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# Metformin and proliferation of cancer cell lines

## Metformina a proliferacja nowotworowych linii komórkowych

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**Abstract**

**Introduction:** Metformin is a widely used drug in treating type 2 diabetes and insulin resistance and nowadays scientists are searching for new potential and multiple roles in prevention and treatment of carcinogenic processes.

**Aim of the study:** The aim of the study was to compare the impact of normoglycemia and hyperglycemia with doses of metformin on vivacity and proliferation of cancer cell lines (MCF-7, MCF-7/DX, A549, CCRF/CEM, THP-1, NHDF).

**Material and methods:** We designed our experiment using raising glucose environment (40 mM, 100 mM, 150 mM, 300 mM) and we added increasing concentrations of metformin (5 mM, 10 mM, 20 mM, 30 mM). We incubated cells for 24 h, 48 h, 72 h, 96 h. In order to measure of viability of cancer cells we use MTT assay – a typical test to mark cytotoxic effects of tested substances.

**Results:** Analysis indicated that populations of cancer cells in our terms was lowering, the incubation of 24 h and 48 h showed favorable results than 72 h and 96 h. In normoglycemic environment (glucose level about 100 mM) and after added metformin in various concentrations we observed decreasing percentage of vivid cells for all cancer cell lines (MCF-7, MCF-7/DX, A549, CCRF/CEM, THP-1).

**Conclusions:** The results of our study showed beneficial effects of metformin on decreasing proliferation of cancer cells. Percentage of vivid populations were lowering and we confirmed anti-cancer effect of this drug.

**Key words:**
cancer cell lines, cell culture, growth, metformin, proliferation.

**Streszczenie**

**Wprowadzenie:** Metformina jest lekiem szeroko stosowanym w leczeniu cukrzycy typu 2 i insulinooporności, a naukowcy koncentrują się obecnie na poszukiwaniu nowych możliwości i wielu ról w profilaktyce i leczeniu procesów nowotworowych.

**Cel pracy:** Porównanie wpływu normoglikemii i hiperglikemii z dawkami metforminy na żywotność i proliferację linii komórek nowotworowych (MCF-7, MCF-7/DX, A549, CCRF/CEM, THP-1, NHDF).

**Materiały i metody:** Eksperyment wykonano z użyciem roztworów glukozy (o stężeniach 40 mM, 100 mM, 150 mM, 300 mM) i dodano zwiększające się stężenia metforminy (5 mM, 10 mM, 20 mM, 30 mM). Inkubowano komórki przez 24 godziny, 48 godzin, 72 godziny i 96 godzin. W celu pomiaru żywotności komórek nowotworowych wykorzystano test MTT – typowy test do oznaczania cytotoksycznego wpływu badanej substancji.

**Wyniki:** Analiza wykazała, że populacje komórek nowotworowych uległy zmniejszeniu, inkubacja 24- i 48-godzinna wykazała korzystniejsze wyniki niż trwająca 72 godziny i 96 godzin. W środowisku normoglikemicznym (stężenie glukozy 100 mM) po dodaniu metforminy w różnych stężeniach zaobserwowano malejący odsetek żywych komórek wszystkich linii nowotworowych (MCF-7, MCF-7/DX, A549, CCRF/CEM, THP-1).

**Wnioski:** Wyniki badania wykazały korzystny wpływ metforminy na zmniejszenie proliferacji użytych komórek nowotworowych. Odsetek żywych komórek w populacji się zmniejszał, potwierdzono również działanie antynowotworowe metforminy w eksperymencie.

**Słowa kluczowe:**
linie komórek nowotworowych, hodowla komórek, wzrost, metformina, proliferacja.

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## Introduction

Nowadays one of the most popular metabolic diseases is diabetes mellitus. It's characterized by hyperglycemia, which can lead to serious damage to the internal organs, like kidneys, heart and blood vessels.

The most common is type 1 and type 2 diabetes, usually in adults (Fig. 1) [1]. In 2020 the World Health Organization informed that about 422 million people worldwide have diabetes and 1.6 million deaths are directly connected to diabetes each year. Unfortunately the number of cases and the prevalence of diabetes have been increasing over the last years [2]. It is estimated that globally diabetes people will reach 439 million in 2030 [2].

Diabetes mellitus is called one of the civilization diseases. In this way we define illness linked with the way people live. Next to hypertension and cancer it is one of the next causes of death in Poland. It's mainly influenced by serious complications that develop as a result of this disease. The Polish National Cancer Registry reports an increasing number of patients each year (150,000 average/per year) [3]. It is estimated that about 20% of cancer patients have co-existing disorders of glucose metabolism [4]. The promoting type is hyperglycemia [5]. It has been recognized that it plays an important role in cancer progression. Hyperglycemia may be also connected with malignant phenotype of cancer cells and cause develop drug resistance [6, 7], but these processes are difficult to define.

Breast cancer is also an epidemiological problem. The incidence of this one is increasing almost everywhere throughout the world, at any latitude. Only in 2018 new cancer cases reached 2 millions (23% of all cancers) and ranked second overall (10.9% of all cancers) [8]. Definitely breast cancer is a multifactorial disease [9]. It can be due to unhealthy lifestyles, obesity after menopause, use of hormone replacement therapy or physical inactivity [10]. The potential of an increased risk of breast cancer for diabetic women has been the subject of a great deal of recent research. One of the potential mechanisms could be connected with hyperinsulinemia, which is a marker of type 2 diabetes [2]. Insulin is a growth-promoting hormone. The mitogenic effects are in normal and malignant breast tissues [4]. Hyperinsulinemia can also be joint with the insulin growth factor 1 (IGF-1) that could be involved in breast cancer [11, 12]. Another reason may be linked in hyperglycemia and the Warburg effect. In this mechanism carcinoma cells produce energy by a higher rate of cytosol glycolysis than by the oxygen-dependent mitochondrial Krebs cycle (Fig. 2) [5].

Human lung cancer is the next common malignancy in patients around the world. It causes increased deaths among the men and women [13]. This cancer is divided into non-small cell carcinoma (NSCC), about 85% all positive diagnosis, and small cell carcinoma (SCLC), 20% of the cases [14, 15]. The co-occurring metabolic disorders are negative prognostic factors in non-small cell lung cancer (NSCLC) [15]. Among patients with lung cancer, comorbidity diseases caused by diabetes mellitus contribute to diminished long-term survival [16]. Some studies with lung cancer patients prove that insulin resistance and hyperinsulinemia, which are characteristic for type 2 diabetes, could activate IGF-1 receptor signal pathway [17]. It plays a main role in cell growth, differentiation and metastasis [18]. Evidence shows that also increased serum hemoglobin $A_{1c}$, C-peptide, and IGF-1 levels are significantly correlated with the carcinogenic processes of lung tissues [14, 19].

Among all types of blood malignancies acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) are the most common. The first one-ALL is a distinctive subtype diagnosed in children [20]. Estimated half of children who are treated for ALL develop treatment-related medical conditions in their lifetime, one of them may be diabetes mellitus [21].

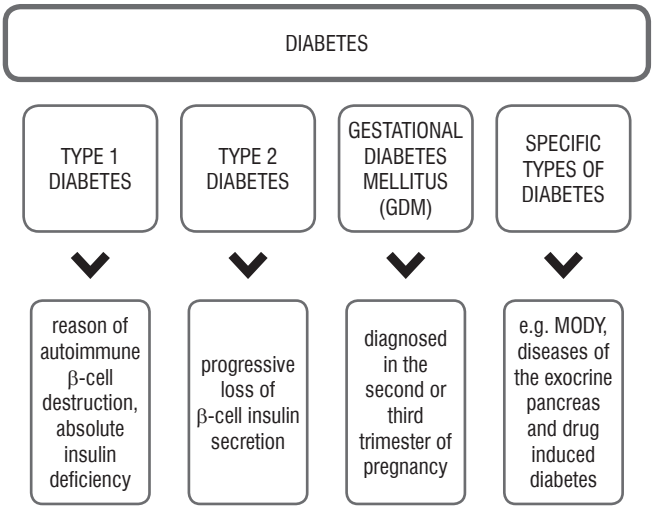


**Figure 1.** Classification types of diabetes

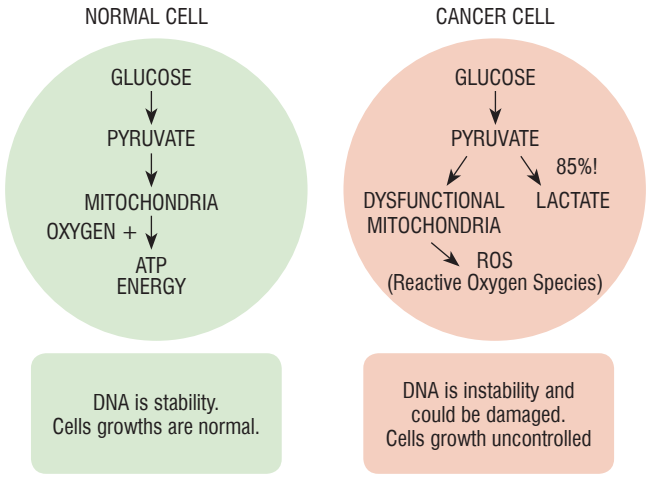


**Figure 2.** The Warburg effect

 

Typical adult diseases is AML. The reasons for acute leukemia causes are unknown. The studies showed that viruses, ionizing radiation, smoking or solvents have been implicated [22]. One of the possibilitation is insulin therapy which causes leukemia directly through growth-promotion effects on normal and cancer cells [23]. Although unlikely, that the high glucose level and metabolic disorders might trigger leukemia, but they are linked with progressing carcinogenic processes strongly.

Metformin (1,1-dimethylbiguanide hydrochloride) is a widely used drug in treating type 2 diabetes and insulin resistance (Fig. 3) [24]. It reduces gluconeogenesis by signaling adenosine monophosphate activated kinase (AMPK). Finally glucose is uptakes in muscle cells, which results in lower glucose and insulin levels. In addition, metformin is a cheap drug and not very harmful or causes minimal side effects [25]. Lowering insulin and IGF-1 levels could be important to stimulate cancer growth [26]. In the last decade, it has been realized that metformin may be linked with modification of cancer cells and their metabolic processes. Nowadays we observe that scientists search potential and multiple roles of metformin in prevention and treatment of carcinogenic processes.

The aim of the study was to compare the impact of normoglycemia and hyperglycemia with doses of metformin on vivacity and proliferation of cancer cells line. We growed cells of normal human dermal fibroblasts (NHDF), human breast cancer (MCF-7 and doxorubicin-resistant MCF-7/DX), human lung adenocarcinoma (A549), acute human lymphoblastic leukemia CCRF/CEM, monocytic human leukemia THP-1 and analysed the potential cytotoxic or therapeutic effect of metformin.

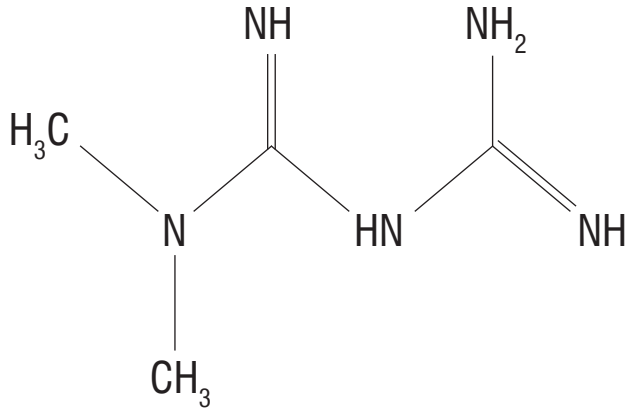


**Figure 3.** Chemical structures of metformin

## Material and methods

*Cell lines*

In study we used 5 different cell lines: normal human dermal fibroblasts NHDF, human acute lymphoblastic leukemia acute line CCRF/CEM (ATCC: CCL-119; suspension cell line), monocytic human leukemia cell line THP-1 (cell suspension line), human lung adenocarcinoma cell line A549 (ATCC: CCL-185; adherent cell line), breast cancer cell line MCF-7 (adherent cell line) and breast cancer doxorubicin-resistant cell line MCF-7/DX (adherent cell line). NHDF call line was used as a benchmark to compare the effects of added metformin concentrations on normal and cancer cells. All cell lines were obtained from the European Collection of Authentified Cell Cultures (ECACC).

Cells were maintained at 37°C, in an incubator humid atmosphere 5% $CO_2$ and full culture medium. They were passaged in accordance with the generally accepted procedure. To neutralize the influence of trypsin – EDTA adherent cells were suspend in Eagle's Minimum Essential Medium (MEM). Cells in suspension were taken from Falcon tubes and centrifuged under conditions of 1000 g for 10 minutes. Finally they were resuspended in complete medium.

All cells were seeded in 96-well plates, precisely 10 000 cells/per well. The culture medium was used in consistent with recommendation for a given cell type. The CCRF/CEM and THP-1 cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 200 mM L-glutamine, 10,000 units/ml penicillin and 10 mg/ml streptomycin. For A549, MCF-7 and MCF-7/DX cell lines was used minimal Eagle Medium (MEM) with supplements as above. Human dermal fibroblast cells were prepared in DMEM medium supplemented with 10% bovine serum.

*Methods*

To investigate whether concentrations of glucose and metformin could affect on proliferation of cancer cells, we treated them with increasing intensity of glucose solution – 40 mM, 100 mM, 150 mM and 300 mM which match for level of blood glucose healthy or diabetic patients. These values correspond to the hypoglycemia (40 mM), normoglycemia (100 mM) and hyperglycemia (150 mM and 300 mM), which occur in diabetes. Metformin is a widely used drug in this disease so we used it in volume 10 ul per well concentrations: 5 mM, 10 mM, 20 mM and 30 mM. Molecule of this drug was synthesized at the Department of Drugs Form Technology of the Faculty of Pharmacy Wroclaw Medical University. Glucose and metformin solutions were prepared in controlled scientific and technical habitat and with appropriate equipment. Cell lines were incubated for 24, 48, 72 and 96 hours and then harvested. Experiment was in 3 independent repetitions for each concentration of mentioned solutions. The MTT assay was according to the protocol [27].

*MTT assay*

To measure the metformin effect on the metabolic activity of cells we choose MTT assay – the most widely used method to analyze cell proliferation and viability. This test measures the reduction of a yellow tetrazolium salt (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)) into insoluble formazan by the mitochondrial enzymes [28]. Only when the membrane is permanently damaged and continuity this structure is lost (what is the consequence of cell destroyed) the dye penetrates into their interiors, enzymes are activated and

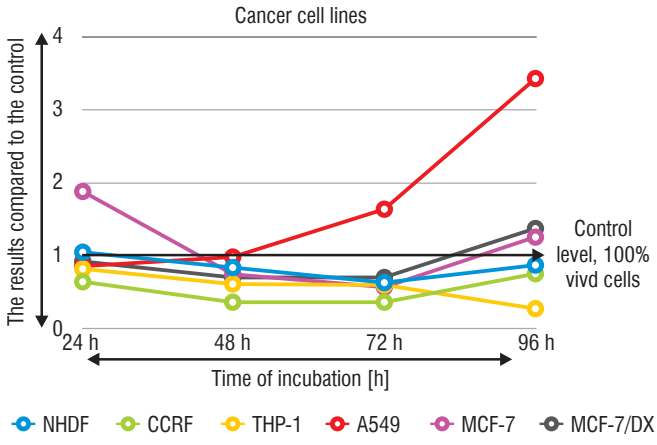


**Figure 4.** Impact of time incubation on level of vivid cells (cell lines signed in color) compared to the control level (1.0–100%). Results for concentration glucose 100 mM and metformin 10 mM

formazan is produced [29]. In the next step insoluble crystals are dissolved by DMSO or isopropanol and the intensity of color is measured. The amount of it produced is proportional to the number of dead cells. Through this assay it is possible to confirm the cytotoxic effect of the tested compound, when more than 30% of the cell population are lifeless. The final colored product is measured quantitatively – spectrophotometric measurement at a wavelength of 570 nm using a Varioskan LUX.

*Statistical analysis*

Statistical significance of the results was calculated using program STATISTICA Enterprise QC. As statistically significant we considered reported value $p < 0.05$.

## Results

The overall study consisted of 6 cell lines: NHDF, CCRF/CEM, THP-1, A549, MCF-7 and MCF-7/DX. They were grown in different concentrations of glucose and metformin. At first we evaluated correlation between the time of incubation and results. The incubation of 24 h and 48 h showed favorable results than 72 h and 96 h (Fig. 4). The results from these incubation intervals were not assessed in this publication because of statistically insignificant results after 3 replications.

The Normoglycemic environment (glucose level about 100 mM) had a favorable effect on the proliferation of all cells in the experiment. It provided them the optimal niche for growth (Fig. 5; 24 h time incubation for all cell lines). At the rising glucose concentrations until to 300 mM we observed an increasing percentage of dead cells.

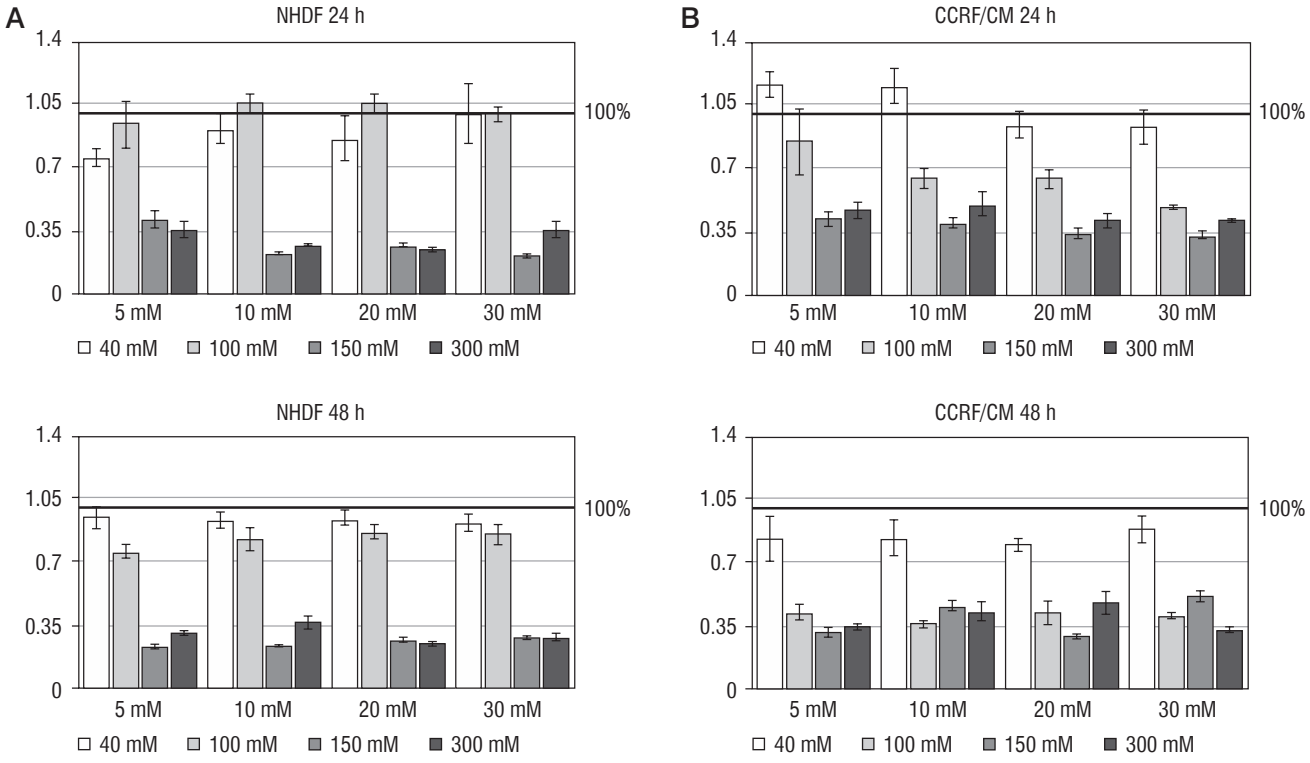


**Figure 5.** Effect concentrations of glucose (axis horizontal) and metformin (signed in color in legend) on the living cells in 24 h and 48 h incubation. The results were compared to the control level. Level of 1.0 indicated 100% of vivid cells

 

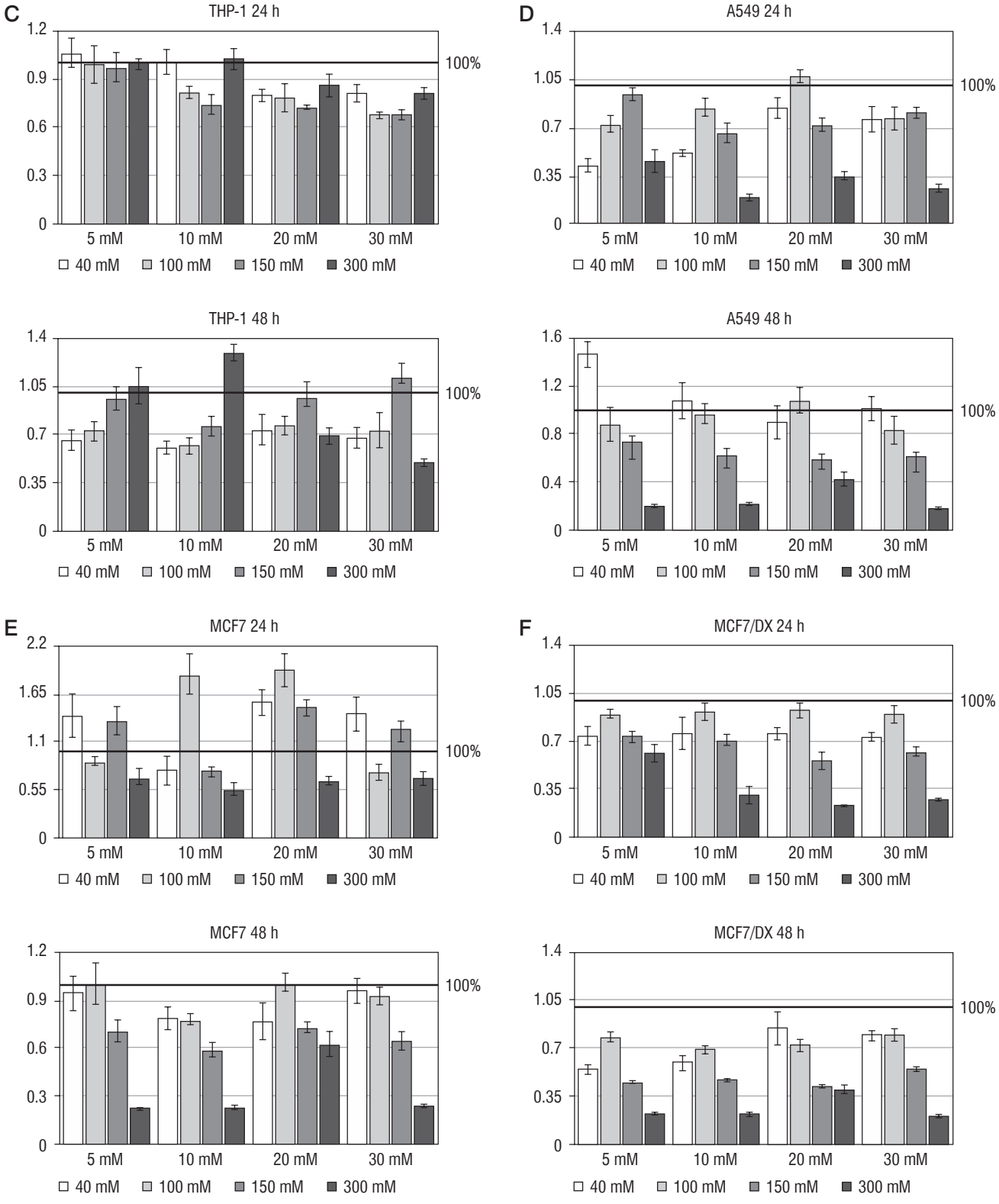


**Figure 5.** Effect concentrations of glucose (axis horizontal) and metformin (signed in color in legend) on the living cells in 24 h and 48 h incubation. The results were compared to the control level. Level of 1.0 indicated 100% of vivid cells (continued)

According to the study conducted on two different cell lines: model of normal cells NHDF and cancer population. After 24 h incubation normal human dermal fibroblasts (NHDF) in hypoglycemic and normoglycemic environments were proliferated and their growth was softly accelerated after we added metformin. In concentration 10 mM population increased about 5.6% compared to control level without metformin. It means that the population of vivid cells was 105.6% in relation 100% at the beginning (before adding metformin). For 20 mM it was growth on the level 4.6% mM. For 30 mM metformin concentration we observed stopping proliferation. Glucose concentration on level 150 mM and 300 mM led to strong dropping the population of vivid cells. After 24 h and 48 h we noticed on average only 26.7% of them as against 100% at the start (Fig. 5; NHDF 24 h, 48 h).

After 24 h incubation of tested metformin with acute lymphoblastic leukemia cells (CCRF/CEM), we observed that all concentrations caused an decrease in cells activity compared to the control. At first we analyzed glucose level 100 mM. Metformin exerted a strong effect, in dose 5 mM population of living cells was about 84.5%, in 10 mM 64.9%, in 20 mM 64.2% and in 30 mM 48.8%. In this case, the reference point was start of incubation, when 100% cells were alive. We confirmed the cytotoxic effect- because more than 30% of cells were dead. During longer incubation – 48 h the influence of metformin was softly and we did not notice results as good as in 24 h time. (Fig. 5; CCRF/CEM 24 h, 48 h; Fig. 5; CCRF/CEM 24 h, 48 h).

Regarding the effectiveness of metformin after adding it to monocytic human leukemia (THP-1) we stated that the percentage of dead cells were strengthened after 48 h compared to 24 h incubation. These results are clearly demonstrated in Fig. 5. For lines THP-1. For example, in environment of 30 mM metformin and 100 mM of glucose only 67.4% of cells were still alive (32.6% has been dead after 24 h). Similarly after 48 h in 30 mM of metformin and 300 mM of glucose we had 50.1% vivid cells compared to control level without metformin (100% vivid).

For breast cancer cells (MCF-7) we noticed that 48 h time incubation is more effective than 24 h in terms of decreasing cancer cells population (Fig. 5; MCF-7). Whereas for breast cancer doxorubicin-resistant cells (MCF-7/DX) the number of vivid ones decreased in 24 h and 48 h time incubation in the same way. We analyzed concentration 10 mM of metformin: after 24 h in habitat 100 mM glucose we measured 92,3% vivid cells in the population and in 150 mM accordingly 70.4%. To compare after 48 h in the same conditions (10 mM metformin) the number of alive cells was 69.6% (100 mM glucose), 47.7% (150 mM glucose). The longer time of incubation had better cytotoxic effect in this case (Fig. 5; MCF-7, MCF-7/DX).

Human lung adenocarcinoma cell line A549 showed favorable results after 48 h from added metformin than 24 h. We analyzed cases of 150 mM glucose with metformin doses equal to 5 mM, 10 mM and 20 mM. After 24 h the number of active cells was: for metformin 5 mM – 94.5%, 10 mM – 65.4% and 20 mM – 71.8%. The population was decreasing, which means that we significantly reduced the number of cancer cells. After 48 h we had for metformin 5 mM – 74.1%, 10 mM – 62% and 20 mM – 60% vivid ones compared to beginning, where it was 100% (Fig. 5; A549).

## Discussion

In our study we try to determine impact metformin and hyperglycemia on cancer cells. We also compared these effects on normal human dermal fibroblasts to confirm that metformin is safe on body cells. Metformin is a well-known natural compound extracted from the plant French lilac. We know mechanisms and action of metformin therapy in patients with hyperglycemia. It reduces glucose levels through activation of the AMP-activated protein kinase (AMPK) pathway and inhibition of hepatic gluconeogenesis [30]. At the molecular level, metformin inhibits the mitochondrial respiratory chain in the liver, leading to activation of AMPK, enhancing insulin sensitivity (via effects on fat metabolism) and lowering cAMP, thus reducing the expression of gluconeogenic enzymes [31]. In cancer it is important mechanism because metformin-activates AMPK activity in tumor cells has a direct impact of decreasing protein and lipid synthesis (via dysregulation of the mTORC1 signaling pathway). It is the reason of their lowering growth and proliferation [30, 32].

From several studies, it is known that metformin improves the prognosis among others in patients with breast cancer and diabetes. Overall, the results suggest in patients with HER2-positive, HR-positive breast cancer and diabetes mellitus that treatment with metformin may improve the survival outcomes associated with diabetes mellitus and taking insulin [33–35].

In our study we noticed that metformin inhibited the growth, proliferation and multiplying potential of the breast cancer cells line. The populations of them are decreasing and the same results were confirmed by other researchers [36, 37]. It is caused by cellular mechanisms that are characteristic of neoplastic processes which can be weakened by blocking the pathways of excessive proliferation [38, 39]. This means that our results are in line with others, and metformin has proven an effect on cancer cells.

Among many factors important to our results was also the selection of the concentration of substances. A glucose level of 300 mM was toxic on every of the cell populations. It is a fact that in this environment any cell can not survive. Another important factor which indirectly influences the cellular response are oncogenic mutations, typical of cancer cells.

Fortunately, the potential of metformin in treatment of hypoglycemia and in cancer diseases is still a subject of discussions and research.

According to the information cumulation in the EU Clinical Trials Register and on the website clinicaltrials.gov the number of completed or not yet clinical trials for metformin and cancer is over 235. The authors suggest that promising results may be focused in nondiabetic patients also. Many tests are still ongoing. Sufficient data from studies on cancer cell lines and animal models suggest that metformin exerts its antineoplastic effect when is used in mix with other agents, especially natural-derived and anticancer drugs [40–42]. That's why it's important to published information on the outcomes of experiments or treatment in groups of patient who takes metformin and anti-cancer drugs in one time. Results of our study responds to this need and provides a new look on world therapeutic models in cancer.

 

We suggest that further research may focus on correlation between time and doses of metformin to contain the most positive therapeutic effects. In this case, it is important to design and select tests to understand the mechanisms that appear in cells. We consider the next step of this study – impact of combination metformin and cytotoxic drugs used in cancer treatment.

## Conclusions

In conclusions, the metformin has shown that as the first-line anti-diabetic drug could be a new chance to reduce mechanisms of cancer growth. For all tested cancer lines was observed positive impact of metformin and lowering proliferation of cells. Our study in preliminary way noticed promising results for monocytic human leukemia cell line (THP-1), human acute lymphoblastic leukemia acute line (CCRF/CEM), human lung adenocarcinoma cell line (A549), breast cancer cell line (MCF-7) and breast cancer doxorubicin-resistant cell line (MCF-7/DX) like other researchers. According to our results metformin is a promising molecule for further research in the context of the antineoplastic activity. It will be important to involve and recognize details of these dependencies. We expect that metformin will join to the group of drugs with unique mechanisms of two-way action. It could be a new field of anticancer drugs.

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