BrdU in 1 mL culture was added to each well and incubated for an hour at the same conditions. Subsequently, the cells were rinsed with phosphate buffered saline (PBS, pH 7.4) and re-incubated with 70% ethanol for 10 min. For denaturation of DNA, the cells were incubated with 2 M HCl at 37°C for 30 min and then neutralized with sodium borate buffer (0.1 M, pH 8.5) for 10 min. The cells were then rinsed twice with PBS. For immunostaining assay, rinsed cells were incubated with anti-BrdU (1:100 diluted (Bu20A): sc-20045, Santa Cruz Biotechnology, Inc.) overnight at +4°C in the dark.

As a chromogen, Amino-ethyl-carbazole (AEC) (Invitrogen) was used. Over several fields of view, greater than 300–350 cells per chamber were counted using a light microscope and the percentages (%) of BrdU-positive cells were assessed according to the literature [16].

Immunocytochemistry for NF-kB, MMP-2, and MMP-9

MCF-7 cells were seeded on cover glass coated in 24-well plates (10^5 cells per well), then incubated with DMEM/F-12 (5 mM glucose). Experimental metformin concentrations were adjusted to 0 mM, 2 mM, 10 mM, and 50 mM in 24-well plates as four groups and the plates were then incubated for 24 h. The cells in well-plates were rinsed with PBS and incubated for 10 min following 70% ethanol addition. After rinsing twice using PBS, the cells were incubated with monoclonal MMP-2 antibody (1:100 diluted) (MMP2/2C1, ThermoFisherSci, Rockford, IL 61105, USA), monoclonal MMP-9 antibody (1:400 diluted) (5G3, ThermoFisherSci, Rockford, IL 61105, USA), polyclonal NF-kB (p65) antibody (1:100 diluted) (ThermoFisher, Sci, Rockford, IL 61105, USA) overnight at +4°C in the dark. Afterwards, indirect streptavidin-biotin-peroxidase method was performed using the Histostain-Plus™ Broad Spectrum Kit (Mouse and Rabbit Specific Detection, ab93705, USA) was applied. AEC staining kit (Invitrogen) was used as the chromogen. Following the incubation for 2 min at room temperature with AEC; the immunoreactivity of the cells were counted under a light microscope (10×) and interpreted semi-quantitatively via a modified H-SCORE analysis assigning numerical scores between 0 and 300 to the immunostaining, a method used by Tombulturk et al. [17].

Statistical analysis

UNISTAT 5.0 for Windows package program was used in the immunostaining statistical analysis of findings given as mean ± SEM. For statistical analysis, one-way ANOVA test for intergroup comparisons and Tukey-Kramer multiple comparisons tests as multiple benchmarks were used. Outcomes with $p<0.001$ and $p<0.05$ significantly were under consideration.

Results

The anti-proliferative effects of metformin on MCF-7 cells

To investigate the anti-proliferative efficacy of metformin, 0 mM metformin (control group), a set of metformin (2, 10 and 50 mM) were tested in vitro normoglycemic conditions for 24 h using MCF-7 cells (see Figure 1). In MCF-7 proliferation percentage, the number of Br-dU positive cells reduced significantly in the groups of 10 mM

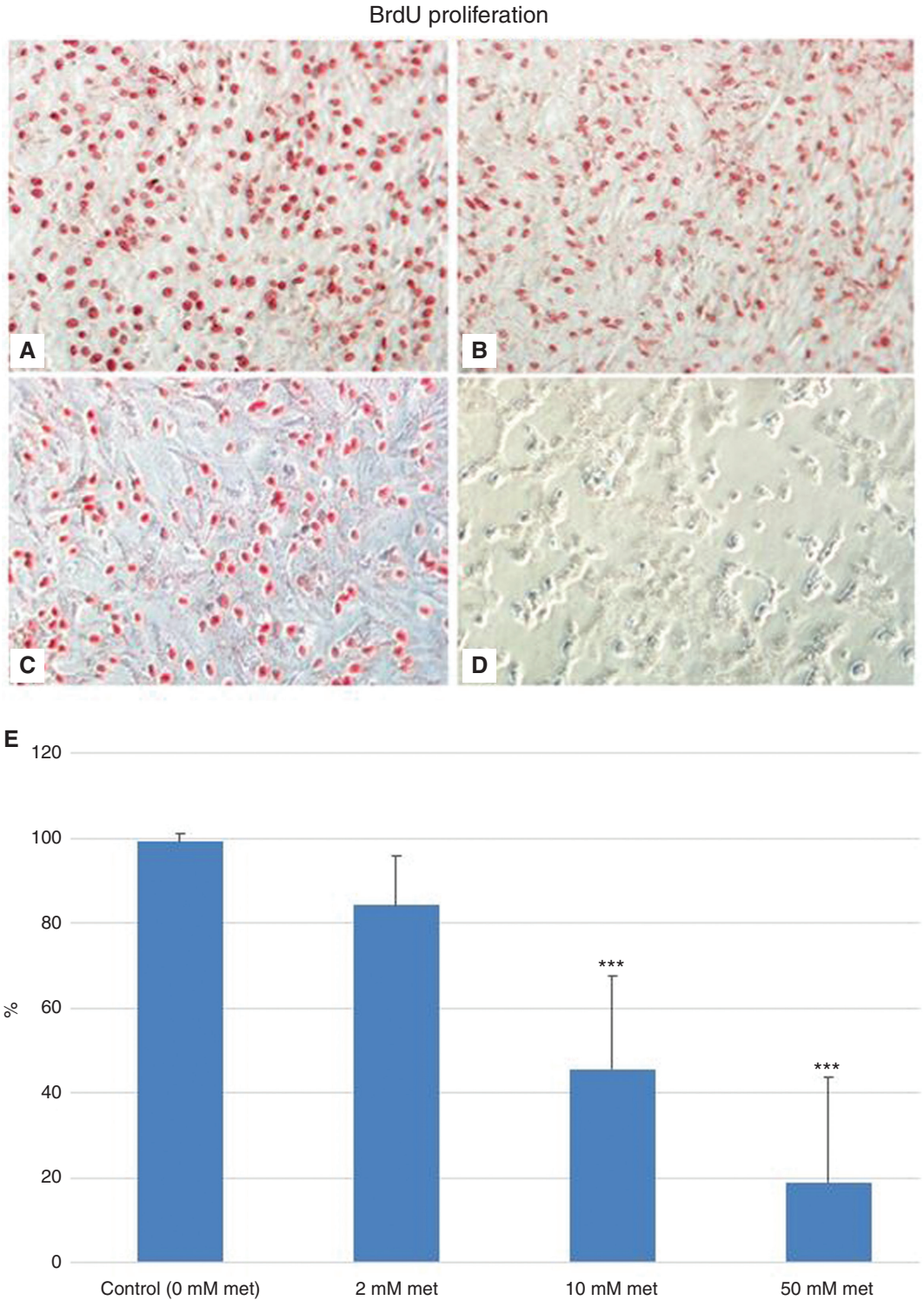

Figure 1: MCF-7 cell number significantly decreases in the presence of increasing metformin concentration in a dose-dependent manner. The micrographs of Br-dU proliferation assay for MCF-7 cells treated by 0 mM (A), 2 mM (B), 10 mM (C), 50 mM (D) doses of metformin (magnified ×40), with statistical findings (E) for % of Br-dU positive cells (***$p < 0.001$).

and 50 mM metformin compared to control and 2 mM metformin groups (***$p < 0.001$). According to these outcomes, the metformin demonstrated a concentration-dependent anti-proliferative activity in MCF-7 cells and a high dose of 50 mM concentration was assumed as an efficient dose of metformin on MCF-7 cell line.

The efficacy of metformin on the NF-kB (p65), MMP-9 and MMP-2 expressions in MCF-7

Expression levels of NF-kB (p65), MMP-2, and -9 in cultured MCF-7 cells were examined at the cellular level by ICC method and analyzed by two independent researchers on randomly selected five areas at ×40 magnification.

MMP-2 immunocytochemical analysis revealed that the immunoreactivity of protein decreased statistically in 50 mM metformin-treated group compared to the control group, and to 2 mM and 10 mM metformin-treated groups (see Figure 2). As an outcome of the evaluation of MMP-2-immunoreactive MCF-7 count (%) in experimental groups, a significant decrease was detected in MMP-2 expression in high dose (50 mM) metformin group compared to the other groups ($p < 0.05$) (see Figure 2).

It is apparent from Figure 3 that the expression of MMP-9 decreased statistically in 10 mM and 50 mM metformin-treated groups compared to the control group and 2 mM metformin-treated group. In parallel with MMP-2 immunoreactivity, we concluded that a significant decrease was detected in MMP-9 expression in 50 mM

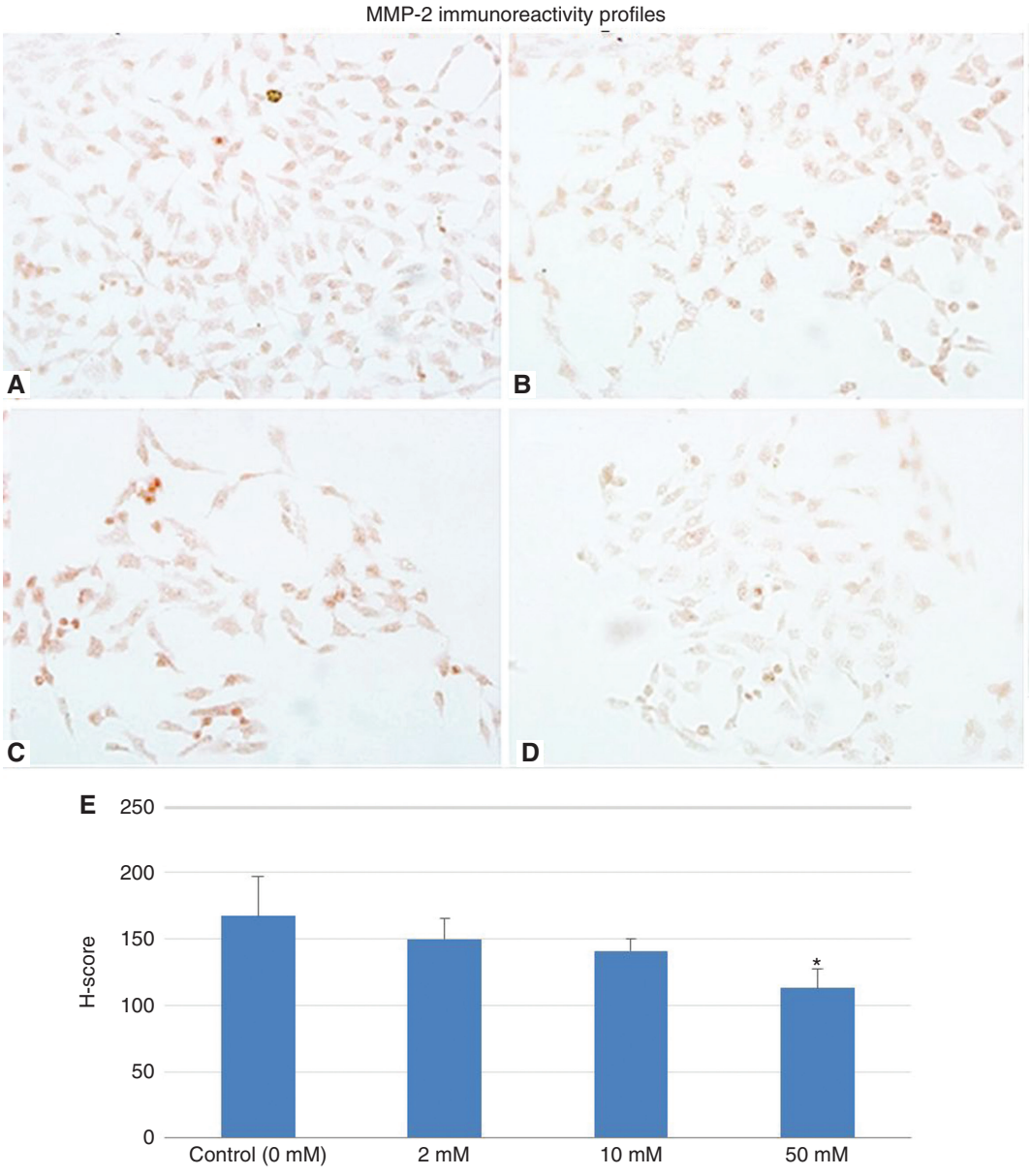

Figure 2: MMP-2 immunoreactivity decreases at 50 mM metformin concentration in MCF-7 cells.
Representative immunocytochemical staining of MMP-2-immunoreactive MCF-7 cells treated by 0 mM (A), 2 mM (B), 10 mM (C), 50 mM (D) doses of metformin (magnified ×40), with statistical findings (E) for MMP-2-immunoreactive cell count (%) (*$p < 0.05$).

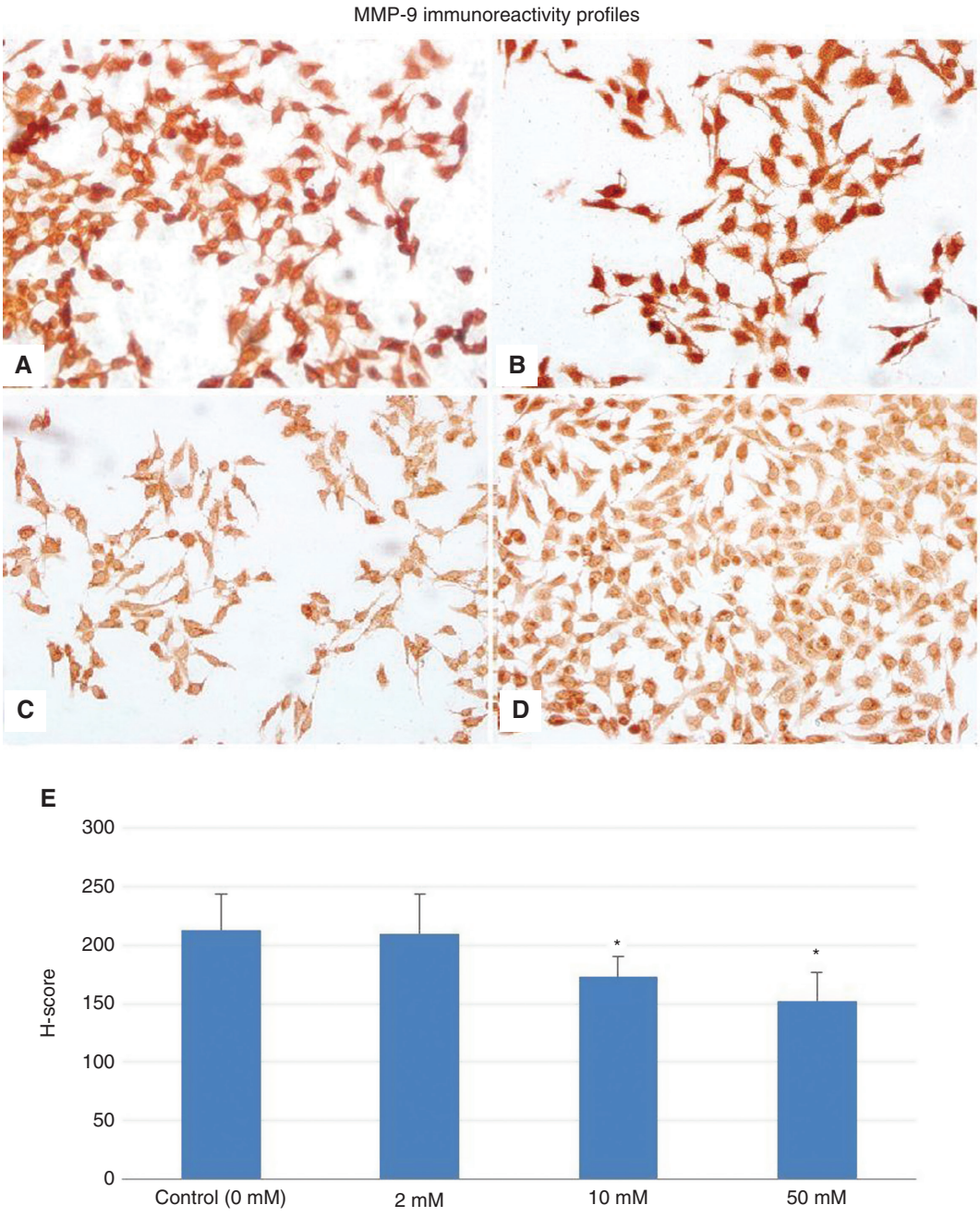

Figure 3: MMP-9 immunoreactivity decreases at both 10 and 50 mM metformin concentrations.
Representative immunocytochemical staining of MMP-9-immunoreactive MCF-7 cells treated by 0 mM (A), 2 mM (B), 10 mM (C), 50 mM (D) doses of metformin (magnified ×40), with statistical findings (E) for MMP-2-immunoreactive cell count (%) (*$p < 0.05$).

metformin group in comparison to the other groups ($p < 0.05$) (See Figure 3).

According to the immunocytochemical findings of NF-kB (p65), the expression of cytoplasmic NF-kB (p65) elevated in the 50 mM metformin-applied group (see Figure 4), and this increase was statistically different from the other groups ($p < 0.05$). Whereas, the nuclear immunostaining for NF-kB in the cells reduced in the highest dose group compared to the other groups ($p < 0.05$) (see Figure 4).

Discussion

There is a growing evidence worldwide either in vivo or in vitro that a T2DM therapy with metformin might be a cure for breast cancer [18]. Nowadays, metformin, with a low market price unlike the other cancer treatment drugs, and its usability with or without chemotherapeutic medications in the cancer therapy, is expected to have benefits economically and to increase the chances of

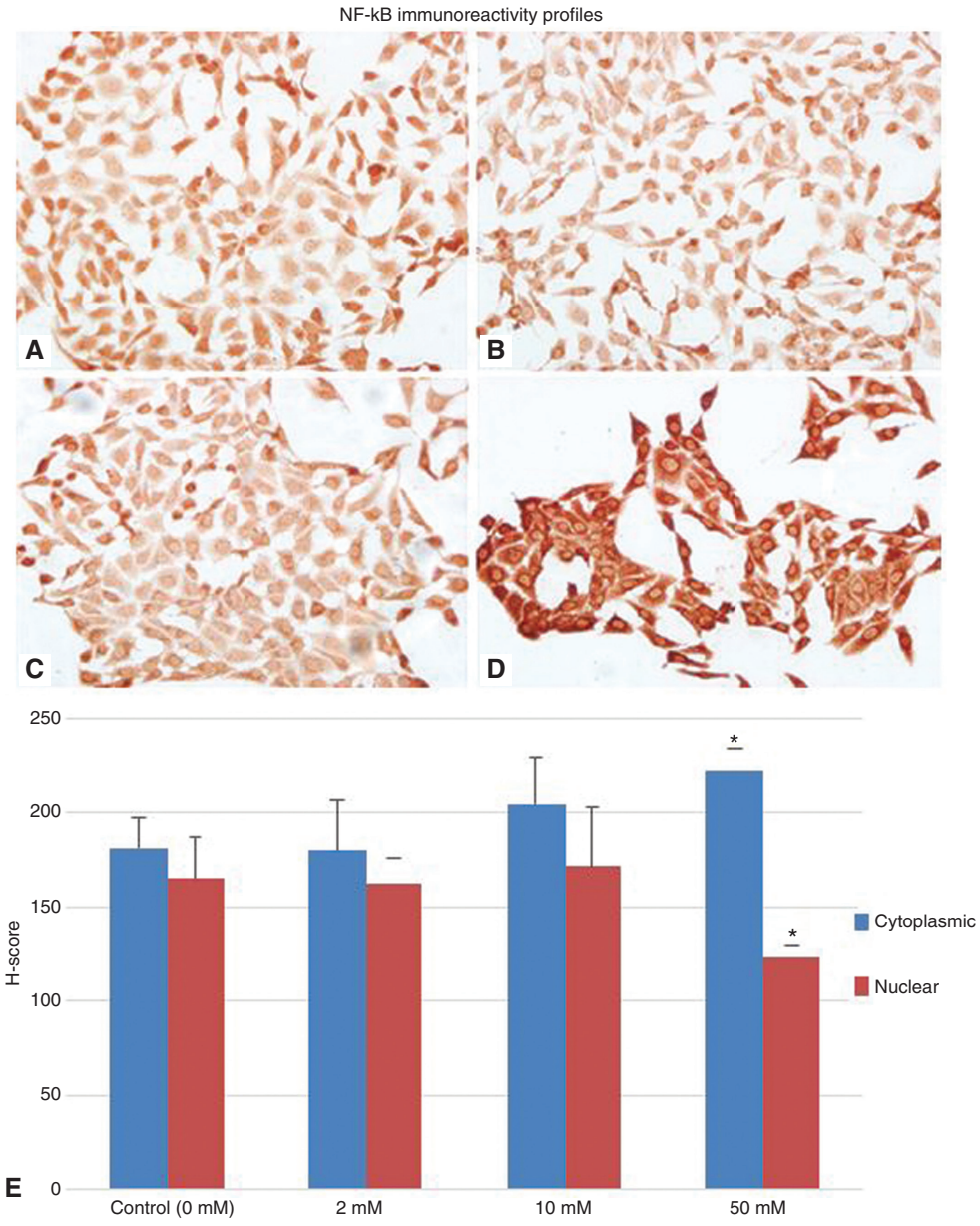

Figure 4: 50 mM metformin significantly increases the amount of cytoplasmic NF-kB whereas decreases the amount of nuclear NF-kB. Representative immunocytochemical staining of NF-kB (p65)-immunoreactive MCF-7 cells treated by 0 mM (A), 2 mM (B), 10 mM (C), 50 mM (D) doses of metformin (magnified ×40), with statistical findings (E) for MMP-2-immunoreactive cell count (%) (*$p < 0.05$).

survival outcomes for cancer patients. Moreover, due to its relative safety, metformin continues to receive intense interest by many researchers regarding the oncological patterns [19, 20].

A numerous amount of researches have been published on this oncological and other possible effects. In a diabetes study, breast cancer patients with a metformin cure have a better prognosis in breast cancer treatment than the patients treated without metformin [21]. Another research reported that metformin showed its anti-proliferative impacts on the breast cancer cells by developing insulin sensitivity not only directly but also indirectly therefore reducing hyperinsulinemia [20, 22]. Mainly, these effects of metformin have been shown to be due to the AMP-activated protein kinase, by which the mammalian target of rapamycin (mTOR) in an LKB1-associated cascade and the cyclin D1- a significant cell-cycle regulator in breast cancer cell lines are blocked, respectively [23–25]. A very recent study by Sharma and Kumar showed that metformin's proliferation inhibitory impact on MDA-MB-231 cell line is correlated with reduced ROS production [26]. In the current study, treating MCF-7 cells by a high metformin dose (50 mM) significantly inhibited the proliferation percentage ($p < 0.001$). These findings are also

in a good agreement with Marinello et al.'s findings [27] in which the cellular metabolic activity of both MDA-MB-231 and MCF-7 cell lines were inhibited dramatically by a variety of metformin concentrations in 24 h and 48 h periods. Alimova et al. studied the activity of metformin against diverse molecular subtypes of breast cancer cell lines [MCF-7, MCF-7/713 (MCF-7 transfected with erbB2), BT-474 and SKBR-3] in vitro [25]. Metformin showed biological activity against all estrogen receptor (ER) positive and negative, and abnormal breast cancer cell lines tested. It inhibited cellular proliferation at a concentration of 50 mM, BT-474 and SKBR-3 cells were 85% growth inhibited, MCF-7 was 57% inhibited and MCF-7/713 cells were 50% growth inhibited, as compared to untreated controls, in these 72 h cell proliferation assays. Thus, MCF-7 without transfection seems to be more resistant to drugs compared with out clones of breast cancer cell lines, hence, we used high doses of metformin to inhibit the proliferation of breast cancer cells [25].

As for breast cancer, MCF-7 cells represent a very important candidate as they are used ubiquitously in research for estrogen receptor (ER)-positive breast cancer cell experiments and many sub-clones, which have been established, represent different classes of ER-positive tumors with varying nuclear receptor expression levels. Different cell lines have shown different reactions to varied doses of metformin in the literature. Sena et al. investigated the effects of different doses of metformin (0, 10, 25, and 50 mmol/L) on NF-κB expression in an epithelial colon cancer cell line (HT29), and found that 50 mM metformin treatment for 24/48 h reduced NF-kB expression significantly. They discussed that the doses of metformin that were shown effective against cancer cells are approximately 300–600 fold (between 20 and 50 mmol/L) greater than the dose routinely administered for diabetic disorders, and about 1000 times higher than what is used in common practice. Their results confirmed that metformin was effective even at low doses in diabetic patients, whereas higher metformin concentrations, being toxic, will lead to apoptosis of cancer cells [28].

Through the immunocytochemical analysis of our study, the expression of NF-kB (p65) activator protein, MMP-2, and -9, which play roles in both pathways of invasion and metastasis in breast cancer, were observed significantly different from the control groups ($p < 0.05$). The NF-kB (p65) protein is known to be the activator protein of MMP-2.-9 [29] and metformin indirectly and negatively affects MMP-2.-9 expressions by preventing the nuclear translocation of NF-kB (p65) from the cytoplasm. In an in vitro study, it has been reported that metformin blocks MMP-9 protein through blocking of AP-1 and reducing the PMA (phorbol 12-myristate 13-acetate)-induced Ca^{2+} influx, which is highly expressed in invasive and metastatic tumor cells of human fibrosarcoma (HT-1080) [30]. Thus, metformin may repress the expression level of proteins that are potentially in charge of cancer invasion and metastasis processes. On the other hand, Sharma and Kumar assessed the role of metformin on the migration by the qRT-PCR (Real-Time Quantitative Reverse Transcription PCR) analysis of MMP-2.-9 in MDA-MB-231 cells, and they observed that 20 mM dose of metformin substantially reduced MMP-2.-9 expressions in this cell line cells, underlining the anti-metastatic feature of metformin [26]. We also detected that the protein level of MMP-2.-9 in MCF-7 cell populations was significantly diminished with an effective dose (50 mM) of metformin compared to the non-treated cells ($p < 0.05$). These findings can be compared with the above-mentioned former studies as a suppressing effect of metformin on MMPs in many cell lines of breast cancer [9, 31].

The results of NF-kB (p65) immunostaining in the current study indicated that a high dose of metformin increased the expression of cytoplasmic NF-kB (p65) but reduced the nuclear immunostaining for NF-kB in MCF-7 cells significantly, suggesting the inhibition of translocation of both subunits to nucleus ($p < 0.05$). Consistent with our findings, Hirsch et al. studied the western blot analysis of NF-kB proteins in nuclear and cytosolic fractions of cancer stem cells treated with a dose (0.1 mmol/L) of metformin, concluded with decreased nuclear NF-κB rates and enhanced cytoplasmic NF-κB proteins and the phosphorylation of IκB in the cytosol [32]. Another study by Kim et al. also reported that the amount of IkB and the NF-kB activity were minimized in case of 1–10 mM metformin dose in MCF-7 cell line [33]. According to our results, metformin suppresses the expression of NF-kB resulting in diminished cell proliferation, as well as decreasing the amount of MMP-9, -2 proteins related to the invasiveness pathway of breast cancer.

Conclusion

Metformin likely acts as a suitable agent against breast cancer in vitro in by taking into account the dose of metformin in accordance with the outcomes of our study. Metformin may potentially have an anti-proliferative effect in MCF-7. In our belief, metformin may act on the process of invasion and metastasis by means of suppressing NF-kB (p65) that is normally expressed in the cancer cells intensely, and by significantly diminishing the expression

of MMP-9 and MMP-2. However, its molecular mechanism has not been totally clear yet.

Given that our findings are based on a limited number methods and limited sample size, with approaching the goal of reaching the data that we aimed in our study, this research has thrown up many questions in need of further investigation in the therapy of breast cancer by detailed investigating laboratory studies.

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Conflict of interest: The authors declare that they do not have any conflict of interest.

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