



Do MCF7 cells cope with metformin treatment under energetic stress in low glucose conditions?

Irem Dogan Turacli¹ · Haldun Umudum² · Arzu Pampal³ · Tuba Candar⁴ · Lara Kavasoglu⁵ · Yaren Sari⁵

Received: 31 October 2017 / Accepted: 30 January 2018 / Published online: 3 February 2018
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Abstract

There is a growing body of evidence about metformin being effective in cancer therapy. Despite controversies about the ways of its effectiveness, several ongoing clinical trials are evaluating the drug when used as an adjuvant or a neo-adjuvant agent. We aimed to investigate metformin's effects on proliferation, metastasis, and hormone receptor expressions in breast cancer cell line MCF-7 incubated in two different glucose conditions. MCF-7 cells were incubated in high or low glucose media and treated with various doses of metformin. The cell viability was studied using MTT test. The Ki-67, estrogen and progesterone receptor expression were evaluated by ICC and galectin-3 expression was evaluated by ELISA or spectrophotometrically. The cell viability following consecutive metformin doses in either glucose condition for 24 and 48 h represented a significant decrease when compared to control. The proliferation detected in low glucose medium following metformin at doses < 20 mM was found significantly decreased when compared to high glucose medium at 48 h. In terms of galectin-3 levels, the increase in high glucose medium treated with metformin and the decrease in low glucose medium were found statistically significant when compared to control. Progesterone receptor staining demonstrated a significant increase in low glucose medium. Our findings represent better outcomes for cancer lines incubated in low glucose medium treated with metformin in terms of viability, receptor expression and metastatic activity, and highlight the potential benefit of metformin especially in restraining the cancer cell's ability to cope energetic stress in low glucose conditions.

Keywords Breast cancer · Estrogen receptor · Galectin-3 · Low glucose · Metformin · Progesterone receptor

Introduction

Hyperinsulinemia is shown to be an independent risk factor for cancer development in type II diabetes mellitus [1]. Hyperinsulinemia due to either insulin resistance or exogenous insulin injections is proven to induce the sensitivity of chemically induced carcinogenesis in animals and exogenous insulin and/or its secretagogues are shown to be

related with increased cancer risk and cancer recurrence in human [2].

Hyperinsulinemia promotes carcinogenesis either directly via insulin receptors on relatively sensitive epithelial cells or indirectly via IGF, sex hormones, inflammatory processes and adipokines [2]. Apart from normal tissues, cancer cells cannot downregulate insulin receptors in case of hyperinsulinemia [2, 3]. Insulin–insulin receptor interaction on cancer cells activates proliferation and anti-apoptotic pathways via PI3K and MAPK pathways [2–4]. IGF-1 receptor (IGF1R) is overexpressed on cancer cells and bound either insulin or IGF-1 [2, 3, 5]. Hyperinsulinemia results elevated circulatory IGFs due to insulin–IGF1R binding and active IGFs result higher mitogenic activity via both IGF1R related pathways and PI3K and MAPK activation [2, 6]. Hyperinsulinemia or insulin resistance decreases the production of sex hormone binding globulins and increases circulatory sex hormones that ease sex-related cancer development like breast cancer. Moreover, insulin and inflammation are interacted in a reciprocal way. Hyperglycemia and insulin

✉ Irem Dogan Turacli
doganirem@gmail.com

¹ Department of Medical Biology, Ufuk University, Ankara, Turkey
² Department of Pathology, Ufuk University, Ankara, Turkey
³ Department of Pediatrics Surgery, Ufuk University, Ankara, Turkey
⁴ Department of Medical Biochemistry, Ufuk University, Ankara, Turkey
⁵ Faculty of Medicine, Ufuk University, Ankara, Turkey

regulates inflammatory response while inflammation provokes insulin resistance. Adipokines like leptin, adiponectin are secreted from macrophages at the tissue level with cytokines and all result in an increased insulin resistance and exacerbated inflammatory response [2, 7].

Metformin is a biguanide antihyperglycemic agent used in the treatment of type II diabetes mellitus. It is known to inhibit the complex I of oxidative phosphorylation in mitochondria and thereby activate AMP-activated protein kinase (AMPK), the central energy sensor of the cell. Its upstream activator liver kinase B1 (LKB1) and AMPK, by increasing the cellular AMP/ATP ratio, inhibit hepatic gluconeogenesis and decrease glucose output [8, 9]. Moreover activation of AMPK, while causing no change at muscular basal glucose level, increases uptake of insulin activated glucose, thereby effecting insulin resistance in a positive manner. For the last decade, the retrospective studies evaluating coexistence of cancer and diabetes exposed a fact that metformin treatment for type II diabetes is related with a reduction of cancer incidence and cancer related mortality [10]. Despite the controversies of its main effect on cancer therapy, hundreds of ongoing clinical trials are evaluating anticancer effects of metformin when used as an adjuvant or a neo-adjuvant chemotherapeutic agent.

To our knowledge metformin, as an anticancer agent, is capable of controlling the growth of various cancer cells both in vivo and in vitro in a time and dose dependent manner. This effect is mainly controlled by upstream activator LKB1 and AMPK [11, 12]. This activation inhibits downstream mammalian target of rapamycin (mTOR) and S6 kinase (S6K1) that regulate the protein translation of cellular growth regulators like cyclinD1, HIF1 α and MYC [13]. mTOR inhibition via metformin also results a decrease in protein HER-2 expression, one of the major actors in cancer cell proliferation [14]. AMPK activation by metformin also activates tumor suppressor gene p53 and induces apoptosis [15]. Moreover, metformin decreases IGF1 levels thereby interrupting the cross talk between insulin, IGF1R and G-protein coupled receptor systems [2, 16–18]. Metformin, even though insufficient to treat cancer alone, is accepted to have cytostatic effects on tumor cells. However, metformin is also accepted to be cytotoxic in particular conditions like glucose deprivation, p53 mutation and synthetically lethal with glucose withdrawal [19, 20].

The aim of the study is to evaluate the effectiveness of metformin depending on low and high glucose conditions on cell viability, galectin 3 levels, estrogen and progesterone receptor expression and Ki-67 staining in MCF7 breast cancer line.

Materials and methods

Cell culture

MCF-7 cells were kindly provided from Prof. Isık Yulug (Bilkent University) and were grown in high (4.5 g/L) or low glucose (1 g/L) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and a 1% penicillin/streptomycin mixture. Cells were incubated at 37 °C in a humidified 5.0% CO₂ atmosphere.

Cell viability

Cells were seeded in 10% FBS containing DMEM at 2×10^4 cells/200 μ L per well in 96 well plates for 24 h at 37 °C. After incubation for 24 h, MCF-7 cells were divided into two groups as; high glucose and low glucose exposed and treated with a series of concentrations of metformin (2.5, 5, 10, 20, 40, 80, 160 mM) for 24 and 48 h. The metabolic activity of living cells, an indication of proliferation and viability, was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After incubation time, 10 μ L MTT solution (0.5 mg/mL) was added to each well. After 4 h of MTT incubation at 37 °C, 100 μ L crystal dissolving buffer was added and the plates were gently shaken on an orbital shaker for 5 min. The absorbance at 570 nm was measured with a microplate reader. Each treatment was repeated at least four times. The mean absorbance of four wells was used as an indicator of relative cell growth.

Immunocytochemistry

Cells were seeded into 6 well plates at $2 \times 10^5/3$ mL per well for 24 h. After 24 h, they were incubated with 5 or 10 mM metformin in high or low glucose containing DMEM media. The cells were washed with ice cold PBS and removed with scrapers at 24 h metformin treatment. Scraped cells were fixed in formalin and embedded in paraffin for immunocytochemical staining protocol. For immunocytochemistry, a four micron thick section was cut from paraffin cell block and taken on poly-L-lysine coated glass slide. Sections were deparaffinized and rehydrated through graded alcohols and rinsed in phosphate buffer saline solution. All processes were performed with DAKO Autostainer (Agilent Technologies, USA). A brief description of immunohistochemistry process is as follows: A prediluted biotinylated antibody for Ki-67 (clone MIB-5), ER (clone EP1), PR (PgR 636) (obtained from Dako) was applied to sections and incubated for 30 min in humid chamber. Hydroperoxide (0.3%) then was applied to inhibit endogenous peroxidase activity.

Sections then incubated with labelled streptavidin biotiny peroxidase (LSAB, obtained from Dako). Reaction products were obtained with 1% solution of 3,3 diaminobenzidine (DAB, Obtained from Dako) in tris buffer. Slides then were counterstained with Mayer's hematoxyline, dehydrated through graded alcohols, cleared in xylene and coverslipped. Positive reaction is identified as brown production on tumor cell nuclei. The magnification was $\times 40$ of all ICC slides.

Protein isolation

Cells were seeded into 6 well plates at $2 \times 10^5/3$ mL per well for 24 h. After 24 h, they were incubated with 5 or 10 mM metformin in high or low glucose containing DMEM media. The cells were washed with ice cold PBS and removed from wells with scrapers at 24 h metformin treatment. For protein isolation, Cell Lysis Buffer (10 \times) (Cell Signaling #9803) was used and equivalent protein was measured with BCA Protein Assay (Thermo Fisher Scientific, USA).

Galectin-3 assay

Architect Galectin-3 was measured from cell protein lysates to measure quantitative galectin-3 by using Chemiluminescent Microparticle Immuno Assay (CMIA) which is the modified and advanced form of the Enzyme Linked Immuno Sorrbant Assay (ELISA) technique. Measurements were done in Abbott *i*1000 autoanalyzer.

Statistical analysis

Data was analyzed statistically by using SPSS 20.0 software (SPSS Inc, Chiago, IL, USA). One Way Anova, Mann–Whitney U and Fisher's Exact Tests were used to analyze the qualitative data. A p value < 0.05 was considered as significant.

Results

Cell viability

The effects of varying metformin concentrations on viability of MCF7 breast cell lines in low and high glucose media for 24 and 48 h are presented as mean $\% \pm$ SD in Fig. 1. Cell viability following increasing doses of metformin in either medium for 24 h represented a decrease when compared to control groups. The viability following consecutive metformin doses in low glucose (1 g/L) medium was even though lower when compared to high glucose (4.5 g/L) medium, the differences were not statistically significant (Fig. 1a). Likewise, the cell viability following increasing doses of metformin in either medium for 48 h represented a

decrease when compared to control groups. The cell viability detected in low glucose medium following metformin at doses < 20 mM was found lower when compared to high glucose medium at 48 h ($p < 0.05$) (Fig. 1b).

Galectin-3 levels

Galectin-3 levels for 5 and 10 mM metformin treatment at 24 h in low and high glucose media are presented as % in Fig. 2. The increase in galectin-3 levels in high glucose medium with either metformin treatment was found statistically significant when compared to control group and the decrease in galectin-3 levels in low glucose medium was found statistically significant only with 10 mM of metformin. The differences between the cells in high and low glucose media with either metformin treatment were also found statistically significant (Fig. 2).

Estrogen receptor (ER), progesterone receptor (PR) and Ki-67 expression

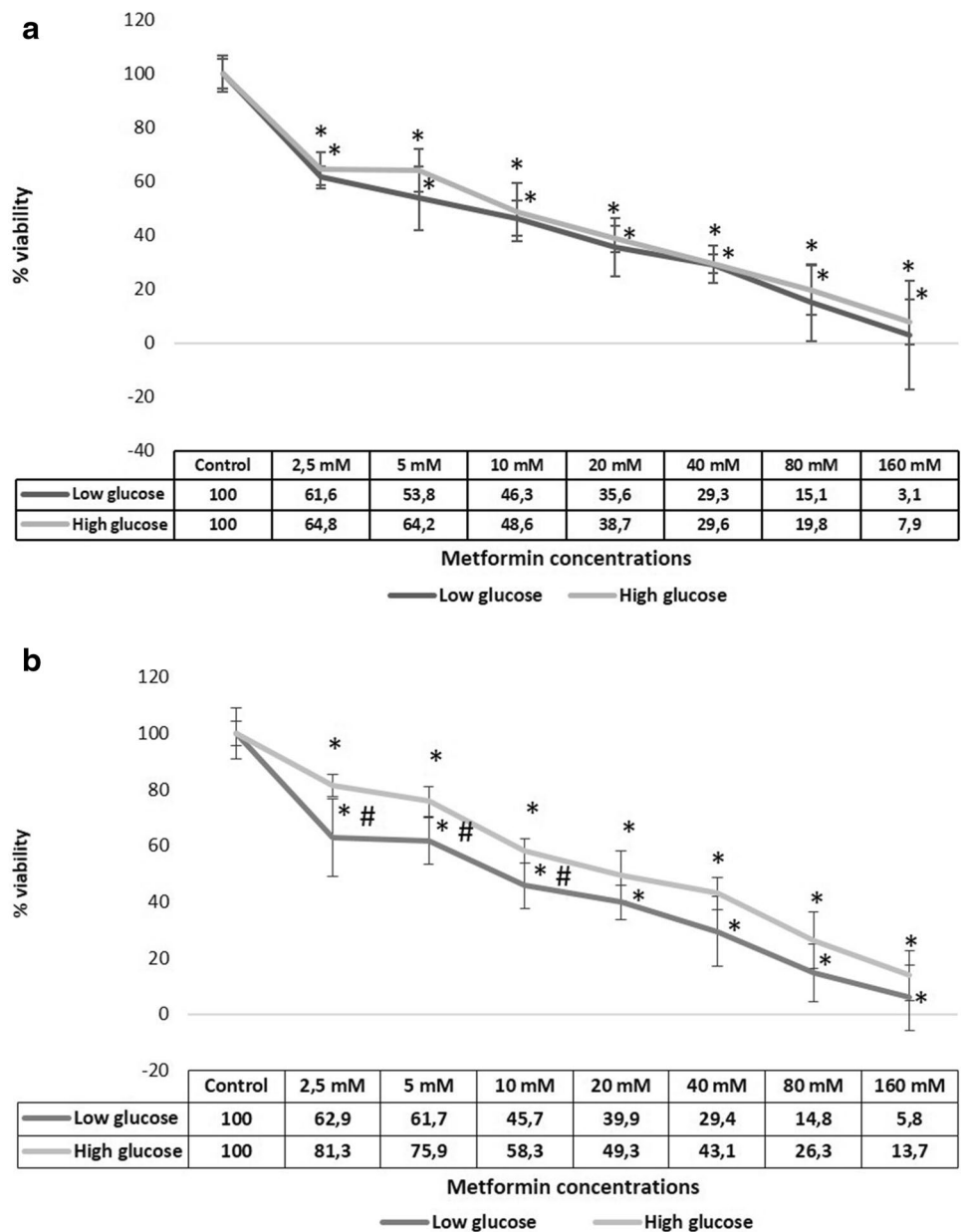
Estrogen and progesterone receptor staining of 5 and 10 mM metformin treatment in low and high glucose media at 24 h are presented as % in Fig. 3. ER positive cells were significantly decreased with metformin treatment in low glucose media and they were significantly increased with only 10 mM metformin treatment in high glucose media. Also, ER positive cells treated with 10 mM metformin in high glucose media were significantly higher when compared to cells of same dose treated in low glucose media (Fig. 3a). The PR positive cells were significantly decreased with metformin treatment in high glucose media. Interestingly, PR positive cells were significantly higher in low glucose media in 5 mM metformin treated group (Fig. 3b). There was no statistically significant difference on Ki-67 staining between metformin treated groups and/or high and low glucose media (Fig. 3c).

Discussion

In this study, we aimed to evaluate the effectiveness of metformin on MCF7 cells incubated in high and low glucose media and found that metformin usage is related with better results in terms of cancer cell growth, proliferation and metastatic activities especially in low glucose medium.

p53 plays an important role as the central regulator of stress response at molecular and biochemical basis. p53 triggers oxidative phosphorylation through upregulation of cytochrome c oxidase and downregulates glycolytic pathway via inducing TIGAR [21]. In our experiments we used p53 wild type cancer cells to see the metformin's effects that does not interfere with mutant p53's effects on proliferation,

Fig. 1 a Cell viability of MCF7 cells treated with consecutive doses of metformin in high and low glucose conditions at 24 h (* and † reflects $p < 0.05$ when compared to control group). **b** Cell viability of MCF7 cells treated with consecutive doses of metformin in high and low glucose conditions at 48 h (* $p < 0.05$ when compared to control group, # $p < 0.05$ when low glucose medium group compared to high glucose medium group)



receptor expression and metastatic activity in low and high glucose settlement.

Glucose is the main source for energy in proliferative cancer cells. The cancer cells possess elevated glucose uptake ability and they tend to make glycolysis instead of oxidative phosphorylation even in the presence of oxygen. This shift from respiration to fermentation in cancer cells results in lactate accumulation leading to acidosis and acidification of the microenvironment allows immune escape of the cancer cells and eases tumor invasion [22]. Otto Warburg posted this activity for the first time in 1920s and so-called Warburg effect is still a matter of debate in the oncology practice [22, 23]. Despite ongoing discussions, tumor cells seem to require ATP, NADH, NADPH in order to support

their anabolic processes, cellular growth and proliferation and glucose is the main source for these activities [24]. Cancer cells incubated in only high glucose medium were shown to increase their viability and proliferation [20, 25–27]. In that manner, cutting off the glucose supply of cancer cells can lead inhibition of cellular growth and proliferation. Retrospective studies evaluating diabetic population associates the use of metformin with decreased cancer incidence and cancer related mortality [28]. With normalization of serum glucose and insulin levels, metformin may serve indirect anticancer effects, as normoglycemia blocks the free glucose use of tumor cells despite their high demand and normal insulin levels inhibits insulin/IGF signaling pathways. There is ample evidence that metformin has also direct anticancer

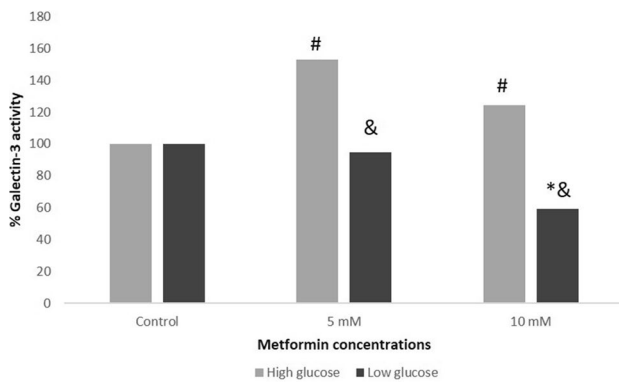


Fig. 2 Galectin-3 levels of MCF7 cells treated with 5 and 10 mM of metformin in high and low glucose conditions (* and # reflects $p < 0.05$ when compared to control group, & reflects $p < 0.05$ when compared to high glucose medium)

effects partially through the activation of AMPK [2, 17, 18]. This activation changes the cells from energy consuming phenotype to energy conserving phenotype with the resultant cytostatic effects of the drug. Even though targeting ATP production at the mitochondrial level seems irrational treatment strategy for cancer cells relying glycolysis for ATP production, in case of limited glucose availability, the cancer cells became dependent on oxidative phosphorylation to produce ATP. In this scenario, inhibition of the mitochondrial metabolism with metformin results an energy crisis at the cellular level and the resultant cytotoxic effect of the drug [29]. However the in vitro studies evaluating the effects of metformin were designed with suprapharmacologic doses of the drug. The drug, used as an oral antidiabetic, is prescribed with the maximal approved daily dose of 2.5 g (35 mg/kg of body weight). After oral administration, metformin is transferred from enterocyte to hepatocytes via portal vein. Even the portal vein plasma concentration of the drug is 2–4 times greater than the systemic plasma concentration, the plasma peak concentration is only as high as 40 μM [30]. Suprapharmacologic doses used in in vitro studies are shown to inhibit respiratory chain complex 1 in mitochondria, increase AMP and suppress adenyl cyclase activity with the resultant blockage of cAMP/PKA pathway [18].

In a study by Zhuang et al., they proposed the protective effect of high glucose against metformin induced cytotoxicity in cancer lines. They stated that high glucose supported the glycolytic activity and ATP production and also both AMPK activation and oxidative phosphorylation blockage due to metformin enhanced glycolytic activity leading to survival. They also stated that lowering glucose levels in the medium potentiated metformin's cytotoxic effect by not only decreasing the ATP levels but also inhibiting the survival signaling pathways [31]. We studied the effects of metformin on MCF7 cell viability, proliferative

activity and found that, even though either medium with metformin represented good results, better results are obtained from cells in low glucose medium. Cell viability significantly affected with lower doses of metformin in low glucose medium when compared to high glucose medium. Metformin and the low glucose level in the medium for cell culture may mimic a clinical scenario of regulated blood sugar levels in a diabetic patient using metformin. And this scenario is compatible with the finding of “less” cancer incidence and cancer related mortality in diabetic population using metformin.

In this study, we evaluated Ki-67 expression, a proliferation marker, and found it to be decreased in either medium with metformin, but the decrease was not statistically significant. Ki-67 is a prognostic factor in breast cancer and its high levels are related with higher relapse risk and worse survival. Hadad et al. demonstrated in vitro and in vivo evidences for AMPK dysfunction for cancer cells and reactivation of AMPK with metformin could have therapeutic potential especially in breast cancer. They attributed the effects of metformin as cytostatic rather than cytotoxic and they demonstrated significant decrease in Ki-67 and cleaved caspase 3 activities [32–34].

Estrogen and progesterone receptor expression of the cancer cells were also evaluated in this study. Estrogen receptors were found decreased after drug incubation in low glucose medium whereas progesterone receptors represented a prominent increase in the same medium. Bernstein et al. demonstrated interesting clinical data about hormone receptor expression and metformin usage in diabetic women with breast cancer. They showed similar estrogen receptors but more progesterone receptors expression in breast tumors of diabetic women treated with metformin when compared to other antihyperglycemic agents. They attributed no change in estrogen receptor and elevation in progesterone receptor to different modes of antidiabetic therapy as the study groups were similar in terms of HER-2/neu expression. They stated that such increase in receptor expression due to metformin can be related to the amelioration of estrogenic signal transduction, tumor sensitivity to hormones and better outcomes [35].

Galectin-3, a member of beta-galactoside-binding protein family, is known to promote neoplastic transformation, ease tumor adhesion to extracellular matrix and enhance metastatic spreading of the tumor [36]. As its overexpression is related with metastasis, we also aimed to evaluate the effectiveness of metformin on galectin-3 levels of MCF7 cells in high and low glucose conditions. We showed galectin-3 levels were significantly decreased with metformin in low glucose medium when compared to control and high glucose medium groups. Interestingly the cells in the high glucose medium represented significantly elevated galectin-3 activity after drug administration.

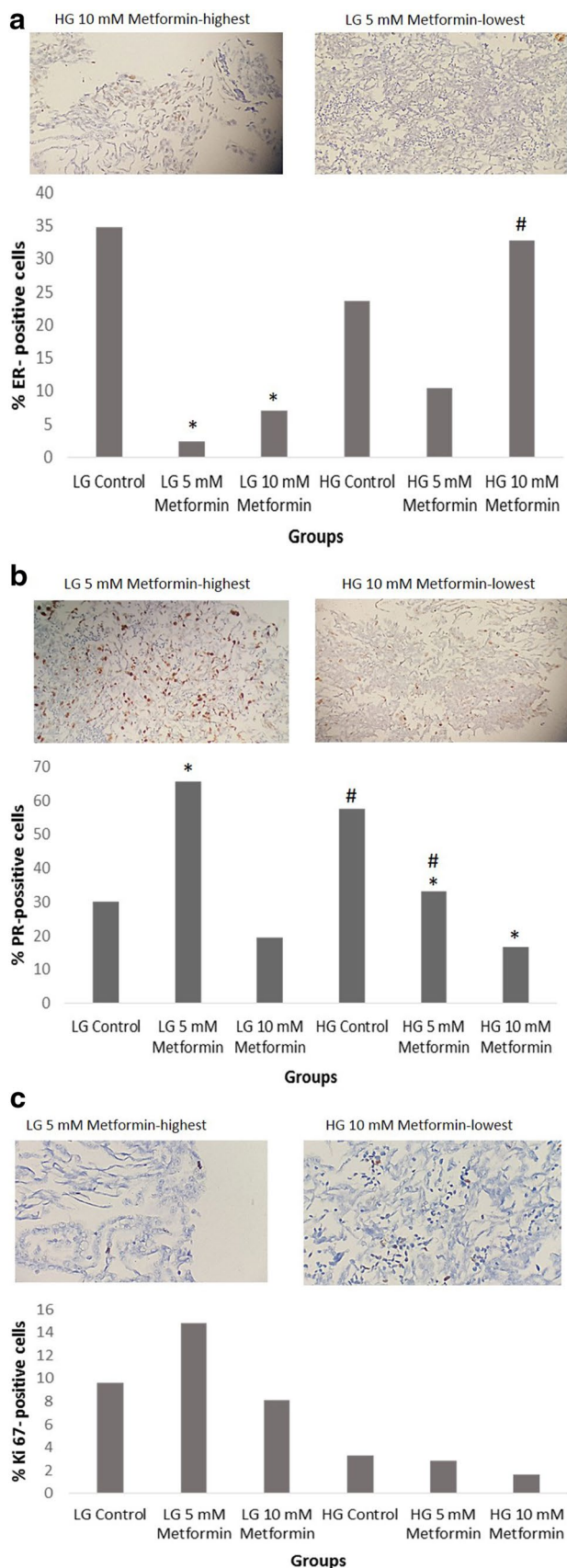


Fig. 3 % ER positive (a), % PR positive (b), % Ki 67 positive (c) MCF7 cells treated with consecutive doses of metformin in high and low glucose conditions at 24 h (* $p < 0.05$ when metformin treated groups compared to control group in LG or HG media, # $p < 0.05$ when low glucose medium group compared to high glucose medium group at the same metformin dose). Axis data represented as log scale. ICC pictures represent the highest and lowest expression within metformin treated groups ($\times 20$)

The overall findings of this study represent better outcomes for MCF7 incubated in low glucose media treated with metformin in terms of viability, receptor expression and metastatic activity. Even metformin blocks oxidative phosphorylation, high glucose inhibits metformin cytotoxicity by providing a power supply for glycolysis in cancer cells. Thus, it will be better to affiliate energetic and microenvironmental changes and incorporate them to the comments of metformin studies in terms of analyzing its antiproliferative and antimetastatic effects. Future research with different cells and animal models will help to better define metformin's role as a potential cancer therapy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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