Combination of high dose of metformin and low dose of cisplatin increases apoptosis in cervical cancer cells line

Ratih Dewi Yudhani,1 Muthmainah,2 Dono Indarto3

ABSTRACT

Background: Cervical cancer is one of the most common gynecological malignancies worldwide including Indonesia which leads to high morbidity and mortality rate. Cisplatin is the first line chemotherapy for treating cervical cancer, but its use is limited because of serious side effects. Our previous study showed that metformin enhanced the anti-proliferative effect of cisplatin in cervical cancer cell line (HeLa). This study aimed to investigate whether or not HeLa cells treated with metformin and cisplatin enhances cell apoptosis.

Methods: HeLa cells were cultured with DMEM medium for 24 hours and then treated with either 60 mM metformin, 40 µM cisplatin, a combination of 30 mM metformin and 6.25 µM cisplatin or 7.5 mM metformin and 12.5 µM cisplatin for 48 hours. Apoptotic and necrotic cells were measured using flow cytometer. Data were analyzed using Kruskal-Wallis test, and the significant value was set up at p < 0.05.

Results: As compared to control group, HeLa cells treated with 30 mM metformin and 6.25 µM cisplatin had higher apoptosis than cells treated with 7.5 mM metformin and 12.5 µM cisplatin. However, apoptosis induction by metformin and cisplatin combination was much lower than that of 60 mM metformin or 40 µM cisplatin alone.

Conclusion: In conclusion, administration of 30 mM metformin and 6.25 µM cisplatin induces higher apoptosis than that of 7.5 mM metformin and 12.5 µM cisplatin in HeLa cells.

Keywords: Metformin, Cisplatin, Apoptosis, HeLa cells

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INTRODUCTION

Based on GLOBOSCAN data published by the International Agency for Research into Cancer (IARC) in 2012, cervical cancer is the third most common malignancy in women worldwide and the fourth leading cause of cancer-related death in women. About 70% of all cases of cervical cancer are found in developing countries with low economic income.1-3 In 2013, cervical cancer is still a health problem in Indonesia because its prevalence is 0.8/1,000 population (98,692 cases) and this disease becomes the highest malignancy.1,4

The current standard therapy for cervical cancer is surgery, radiation, chemotherapy and targeted therapy. Surgery is only an effective therapy in early-stage cancer whereas radiation and chemotherapy can be administered in all stages of cancer.5 However, the use of radiation and chemotherapy for a long time often cause severe side effects.6 Chemotherapy is a cytotoxic agent that kills not only dividing cancer cells but also normal cells with high proliferation rate such as hair, mucosal layer, skin and bone marrow.7 Cisplatin, for instance, is the first-line chemotherapy agent for cervical cancer, but has unwanted side effects such as neurotoxicity, nephrotoxicity, and bone marrow suppression.8-10

Current research is directed to the development of co-chemotherapy to overcome the side effects of chemotherapy. Co-chemotherapy is combining a compound with the standard chemotherapy agent as a new strategy for cancer therapy, which aims to improve the effectiveness of chemotherapy agents and to minimize their side effects.10 Metformin (N1,N1-dimethyl biguanide) is widely used as a type II diabetic therapy.11 According to Evans et al. in 2005, metformin has a potential anticancer effect.12 After several years later, metformin has been proved to inhibit cancer cell growth in vitro and in vivo in mouses model with cancer. In culture cells, metformin inhibits the proliferation of certain types of cancer cells, such as breast, prostate, colon, endometrial, ovarian and glioma cells.13

To date, there have been several studies examining the effectiveness of the potential cytotoxicity of metformin combinations with several chemotherapeutic agents. Hirsch et al. reported that a combination of metformin and doxorubicin kill cancer cells, including cancer stem cells in certain types of breast cancer cell lines. In addition, studies with model mice showed that the combination of metformin with some standard chemotherapy (doxorubicin, paclitaxel, and carboplatin) was effective in
inhibiting growth and preventing the recurrence of some cancers including breast, prostate and lung cancer.\textsuperscript{14} This indicates that metformin can work synergistically with some standard chemotherapy agents.\textsuperscript{15} In addition, Rattan et al. (2011) reported that metformin increases the cisplatin cytotoxicity capacity to suppress the growth of ovarian cancer cells by up to 90%.\textsuperscript{16}

Our previous study also showed that in particular combinational dosage, metformin enhanced the anti-proliferative effect of cisplatin in cervical cancer cell line (HeLa). The combination of 30 mM metformin and 6.25 µM cisplatin based on combination index showed synergistic antiproliferative effect. Meanwhile, a combination of 7.5 mM metformin and 12.5 μM cisplatin showed antagonist antiproliferative effect.\textsuperscript{17} This study aimed to investigate whether or not HeLa cells treated with metformin and cisplatin enhances cell apoptosis.

**MATERIALS AND METHODS**

**Cell Line and Drugs**

Metformin was obtained from Sigma-Aldrich while Cisplatin was from Kalbe. HeLa cell line was supplied by Parasitology Laboratory, Medical Faculty, Gadjah Mada University, Yogyakarta. HeLa cells were cultured in six-well plates (2x10\(^5\) cells per well) using DMEM (Gibco\textsuperscript{\textregistered}) supplemented with 10% fetal bovine serum (FBS) (Gibco\textsuperscript{\textregistered}), fungizone 0.5% (Gibco\textsuperscript{\textregistered}), and penicillin-streptomycin 2% (Penstrep; Gibco\textsuperscript{\textregistered}). After 24 hours incubation and 80% confluency was achieved, cells were harvested with conventional trypsinization procedure using 0.25% Trypsin-EDTA (Gibco\textsuperscript{\textregistered}). In this fashion, cells were ready to be used for the experiment.

**Treatment and Apoptosis Analysis by Flow cytometry**

Before treatment, culture media was removed, and cells were washed with Phosphate Buffer Saline (PBS) (Invitrogen\textsuperscript{\textregistered}). Cells were then treated with either 40 µM cisplatin (IC\textsubscript{50}), 60mM metformin (IC\textsubscript{50}), 30 mM metformin and 6.25 µM cisplatin or 7.5 mM metformin and 12.5 µM cisplatin followed by incubation in 5% CO\textsubscript{2} at 37°C for 48 hours. Cells were stained with Annexin V Fluos (Roche\textsuperscript{\textregistered}), and apoptosis analysis was done by flow cytometry using FACS Calibur (Becton-Dickinson).

**Statistical Analysis**

Data were analyzed using Kruskal-Wallis Test with SPSS for Windows Release 22.0. The significance level was set at \(P< 0.05\).

**RESULTS**

Figure 1 shows that HeLa cells in control group receiving no treatment stayed confluence and viable with normal morphology (a). In contrast, the positive control group treated with either 40 µM cisplatin or 60 mM metformin (b and c) had lower cell confluency because a large number of cells were shrunk and dead.

As compared to control group (a), administration of cisplatin in combination with metformin (d and e) increased cell death. However, the number of viable cells with normal morphology in both treatment groups were higher than those in the positive control group (b and c). It is likely because the dose of cisplatin and metformin applied was less than their IC\textsubscript{50}.

Apoptosis analysis using Flow cytometry revealed that the number of apoptotic cells was higher after treatment with 30 mM metformin and 6.25 µM cisplatin (10.26 ± 7.72 %) as compared to control group (2.72 ± 2.30 %). However, this
difference is not statistically significant (p=0.26). On the other hand, cell apoptosis decreased in the group which is treated with a combination of lower dose metformin (7.5 mM) and higher dose cisplatin (12.5 µM). This combination induced less apoptosis at only 4.73 ± 1.32% as compared to the group with higher dose combination of metformin (30 mM) and a lower dose of cisplatin (6.25 µM). Unfortunately, apoptosis induction by both of these regimes could not surpass the potential of 60 mM metformin or 40 µM cisplatin alone in inducing cell death although statistical analysis did not show any significance on their different properties (p=0.26). The percentage of the apoptotic cell after treatment in each group is described in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Average number of apoptotic cell % ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 mM</td>
<td>2.72 ± 2.30</td>
</tr>
<tr>
<td>Cisplatin IC50</td>
<td>40 µM</td>
<td>30,32 ± 22.54</td>
</tr>
<tr>
<td>Metformin IC50</td>
<td>60 mM</td>
<td>14.80 ± 3.61</td>
</tr>
<tr>
<td>Metformin + Cisplatin</td>
<td>30 mM + 6.25 µM</td>
<td>10.26 ± 7.72</td>
</tr>
<tr>
<td>Metformin + Cisplatin</td>
<td>7.5 mM + 12.5 µM</td>
<td>4.73 ± 1.32</td>
</tr>
</tbody>
</table>

DISCUSSION

Multicellular organism maintains its homeostasis by keeping the balance among cell proliferation, differentiation, and cell death. Disruption in either of these processes can lead to cancer development. Apoptosis and necrosis are two primary mechanisms of cell death. Apoptosis also is known as programmed cell death is crucial to eliminate abnormal cells and control excessive cell proliferation leading to neoplastic transformation.18 In this study, we investigated the potential role of metformin and cisplatin as an apoptosis inducer. The main finding of this study is a combination of metformin and cisplatin tend to enhance apoptosis in HeLa cells, and the rate of apoptosis was increased when a higher dose of metformin and a lower dose of cisplatin was used.

There have been controversial findings in studies evaluating the anticancer effect of metformin/cisplatin co-chemotherapy. Some studies reported synergistic effect while others revealed antagonistic effect. Uehara et al. reported that when combined with cisplatin, metformin has an additive anti-cancer effect in endometrial cancer cell line as shown by reduced cell proliferation and increased Caspase activity. This effect was even more prominent when a higher concentration of metformin was used.19 In addition, a combination of metformin and cisplatin showed a synergistic anticancer effect in lung cancer xenografts mouse model by inhibiting the expression of survivin, matrix metalloproteinase-2 (MMP-2) required for tumor growth and metastasis, vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor receptor-3 (VEGFR-3) which are essential for angiogenesis.20 On the other hand, other studies revealed that instead of potentiating cisplatin-induced apoptosis, metformin antagonized cisplatin effect in MKN-45 gastric cancer cell line. This antagonistic effect was associated with upregulation of survivin, and Akt.21,22 Antagonist effects were also found in experiments using soft tissue, blood, and brain tumor cells.19

One of the possible mechanisms in which metformin increases the pro-apoptotic property of cisplatin is through regulation of the level of expression of survivin and Akt. In breast cancer cell line, this combination can activate AMP-activated Protein Kinase (AMPK). In its phosphorylated form, AMPK inhibits mTOR signaling resulting in down-regulation of survivin, a protein that regulates G1 to S phase transition and acts to inhibit apoptosis.23 Rogalska et al. reported that when given individually, metformin significantly increased apoptosis in ovarian cancer cell line. Increased apoptosis was associated with decreased expression of survivin and Akt.
of BIRC5 gene (baculoviral IAP repeat-containing 5) which encodes survivin. Akt is a protein kinase that plays a significant role in apoptosis and cell survival. Increased expression of Akt leads to apoptosis inhibition. The anti-apoptotic property of survivin and Akt has been associated with chemotherapy resistance in certain cancers.21,23 Our data suggest that combination of metformin and cisplatin tend to increase apoptosis in Hela cancer cell line as compared to control. This pro-apoptotic potential is not as strong as when cisplatin and metformin were administered alone. Considering the fact that the doses we used in the combination were lower than the IC50 used in the positive control groups and the likelihood that anti-cancer effect of metformin is cancer-type specific, further experiments are needed to justify whether the combination of metformin and cisplatin can be better used for cervical cancer therapy.

CONCLUSION
Administration of 30 mM metformin and 6.25 µM cisplatin induces higher apoptosis than that of 7.5 mM metformin and 12.5 µM cisplatin in HeLa cells.

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REFERENCES

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