COMPARISON OF BLADDER CANCER CELLS APOPTOSIS INDUCED BY METFORMIN, CISPLATIN, AND COMBINATION OF METFORMIN-CISPLATIN: IN VITRO STUDY

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ABSTRACT

Objective: *To evaluate the effect of metformin, cisplatin, and their combination on apoptosis of bladder cancer cells.* ***Material & Methods:*** *Urothelial cell lines 5637 were cultured until 80% confluence. Cells were exposed to metformin and cisplatin at certain doses for 24 and 48 hours. Cytotoxicity test was carried out by calculating the viability of bladder cancer cells using the MTT assay until IC_{50} of each drug was obtained. IC_{50} Metformin and Cisplatin obtained from the cytotoxicity test were used to induce apoptosis in bladder cancer cells using TUNEL assay. Additional combination doses of Metformin and cisplatin used to induce apoptosis were ¼ IC_{50} (metformin + cisplatin), ½ IC_{50} (metformin + cisplatin), and IC_{50} (metformin + cisplatin).* ***Results:*** *IC_{50} of metformin was 15 uM while cisplatin was 18 uM with a 48-hour exposure. There was a difference in the mean value of the apoptosis index in all treatment groups compared to control except for the group exposed to IC_{50} metformin and ¼ IC_{50} (metformin + cisplatin).* ***Conclusion:*** *Metformin administration solely is not able to increase bladder cancer cell apoptosis. Conversely, the administration of Cisplatin can significantly increase bladder cancer cell apoptosis. The combination of Metformin and Cisplatin can significantly increase bladder cancer cell apoptosis. The rate of apoptosis in line with an increase dose of the combination of these two drugs.*

Keywords: *Metformin, cisplatin, cytotoxicity, apoptosis.*

ABSTRAK

Tujuan: *Mengetahui efek metformin, cisplatin, dan kombinasi keduanya dalam menginduksi apoptosis sel kaker buli.* ***Bahan & Cara:*** *Sel kanker buli (urothelial cell line 5637) dikultur hingga kepadatan 80%. Sel dipaparkan dengan metformin dan cisplatin dengan dosis tertentu selama 24 dan 48 jam. Uji sitotoksisitas sel dilakukan dengan menghitung viabilitas sel kanker buli menggunakan metode MTT assay. Data IC_{50} metformin dan cisplatin yang didapatkan dari uji sitotoksisitas selanjutnya digunakan untuk uji apoptosis dengan metode TUNEL assay. Tambahan dosis kombinasi yang digunakan dalam uji apoptosis yaitu: ¼ IC_{50} (metformin + cisplatin), ½ IC_{50} (metformin + cisplatin), and IC_{50} (metformin + cisplatin).* ***Hasil:*** *IC_{50} metformin adalah sebesar 15 uM sedangkan IC_{50} cisplatin sebesar 18 uM dengan waktu paparan selama 48 jam. Pada uji apoptosis ditemukan adanya perbedaan nilai rerata index apoptosis pada semua grup perlakuan dibandingkan dengan kontrol kecuali pada grup yang terpapar dengan IC_{50} metformin dan ¼ IC_{50} (metformin + cisplatin).* ***Simpulan:*** *Metformin dosis IC_{50} tidak dapat meningkatkan apoptosis sel kanker buli. Sementara itu, cisplatin dosis IC_{50} dapat meningkatkan apoptosis sel kanker buli secara signifikan. Kombinasi metformin dan cisplatin juga dapat meningkatkan apoptosis sel kanker buli. Peningkatan dosis kombinasi metformin dan cisplatin sejalan dengan besaran apoptosis sel kanker buli.*

Kata Kunci: *Metformin, cisplatin, sitotoksisitas, apoptosis.*

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INTRODUCTION

Bladder cancer is one of the most prevalent cancers in the urinary tract and is the 9th most malignancy of all malignancies in the world.[1] In men, bladder cancer is the 7th most prevalent malignancy with an incidence of 10.1 per 1.000.000 population, while in women it ranks 17th with an incidence of 2.5 per 1.000.000 population.[2,3] About 2/3 of cases of bladder cancer are non-muscle invasive bladder

cancer (NMIBC) and about one-third of them are muscle-invasive bladder cancer (MIBC).[1] Currently, the standard treatment for bladder cancer is transurethral resection of bladder tumor (TURB), followed by intravesical instillation in the case of NMIBC and radical cystectomy or chemotherapy with radiotherapy in case of MIBC. However, the progression and recurrence of bladder cancer is still common.[4,5]

Cisplatin-based chemotherapy is commonly used for MIBC, which is combined with other chemotherapeutic agents. However, the success rate of the combination chemotherapy is still low.[6] The response rate for MVAC (Methotrexate, Vinblastine, Adriamycin, and Cisplatin) is 46% while GC (Gemcitabine and Cisplatin) is 49%. The survival rate of MVAC and GC are 14.8 and 13.8 months respectively. This condition shows the lack of efficacy of standard chemotherapy currently available. In addition, the side effects of each of this chemotherapy are also factors need to be considered.[7]

Metformin is one of the type II diabetes mellitus (DM) drug. Metformin has an ability to reduce the incidence and prevalence of various types of cancer in animal model.[8-11] Metformin is also able to reduce the progression and recurrence of various types of cancer in the animal models. In particular, metformin is also able to reduce the incidence of bladder cancer in diabetic patients.[12,13] In addition, metformin can also improve recurrence-free survival and bladder cancer-specific survival of diabetic patients after radical cystectomy. At the cellular level, metformin is also capable of inducing apoptosis and decreasing bladder cancer cell proliferation.[14]

Several studies have shown a synergistic effect on the combination of metformin and cisplatin in bladder cancer.[15] However, there is still lack of data on the effectiveness of the combination of the two drugs. Therefore, the potential of metformin as a therapeutic agent for bladder cancer has to be explored.

OBJECTIVE

To evaluate the effect of metformin, cisplatin, and their combination on apoptosis of bladder cancer cells.

MATERIAL & METHODS

The bladder cancer cell used was cell line 5637 obtained from the American Type Culture Collection (ATCC® HTB-9TM) Manassas, Virginia. These cells were bred in the laboratory of Stem Cell Research and Development Center, Airlangga University, Surabaya. Cell line 5637 used in this research was grade II urothelial cell carcinoma taken from a 68-year-old Caucasian man. The culture medium used was RPMI 1640. It is needed 100 mL of RPMI 1640 media added with PBS 10%, penicillin-streptomycin 1% and function 1% to make RPMI 1640 serum media.

Activation of bladder cancer cells is done by taking cells from the liquid nitrogen tank, then diluting them using a water bath at 37°C. Then, the cells were inserted into a centrifugation tube containing 10 mL of 1640 serum RPMI media and centrifuged at 2500 rpm for 5 minutes. The supernatant was removed, the precipitate formed was added to RPMI 1640 serum. After which the cells were centrifuged at a speed of 1200 rpm for 5 minutes. The supernatant is removed and left with 1 mL for resuspension. The cell suspension was raised in TCF (Tissue Culture Flask) with growing media containing FBS 10%. The cells were observed using an inverted microscope. The cells were then incubated in a CO2 5% incubator at 37°C with a loose lid.

Trypsinization was carried out to release cells from TCF media by adding trypsin 0.05%. The released cells were put into a centrifugation tube and RPMI 1640 was added and then centrifuged at 1200 rpm for 10 minutes. The supernatant is removed, then 1 mL of media is added to cell resuspension. Furthermore, the cell suspension was put into a CO2 incubator at 37°C. Cell density was calculated by taking a cell suspension of 20 µL, then the cell was calculated with the help of a hemocytometer in the contrast phase of the microscope. In cytotoxicity tests, each well requires 5.000 cells whereas for apoptosis tests, each well requires 250.000–300.000 cells.

Cytotoxicity tests were carried out using 96 microculture plates. Cells were incubated for 24 hours in CO2 incubator at 37°C. After incubation, the media for each well was discarded, then replaced with new media containing FBS 10% and then treated using certain metformin doses of 1 mM, 2 mM, 5 mM, 10 mM, and 20 mM. Besides metformin, cisplatin was also used with several doses, namely 1 mM, 3 mM, 6 mM, 12 mM and 18 mM. Micro-cultures were then re-incubated for 24 hours and 48 hours in CO2 incubators at 37°C. The medium was removed, then each well was added with 100 µL of new medium and 25 µL of MTT reagent (25 µL/100 µL per well). It was then incubated for 4 hours in

CO2 incubator at 37°C and added with 100 uL DMSO. The absorbance of the microculture plate is read using an ELISA reader at a wavelength of 595 nm. The percentage of cell viability is obtained by the formula:

$$\% \text{ Cell viability} = \frac{(\text{treatment absorbancemoderate absorbance}) \times 100\%}{(\text{control of cell absorbance medium absorbance})}$$

The apoptosis test consisted of five groups, namely one control group (without treatment) and five treatment groups given metformin IC_{50}, cisplatin IC_{50}, combination ¼ IC_{50} (metformin and cisplatin), ½ IC_{50} (metformin and cisplatin), and IC_{50} (metformin and cisplatin) that obtained from previous cytotoxicity tests. Microplate 96 wells are used as a container for the growth of bladder cancer cells in the amount of 250.000–300.000 in each well. Exposure to metformin in bladder cell culture was carried out for 48 hours. The first apoptosis test was performed by the deparaffinization of the specimen (paraffin block) with xylene 3 times each for 3 minutes. The specimen was then rehydrated using 100% ethanol, 95% ethanol and 70% ethanol for 2 minutes, 2 minutes, and 1 minute, respectively. Thus, the specimen was rehydrated with sterile aquabidest. The specimen was then dropped with 50 ml of the TUNEL labeling mix (consisting of 5 ml of enzyme terminal deoxynucleotidyl transferase and 45 uL fluorescein-dUTP). After that, the specimen was closed with siliconize cover slip.

The specimen was incubated at 37^0C for 30 minutes in the moist chamber, then washed with PBS (Phosphate Buffer Saline) 3 times. The next step was incubating the specimen with RNase Solution at 37^0C for 30 minutes. The specimen was then washed with PBS 3 times. The specimen with propidium iodide was then incubated at room temperature for 10 minutes. Lastly, the specimen was washed with PBS 3 times, then covered with slide cover diameter of 18 mm. Observation of the apoptotic cells was done using a light microscope with 1000x magnification (green fluorescence).

RESULTS

The viability of bladder cancer cells was observed using the MTT assay. Cell culture replication was carried out 5 times in each treatment group. Urothelial cell lines were cultured and incubated for 24 hours then observed until they reached a density of 50%. Then trypsinization is carried out to release cells from the culture plate. The urothelial cell subculture is done on the 96 well plates. Furthermore, each well is exposed to metformin according to the treatment dose. Cells are re-bred for 24 and 48 hours. The cells were then terminated. The cell metabolite results were read using the MTT assay.

The MTT results showed that the percentages of bladder cancer cells living with metformin 1 mM, 5 mM, 10 mM, and 20 mM for 24 hours were 86.044%, 81.889%, 77.959%, 69.514% and 54.193% respectively (Table 1).

Additional exposure of metformin for 48 hours showed that the percentages of bladder cancer cells living with metformin concentration of 1 mM, 2 mM, 5 mM, 10 mM, and 20 mM were 69.195%, 63.854%, 58.686%, 54.024%, and 47.163% respectively (Table 2). From the analysis of the results of the metformin cytotoxicity test with exposure time of 24 and 48 hours, IC_{50} metformin in bladder cancer cells was found to be 15 mM with 48-hour exposure time (Figure 1).

Table 1. Metformin cytotoxicity test at 24 hours.

No	K. Med	K. Sel	1 mM	2 mM	5 mM	10 mM	20 mM
1	0.095	0.870	0.768	0.727	0.704	0.647	0.514
2	0.096	0.879	0.770	0.734	0.696	0.626	0.510
3	0.094	0.865	0.769	0.726	0.711	0.630	0.528
4	0.096	0.882	0.776	0.749	0.708	0.641	0.525
5	0.098	0.866	0.737	0.723	0.687	0.634	0.506
Total	0.478	4.362	3.820	3.659	3.506	3.178	2.583
Mean	0.096	0.872	0.764	0.732	0.701	0.636	0.517
% Of living cells			86.044	81.899	77.959	69.514	54.193

Table 2. Metformin cytotoxicity test at 48 hours.

No	K. Med	K. Sel	1 mM	2 mM	5 mM	10 mM	20 mM
1	0.091	1.462	1.040	0.949	0.896	0.847	0.738
2	0.096	1.510	1.020	0.985	0.913	0.839	0.743
3	0.094	1.487	1.100	0.992	0.888	0.835	0.749
4	0.093	1.465	1.050	0.967	0.915	0.851	0.757
5	0.096	1.454	1.040	0.988	0.912	0.830	0.741
Total	0.470	7.378	5.250	4.881	4.524	4.202	3.728
Mean	0.094	1.476	1.050	0.976	0.905	0.840	0.746
% Of living cell			69.195	63.854	58.686	54.024	47.163

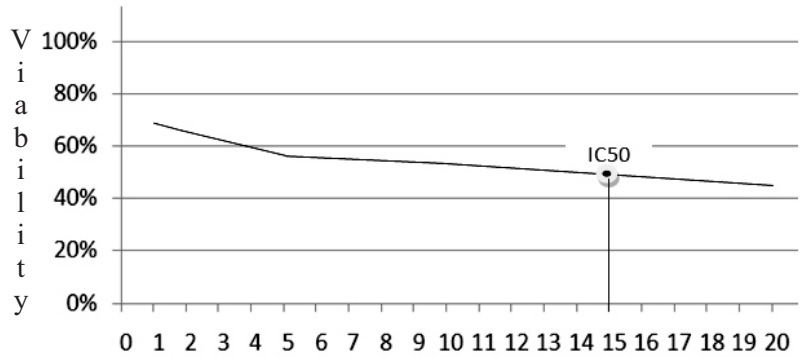


Figure 1. Graph of Metformin concentration on cell viability.

Observation of bladder cancer cells viability using the MTT assay was carried out in the treatment group with exposure to cisplatin. There were 5 treatment groups with cisplatin exposure dose of 1 uM, 3 uM, 6 uM, 13 uM, and 18 uM. The percentages of each bladder cancer cell group living with a 24-hour cisplatin exposure were 87.598%, 82.182%, 77.985%, 73.041%, and 70.080% (Table 3).

The percentages of each bladder cancer cell group living with a 48-hour cisplatin exposure were 63.636%, 60.090%, 57.180%, 52.895%, and 50.318% (Table 4).

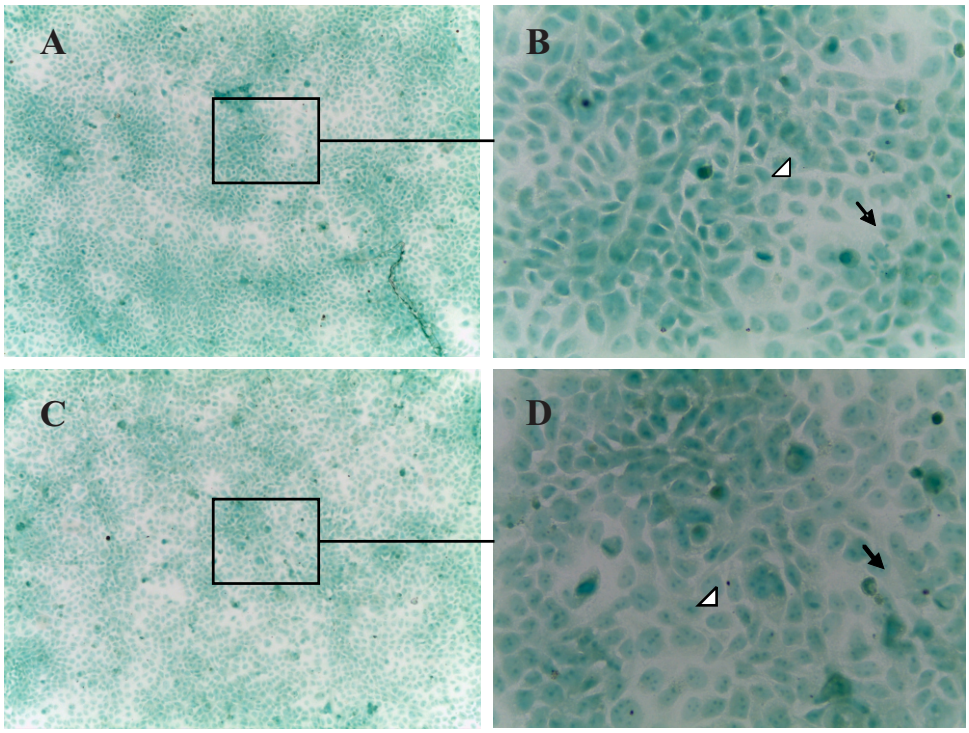
Table 3. Cisplatin cytotoxicity test at 24 hours.

No	K. Med	K. Sel	1 uM	3 uM	6 uM	13 uM	18 uM
1	0.095	0.870	0.784	0.732	0.697	0.654	0.631
2	0.096	0.879	0.763	0.727	0.704	0.671	0.640
3	0.094	0.865	0.789	0.730	0.710	0.646	0.638
4	0.096	0.882	0.770	0.743	0.683	0.665	0.647
5	0.098	0.866	0.774	0.738	0.713	0.679	0.644
Total	0.478	4.362	3.880	3.670	3.507	3.315	3.200
Mean	0.096	0.872	0.776	0.734	0.701	0.663	0.640
% Of living cells			87.589	82.182	77.985	73.041	70.080

Table 4. Cisplatin toxicity test at 48 hours.

No	K. Med	K. Sel	1 uM	3 uM	6 uM	13 uM	18 uM
1	0.095	0.870	0.784	0.732	0.697	0.654	0.631
2	0.096	0.879	0.763	0.727	0.704	0.671	0.640
3	0.094	0.865	0.789	0.730	0.710	0.646	0.638
4	0.096	0.882	0.770	0.743	0.683	0.665	0.647
5	0.098	0.866	0.774	0.738	0.713	0.679	0.644
Total	0.478	4.362	3.880	3.670	3.507	3.315	3.200
Mean	0.096	0.872	0.776	0.734	0.701	0.663	0.640
% Of living cells			87.589	82.182	77.985	73.041	70.080

Using the cytotoxicity test data, IC_{50} Cisplatin value was 18 uM with 48-hour exposure time. Apoptosis test in this study was observed using TUNEL Assay. This apoptosis test consists of six groups, namely: 1 control group (without treatment) and 5 treatment groups. The treatment group was exposed to IC_{50} metformin, IC_{50} cisplatin, ¼ IC_{50} (metformin + cisplatin), ½ IC_{50} (metformin + cisplatin), and IC_{50} (metformin + cisplatin) obtained from prior cytotoxicity tests. Observations were made by counting fluorescence using a light microscope. The means of apoptosis are 0.24, 0.34, 3.52, 1.02, 2.72, 4.42 for control, metformin IC_{50} (15 mM), cisplatin IC_{50} (18 uM), cisplatin ¼ IC_{50} (4.5 uM) + metformin ¼ IC50 (3.75 mM), cisplatin ½ IC50 (9 uM) + metformin ½ IC_{50} (7.5 mM), and cisplatin IC_{50} (18 uM) + metformin IC_{50} (15 mM) group respectively (Table 5). The picture of the apoptosis of each group can be seen in Figure 2(A-L) below.



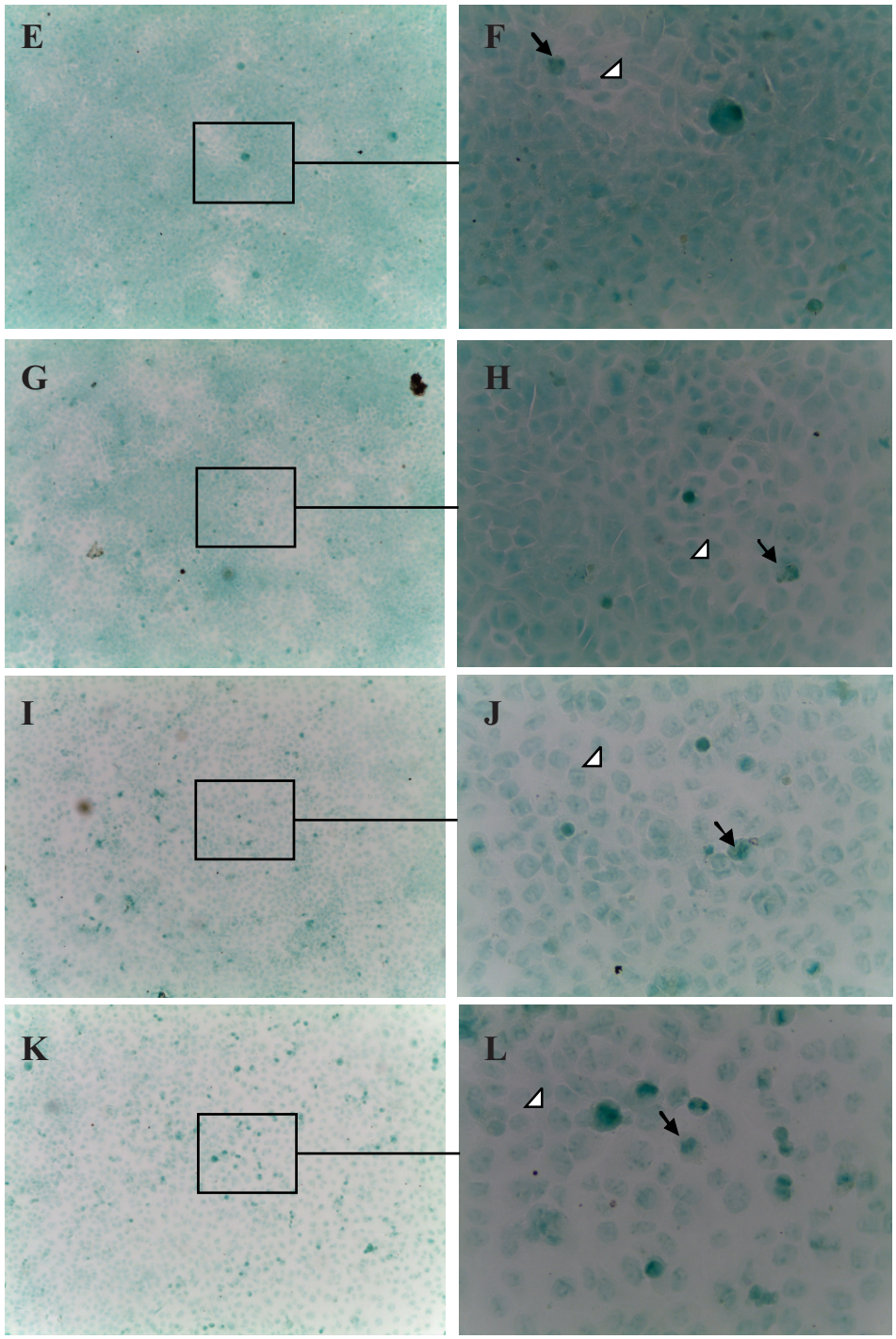


Figure 2. Apoptosis of bladder cancer cells (Urothelial cell line 5637) by TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling) Assay. Apoptotic cells are indicated with an arrow while the non-apoptotic cells are indicated with an arrow head. A. Control (100x magnification), B. Control (400x magnification), C. Metfotmin 15 mM (100x magnification), D. Metformin 15 mM (400x magnification), E. Metformin 3.75 mM + Cisplatin 4.5 uM (100x magnification), F. Metformin 3.75 mM + Cisplatin 4.5 uM (400x magnification), G. Metformin 7.5 mM + Cisplatin 9 uM (100x magnification), H. Metformin 7.5 mM + Cisplatin 9 uM (400x magnification), I. Cisplatin 18 uM (100x magnification), J. Cisplatin 18 uM (400x magnification), K. Metformin 15 mM + Cisplatin 18 uM (100x magnification), L. Metformin 15 mM + Cisplatin 18 uM (400x magnification).

From the normality test using Kolmogorov Smirnov's One sample, the data obtained from observation of bladder cancer cell apoptosis were not normally distributed ($p<0.05$) (Table 5).

The next statistical analysis was done using the Kruskal-Wallis non-parametric test. There was a significant difference in the mean apoptosis index in each treatment group with a value of $p<0.0001$. Further analysis was carried out with Post Hoc Dunn's multiple comparisons test which showed a significant difference in the apoptosis index in each treatment group compared to the control group except in the group that received exposure to IC_{50} Metformin and ¼ IC_{50} (Metformin + Cisplatin) (Table 6).

Table 5. Bladder Cancer Cell Apoptosis Test.

Group	N	Mean (Min-Max)	p value
Control	5	0.24 (0.129 – 0.351)	0.009*
Metformin 15 mM	5	0.34 (0.114 – 0.565)	0.0234*
Cisplatin 18 uM	5	3.52 (3.211 – 3.829)	<0.0001*
Cisplatin 4.5 uM + Metformin 3.75 mM	5	1.02 (0.633 – 1.407)	0.0022*
Cisplatin 9 uM + Metformin 7.5 mM	5	2.72 (1.909 – 3.531)	0.0008*
Cisplatin 18 uM + Metformin 15 mM	5	4.42 (3.767 – 5.073)	<0.0001*

Table 6. Comparison of the Apoptosis index.

Group	p value
Control vs Metformin 15 mM	>0.9999
Control vs Cisplatin 18 uM	0.0081*
Control vs Cisplatin 4.5 uM + Metformin 3.75 mM	0.6294
Control vs Cisplatin 9 uM + Metformin 7.5 mM	0.0428*
Control vs Cisplatin 18 uM + Metformin 15 mM	0.0001*

DISCUSSION

Bladder cancer has an increasing incidence worldwide.[2] Chemotherapy is usually used as adjuvant therapy, especially for advanced bladder cancer.[16,17] However, several tumor cells can become resistant to chemotherapy resulting in recurrence and/or progression to more severe cancer. For example, bladder cancer cells that were initially sensitive to cisplatin-based combination chemotherapy could become resistant to these chemotherapeutic agents.[18]

Cytotoxic tests in this study using the MTT assay method proved that metformin was able to reduce bladder cancer cell proliferation (Table 1 and 2). Decreasing proliferation of bladder cancer cells after exposure to metformin indicates that the cytotoxicity effects of metformin depend on the dose and duration of exposure given. Several studies have shown that Metformin has an anti-cancer effect and is effective in several types of cancer. The incidence of bladder cancer in patients with type II DM who routinely consume metformin was much lower than normal patients [8-11,14,15,19,20] However, there are several studies reported that metformin has a negative effect on some types of cancer.[21]

Cisplatin exposure to bladder cancer cells also has a similar effect on metformin. The greater and longer exposure to cisplatin, the lower the bladder cancer cell proliferation (Table 3 and 4). Furthermore, the dose of metformin needed to achieve 50% cell viability (IC_{50}) is much greater than the dose of cisplatin. In this study, the IC_{50} for metformin was 15 mM, while cisplatin was 18 uM. In conclusion, cisplatin is a very toxic drug for bladder cancer cells because only a small dose is needed to kill these cells, in contrast to metformin which is not toxic to bladder cancer cells. However, the in vitro dose must be tested preclinically with in vivo research before finally being able to proceed to clinical trials in humans.

This study also observed apoptosis of bladder cancer cells after exposure to metformin or cisplatin both as a single agent and a combination of different doses. This becomes very crucial because in the cytotoxicity test, it is not known whether cell death is induced by apoptosis or necrosis. In this study, it was proven that single Metformin was not

able to significantly increase bladder cancer cell apoptosis compared to control group. In contrast, cisplatin administration significantly improved bladder cancer cell apoptosis compared to control. The result breaks the previous theory which explains that Metformin can increase bladder cancer cell apoptosis.

The addition of combination dose of Metformin + Cisplatin showed a significant increase in the number of apoptosis, known from Post Hoc Analysis where the combination group Metformin + Cisplatin dose ¼ IC_{50} initially did not differ significantly from the control group, to become significantly different when the dose was increased to ½ IC_{50} and IC_{50}. The results showed a dose-related effect on the combination of Metformin + Cisplatin. However, whether the increase in apoptosis that occurred was caused by metformin, cisplatin, or both is not the focus of this study and cannot be proven through this study. For this reason, further research is needed to determine the synergistic effects and roles of each of these drugs.

CONCLUSION

Metformin administration solely was not able to increase bladder cancer cell apoptosis. Conversely, the administration of Cisplatin alone or its combination with Metformin can significantly increase bladder cancer cell apoptosis, while the apoptosis rate was higher for the combination group.

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