In vitro assessment of cytotoxic, apoptotic and genotoxic effects of metformin

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ABSTRACT

Background and Aims: Recent studies have shown the anticancer properties of metformin, which is widely used in diabetes mellitus. The possible mechanisms of anticancer effects of metformin have not been fully elucidated. We aimed to investigate the cytotoxic, genotoxic, and apoptotic effects of metformin in HepG2 and HeLa cells.

Methods: The cytotoxicity, genotoxicity, and apoptotic effects were determined by MTT method, Comet assay, and FACS assay, respectively.

Results: Metformin significantly decreased cell viability above 4 and 32 mM in HepG2 and HeLa cells, respectively, for 48 h. The IC50 values were 57.3 mM (HepG2) and 76.9 mM (HeLa). Metformin (5-1000 µM) alone did not increase DNA damage in all cells. It did not change oxidative DNA damage in HepG2 cells but induced oxidative DNA damage in HeLa cells. HepG2 cells treated with only 32 mM metformin revealed 10% apoptosis. G0/G1 phase accumulation was statistically higher in the cells treated with 4, 8, and 64 mM metformin (91%, 99%, and 97% respectively) than in (-) control (80%). HeLa cells revealed apoptosis of 30%, 39%, 27% at 4, 32, and 64 mM concentrations, respectively. The results implicate that the inhibition of HepG2 cell viability may be due to the arrest of cell cycle in G0/G1 phase and apoptosis, whereas apoptotic response is mainly responsible for the cytotoxicity of metformin in HeLa cells.

Conclusion: Metformin may not induce DNA damage at non-cytotoxic high doses and lead to apoptosis, even if compatible with previous data. This study provides important information that metformin may play an essential role in apoptosis and cell cycle progression in carcinoma cell lines, which can explain the anticancer effects of metformin, but further studies are needed to support these results.

Keywords: Metformin, genotoxicity, apoptosis, HepG2 cells, HeLa cells

INTRODUCTION

Despite advancements in diagnosis and medical care, cancer remains as the leading reason for death worldwide. While the most common cancers in males are prostate, lung and bronchus and colorectal; the most common cancers in females are breast, lung and bronchus and colorectal (Siegel, Miller & Jemal, 2019). Antidiabetic drugs are known to influence cancer progression, as high glucose level is a risk factor for both cancer and diabetes. Metformin (1,1-dimethylbiguanide) is a product of French lilac (Galega officinalis L.). It is an oral biguanide functioning as a hypoglycemic agent (Kamarudin, Sarker, Zhou, & Parhar, 2019). This drug lowering the blood glucose level is widely used for the treatment of type 2 diabetes. It is responsible for the activation of the energy sensor AMP-activated protein kinase (AMPK), and it has been associated with the inhibition of glucose production in primary hepatocytes (Kim et al., 2019). It is well-described in the literature that metformin suppresses
cancer by inhibiting the transdifferentiative and hyperproli-
ferative processes with anti-angiogenesis, radio-chemo-
sensitizer and anti-metabolic effects (Salani, Del Rio, Marinii, Sam-
bucetti, Cordera, & Maggi, 2014; Leone, Di Gennaro, Bruzzese, Au-
vallone, & Budillon, 2014; Jalving et al., 2010).

Cell checkpoints and apoptosis, which are important molecu-
lar pathways in anticancer effect, are well known for playing a vital
role in regulating growth, development, and immune
response, therefore removing cancerous cells. The avoidance of
apoptosis is an important hallmark of cancer; thus, the abil-
ity to induce apoptosis and suppress cell growth is a promising
therapeutic approach in cancer research. Although chemothera-
peticants can be used to achieve this, their use is associated
with high levels of toxicity. In several studies, the anticancer
effect of metformin has been the focus of attention. Usefulness
of metformin in reducing the risk for diabetes related can-
cers as well as breast cancer, cervix cancer, pancreas cancer,
prostate cancer and colorectal cancer have been investigated
in numerous studies (Kamarudin, Sarker, Zhou, & Parhar, 2019;
Kim et al., 2019; Lopez-Bonet, et al., 2019; Donadon, Balbi, Casa-
rin, Vario, & Alberti, 2008; Giovannucci & Michaud, 2007; Dom-
browski, Mathieu, & Evert, 2006).

The antitumor effects of metformin on Hela and HepG2 cells
and the mechanisms underlying apoptosis, and their cell cycle
regulation remain elusive. There are conflicting results regard-
ing the effects of metformin on genotoxicity. It seems that
the mechanistic studies on anticancer effects are required to
evaluate how metformin affects the apoptotic pathways and

genotoxicity together. In this study, we aimed to investigate the
cytotoxic, genotoxic and apoptotic effects of metformin
on human hepatoma cells (HepG2) and cervical cancer cells
(HeLa).

MATERIALS AND METHODS

Chemicals

Metformin was obtained from Sigma Aldrich (St. Louis, USA).
Also 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-
mine (MTT), dimethyl sulfoxide (DMSO), Dulbecco’s modified
Eagle’s medium (DMEM), ethanol, ethidium bromide (EtBr),
ethylenediamine tetra acetic acid disodium salt dihydrate
(Na2-EDTA), fetal bovine serum (FBS), hydrogen peroxide (35%)
(H2O2), low melting point agarose (LMA), methanol, N-lauroyl
ethylenediamine tetra acetic acid disodium salt dihydrate
(Na2-EDTA), fetal bovine serum (FBS), hydrogen peroxide (35%)
(H2O2), low melting point agarose (LMA), methanol, N-lauroyl

analyses of genotoxicity (Comet assay)

HeLa and HepG2 cells (4x10⁶ cells/well) were plated in 12-
well plates. The cells were incubated with the non-cytotoxic
doses of metformin (5-1000 μM) for 48 h. The Trypan Blue dye
exclusion test was applied to determine the viable cells and the cell viability was above 80% in the comet method. After the pretreatment of metformin for 48 h, oxidative damage was induced by replacing the medium with PBS containing 50 µM H₂O₂ and then incubating for 5 min on ice to assess the effect against oxidative DNA damage. At the end of the incubation period to examine the effects of DNA damage, the cells were trypsinized with trypsin-EDTA and washed with PBS. The cell suspension (50 µL) mixed with 100 µL 0.5% LMPA melted at 37°C±0.5°C was spread on agar-coated slides previously immersed in 1% NMPA solution and the coverslip was closed. Then they were placed into the electrophoresis solution for 20 min, at 25 V and 300 mA. After electrophoresis, the slides were stained with 50 µL ethidium bromide (20 µg/mL). 100 random cell analyzes were performed using fluorescence microscope (Leica) for each sample by using the comet computerized imaging system (Comet Analysis Software, version 3.0 Kinetic Imaging). The medium was used as a negative control and 50 µM H₂O₂ was used as a positive control.

**Statistical analysis**
All of the experiments were performed three or four times. The data are presented as means±standard deviation. Whether the data was normally distributed or not, it was evaluated using the Kolmogorov-Smirnov test and histograms. The differences between the groups were determined by one-way analysis of variance (ANOVA), LSD test. In all analyzes, statistical significance level was accepted as p<0.05.

**RESULTS**

**Effects of metformin on cell viability**
We used MTT assay to determine the cytotoxic effect of metformin on HepG2 and HeLa cells treated with a wide range doses of metformin.

It was observed that metformin did not have a significant cytotoxic effect in HepG2 cells at the concentration range of 0.5-2 mM after 48 h incubation when compared to negative control (PBS), but it produced a statistically significant decrease in cell viability at concentrations of 4 mM and above in a dose-dependent manner (p<0.05) (Figure 1). IC₅₀ was found to be 57.3 mM in HepG2 cells exposed to metformin for 48 h.

Metformin did not produce a significant cytotoxic effect in HeLa cells at the concentration range of 0.5-16 mM after 48 h incubation when compared to negative control (PBS), but it significantly reduced cell viability at 32 mM and 64 mM concentrations in a dose-dependent manner (p<0.05) (Figure 2). IC₅₀ value was found to be 76.9 mM in HeLa cells exposed to metformin for 48 h.

**Effects of metformin on cell cycle and apoptosis**
The changes in cell cycle progression were evaluated using the flow cytometry method to determine the growth inhibition in the metformin treated HepG2 cells.

In cell cycle analysis with HepG2 cells, 10% apoptosis was observed in the cell population at a concentration of only 32 mM (Figure 3). The statistically significant increases in the accumulation of G0/G1 phase at 4, 8, and 64 mM concentrations (91%,...
In the cell cycle analysis with HeLa cells, 30%, 39%, and 27% apoptosis were observed at 4, 32, and 64 mM concentrations of metformin, respectively. A statistical increase in the accumulation of G0/G1 phase was found at only 2 mM of metformin (88%) when compared to (-) control (78%), but proliferation occurred at this concentration. The accumulation in S phase at 16 and 32 mM concentrations (47% and 26%, respectively) were found to be statistically higher than the (-) control (22%). On the other hand, the accumulation in the G2/M phase was only observed at the concentration of 0.5 mM metformin (18%) (Figure 4).

Effects of metformin on apoptosis and cell cycle in HeLa cells. Values were given as the mean±standard deviation. *p<0.05, statistically different from negative control (PBS). #p<0.05, statistically different from positive control (50 µM H2O2).

Figure 4. Effect of metformin on apoptosis and cell cycle in HeLa cells. Values were given as the mean±standard deviation. *p<0.05, statistically different from negative control (PBS).

**DISCUSSION**

Day by day cancer has become one of the most serious mortalities and morbidity causes in the world. Therefore, cancer treatment has begun to come to the fore. Current chemotherapeutic drugs cannot effectively control tumor progression. Resistance to drug inhibiting therapy is common, which increases the trend towards new approaches. New therapy initiatives are needed to increase the overall survival rate of cancer patients. Recently, some studies have provided preliminary evidence that metformin can reduce the risk of cancer and improve prognosis in diabetic patients. For this purpose, *in vitro* and *in vivo* studies have become widespread in various types of cancer related to metformin. The results are sometimes contradictory in the studies conducted. Some studies show that metformin has an inhibitory effect on the growth of various human cancer cells. However, data on whether these growth-inhibiting effects alone cause arrest of cell apoptosis or alter the cell cycle are not well known. Although some of these studies suggest several possible mechanisms, the detailed molecular basis is largely unknown. On the other hand, many studies have shown that metformin inhibits cell proliferation by causing apoptosis and can lead the cell to death (Will, Pa-
Our data shows that the IC$_{50}$ value was determined to be 57.3 mM for HepG2 cells at 48 h exposure. It was observed that metformin did not produce a significant cytotoxic effect in HepG2 cells in the concentration range of 0.5-2 mM, but it caused a statistically significant decrease in cell viability at the concentrations of 4 mM and above in a dose-dependent manner. Research on cell viability in HepG2 cells has also shown that metformin reduces dose-dependent cell viability, in accordance with our study (Zhang et al., 2018; Sun et al., 2016; Cai et al., 2013).

In our study, we found that metformin alone did not induce DNA damage and also did not change oxidative DNA damage in all studied non-cytotoxic concentrations (5-1000 µM) in HepG2 cells (p>0.05). These results suggest that metformin does not bring about DNA damage in HepG2 cells. In a study, metformin was shown to reduce ROS accumulation, DNA damage and mutations in experimental systems containing mitochondrial toxins (Algire et al., 2012). Although metformin causes a decrease in DNA damage, various mechanisms are emphasized, and the decrease in ROS level has been revealed in many studies conducted in different cell lines (Piro, Rabazzo, Renis, & Purello, 2012; Kane et al., 2010; Ouslimani, Peynet, Bonnefont-Rousselot, Therond, Legrand, & Beaudex, 2005).

DNA damage formation, inhibition of transcription and replication, and induction of apoptosis may produce different results that mediate cell death. Increased DNA damage and accumulation of these damages can lead to cell death or pause in different phases of the cell cycle. In the literature, it was observed that metformin induced apoptosis different cell lines including HepG2 cells. After longer exposures (3–14 days), metformin (≥40 µM) induced a dose-and time-dependent increase in the number of apoptotic β-cells (Kefas et al., 2004). It was found that metformin did not induce apoptosis but blocked cell cycle in G0/G1 in human prostate cancer cells (DU145, PC-3 and LNCaP cancer cells). This blockade was accompanied by a strong decrease of cyclin D1 protein level, pRb phosphorylation and an increase in p27kip protein expression (Ben Sahra et al., 2008). Metformin suppressed cell growth and induced apoptosis in a dose-dependent manner in hepatocellular carcinoma cells (Saito et al., 2013). In our study, metformin significantly reduced cell viability above 4 mM in HepG2 cells in a dose-dependent manner (p<0.05). We found that the accumulations in the G0/G1 phase at the concentrations of 4 mM, 8 mM and 64 mM metformin and the accumulations in the S phase statistically increased when compared to the negative control. Metformin caused 10% apoptosis in HepG2 cells at a concentration of 32 mM. It seems that metformin induced significant growth inhibition of HepG2 cells through the induction of G0/G1 phase and S phase cell-cycle arrest (Kefas et al., 2004; Ben Sahra et al., 2008; Saito et al., 2013).

In our results, it was observed that metformin did not have a significant cytotoxic effect in HeLa cells in a range of 0.5-16 mM concentration, but significantly reduced cell viability at 32 mM and 64 mM concentrations in a dose-dependent manner (p<0.05). We also found that metformin alone did not reveal DNA damage at the non-cytotoxic concentrations (5-1000 µM) (p>0.05), however it induced oxidative DNA damage in HeLa cells at all studied doses (p<0.05). In the cell cycle analysis with HeLa cells, 30%, 39%, 27% of apoptosis were observed in the cell population at concentrations of 4 mM, 32 mM and 64 mM, respectively. Few studies relating the effects of metformin on HeLa cells viability have similarly shown that metformin reduces cell viability and induces apoptosis (Xia et al., 2017; Tyszka-Czochara, Konieczny, & Majka, 2017; Tyszka-Czochara, Bukowska-Strakova, & Majka, 2017).

Different IC$_{50}$ metformin levels reported in previous studies appear to be due to differences in cell types, cytotoxicity tests, and treatment times. The effects of metformin on the proliferation of esophageal squamous cell carcinoma cells (ESCC, EC109, and EC9706) treated with different concentrations were investigated for 24 h to 72 h using MTT assay. Cell viability decreased depending on the dose and time, consistent with our study. For 24 h, the significant decrease in cell viability was observed only at 20 mM metformin (about IC$_{50}$). A dramatic suppression in the growth of the EC109 cell lines

Figure 6. Genotoxicity of metformin in HeLa cells. DNA damage expressed as DNA tail intensity (A), DNA tail moment (B), DNA tail migration (C) in HeLa cells. Met= metformin. Values were given as the mean±standard deviation. *p<0.05, statistically different from negative control (PBS). #p<0.05, statistically different from positive control (50 µM H$_2$O$_2$).
was observed after metformin (20 mM) treatment for 72 h (Cai et al., 2015).

Metformin inhibited the proliferation of esophageal carcinoma cell lines (TT, KYSE30 and KYSE70 cells) as shown by WST-8 test, an MTT-like test. Metformin led to a dose-dependent and strong inhibition of cell proliferation. In KYSE70 cells, although 5 mM metformin did not affect the proliferation of cancer cells, treatment with 10 mM metformin inhibited the proliferation of cells. The highest dose (10 mM) of metformin significantly increased the cell proliferation for 24 h (Kobayashi et al., 2013).

Zhang et al. investigated the effects of metformin and curcumin on proliferation, apoptosis, invasion, metastasis and angiogenesis of hepatocellular carcinoma cells in vitro and in vivo. The IC50 values of metformin were 53.72 mM, 23.46 mM, 8.52 mM for 24 h, 48 h and 72h, respectively. In HepG2 cells by CCK-8 assay, 10 mM metformin also significantly increased the apoptotic effects of curcumin about two times in HepG2 cells. Metformin was also found to be involved in down-regulation of MMP2 and MMP9 (well-known proliferation/metastasis proteins) (Zhang et al., 2018).

Yudhani et al. reported that metformin enhanced the anti-proliferative effect of cisplatin in cervical carcinoma cell lines. Treatment of 10 mM metformin showed inhibition of HeLa cell proliferation and IC50 was reported to be 60 mM by MTT assay, which was like our result. Combination of 30 mM metformin and 5 µM cisplatin indicated the strongest anti-proliferative effect on HeLa cells (Yudhani, Pesik, & Indarto, 2016).

Wang et al. investigated the anti-myeloma effects of metformin in myeloma cells (RPMI8226 and U266). Cell viability was assessed with CCK8 cytotoxicity assay. The cell viability decreased with increasing concentrations of metformin and with increasing duration of treatment. The IC50 of metformin was reported to be 20.2 mM and 17.9 mM in RPMI8226 cells and U266 cells, respectively, for 48 h (Wang et al., 2018).

Xia et al. reported that metformin inhibited cervical carcinoma cells (HeLa and SiHa) proliferation, cervical cancer xenograft growth, expression of PCNA, p-Pi3K and p-Akt. It induced apoptosis and caused cancer cell cycle arrest and also upregulated the expression of DDR-1 and p53. Metformin also regulated the mRNA and protein expression of MICA and HSP70 on the surface of human cervical cancer cells via the Pi3K/Akt pathway, enhancing NK cell cytotoxicity. Metformin was reported to inhibit cervical carcinoma cells proliferation in a time-dependent manner for 24 h, 48 h and 72 h by using CCK-8 test, a MTT-like test. The IC50 values of metformin for 72 h were 25.13 mM and 19.43 mM in HeLa and SiHa cells, respectively, which were about three times lower than our results. The apoptosis ratio of the cells treated with 20 mM metformin for 48 h were found to be increased from 11.61 to 39.04% and 5.69 to 12.31% for HeLa cells and SiHa cells, respectively. The percentage of G0/G1 phase increased and the percentage of S phase cells decreased when HeLa and SiHa cells treated with 20 mM metformin for 48 h (Xia, Liu, He, Cai, & Chen, 2020).

Studies on the cell cycle analysis in its anticancer effect has shown that other different pathways may also be responsible for this effect. It was reported that metformin was effective in blocking the cell cycle in G0/G1, but not in the induction of apoptosis in human prostate cancer cells (DU145, PC-3 and LNCaP) treated with metformin (1 and 5 mM) (38-54% decrease in cell viability, in a dose-dependent manner). It inhibited cyclin D1 expression and pRb phosphorylation independently of the sensor pathway AMPK (Ben Sahra et al., 2008). It was observed that metformin (10 mM) blocked the cell cycle in G0/G1 for 24 h. This blockade was accompanied by a strong decrease of G1 cyclins, especially cyclin D1, as well as decreases in cyclin-dependent kinase (Cdk)4, Cdk6 and phosphorylated retinoblastoma protein (Rb). In addition, the expression of miRNAs was markedly altered with the treatment of metformin in vitro. Metformin inhibited the growth of esophageal carcinoma cells, and this inhibition may have involved reductions in cyclin D1, Cdk4 and Cdk6 (Kobayashi et al., 2013).

Wang et al. revealed the accumulation of cells in the G0/G1 phase, while the fraction of cells in the S phase decreased in the cells treated with 5 mM and 20 mM metformin for 24 h. They concluded that metformin effectively inhibited the cell proliferation, which is associated with the induction of autophagy and G0/G1 cell cycle arrest, but not apoptosis. They suggested that the molecular mechanism of metformin is also involved in AMPK activation (Wang et al., 2018). Kheirandish, Mahboobi, Hiyazdanparast, Kamal & Kamal (2018) stated that AMPK-dependent (decreases in folate level, c-Myc and NF-κB; increases in p53 phosphorylation ) and AMPK-independent (decreases in ROS and cyclin D1; increases in mTORC1) pathways may be responsible for the anticancer effects of metformin. Metformin also decreases both pro-inflammatory cytokines and improves the immune response to cancer cells.

Cell cycle progression, involved in cell division and replication, can be restricted under conditions such as DNA replication error, nutrient depletion, DNA damage and low growth factor. Cell cycle regulatory functions are usually impaired in cancer cells. Therefore, the improvement in cell cycle progression might be an effective strategy for the treatment of carcinomas. Cyclin, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs) in the G1 phase interact with each other to regulate cell-cycle transitions and cell division. It was reported that the anti-proliferative action of metformin on esophageal squamous carcinoma cell lines (ESCC) was partially mediated by AMPK. Moreover, it was observed that metformin induced G0/G1 phase arrest accompanied by the up-regulation of p27kip1 and p27kip1. The results indicate that metformin may inhibit carcinoma cell growth via causing cell cycle arrest and delaying tumorigenesis (Cai et al., 2015).

In some reports it has been concluded that metformin might induce DNA damage, however some of reports indicated no genotoxic effects (Janjetovic et al., 2011; Attia, Helal, & Alhaider, 2009; Onaran, Guven, Ozdas, Kanigur, & Vehid, 2006). In one study, metformin was found to increase DNA damage at the dose of 114.4 µg/mL (882, 6 µM) in Chinese hamster ovary cells for 24 h using comet assay (Amador, Longo, Lacava, Dórea, &
In our study, metformin was found to be responsible for the changes in the cell cycle arrest, with differences between HepG2 and HeLa cells. In HepG2 cells, the G0/G1 phase accumulation may be mainly responsible for the regulation of cell proliferation. Moreover, G2/M phase accumulation was also shown, being greater at lower doses (33% and 22% for 0.5 and 1 mM, respectively). It is assumed that the G0/G1 phase and the G2/M phase play a role against cytotoxicity and maintaining cell viability, respectively, in HepG2 cells, whereas in HeLa cells, apoptosis rather than the G0/G1 and G2/M phases seems to be primarily responsible for the effect of metformin on cell proliferation.

CONCLUSION

In recent studies, metformin has been shown to be a promising anticancer drug. The number of studies on combinations and effects of metformin with various anticancer drugs is increasing. Our study showed that metformin decreased cell viability in a dose-dependent manner and it did not induce DNA damage in HepG2 and HeLa cells at non-cytotoxic doses, but did lead to a significant change in apoptosis at high doses. In conclusion, we suggest that metformin may not cause DNA damage but lead to apoptosis. This study provides important information that metformin may play an essential role in the apoptosis and cell cycle progression in carcinoma cell lines, which can explain the anticancer effect of metformin. Although there are various studies in the literature on which pathways metformin produce these effects, it is not certain yet. For this reason, further studies are needed to clarify these pathways.

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