COMPARISON BETWEEN GEMCITABINE-CARBOPLATIN AND COMBINATION OF GEMCITABINE-CARBOPLATIN AND CELECOXIB IN UROTHELIAL CARCINOMA CELL APOPTOSIS

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ABSTRACT

Objective: *To determine the combination effect of celecoxib and gemcitabine-carboplatin chemotherapy to the apoptosis of cultured-urothelial cancer cell line.* ***Material & Methods:*** *Urothelial carcinoma cell line originating from Bladder Ca 5637 was cultured and used in this in vitro study. Three-groups of cultured-urothelial cancer cell line consisted of (1) control (C), (2) gemcitabine-carboplatin treated group (GC) and (3) gemcitabine-carboplatin and celecoxib treated group (GCC) were treated with 0.086 μM gemcitabine, 290 μM carboplatin and 25 μM celecoxib. All groups were evaluated at 24 hours following treatment, and the apoptotic index (AI) measured accordingly.* ***Results:*** *Significant mean apoptotic index differences were found between the C and GC group; and between C and GCC groups at 24 hours following treatment. However, the AI of GCC was lower than the GC group although not statistically significant (p>0.05).* ***Conclusion:*** *Our study have shown that gemcitabine-carboplatin and the combination of both with celecoxib may increase the apoptosis of urothelial cancer cell line. The role of celecoxib in the addition of gemcitabine-carboplatin to treat urothelial cancer cell line needs to be elucidated further.*

Keywords:*Gemcitabine, carboplatin, celecoxib, bladder carcinoma, apoptosis.*

ABSTRAK

Tujuan: *Mengidentifikasi efek kombinasi celecoxib dengan regimen kemoterapi gemcitabine-carboplatin untuk apoptosis sel kultur karsinoma urothelial.* ***Bahan & Cara:*** *Desain penelitian ini adalah penelitian eksperimental in vitro menggunakan sel kultur karsinoma urotelial. Sel karsinoma urotelial ini berasal dari sel kanker buli-buli tipe 5637. Ada tiga kelompok, kelompok pertama adalah kelompok kontrol, kelompok kedua diberikan paparan gemcitabine dan carboplatin, dan kelompok ketiga diberikan paparan gemcitabine, carboplatin dan celecoxib. Dosis obat yang digunakan adalah 0.086 μM untuk gemcitabine, 290 μM untuk carboplatin dan 25 μM untuk celecoxib. Semua kelompok diberikan paparan selama 24 jam dan setelah itu indeks apoptosis untuk setiap kelompok diukur.* ***Hasil:*** *Terdapat perbedaan yang signifikan pada rerata indeks apoptosis antara kelompok kontrol, kelompok gemcibatin carboplatin dan kelompok gemcitabine-carboplatin dan celecoxib setelah 24 jam paparan. Rerata indeks apoptosis kelompok gemcitabine-carboplatin dan celecoxib lebih rendah dibandingkan dengan kelompok gemcitabine-carboplatin tetapi tidak berbeda signifikan (p>0.05).* ***Simpulan:*** *Gemcitabine-carboplatin dan kombinasi gemcitabine-carboplatin dan celecoxib dapat meningkatkan apoptosis sel kultur karsinoma urothelial. Akan tetapi indeks apoptosis pada kelompok kombinasi gemcitabine-carboplatin dan celecoxib lebih rendah dibandingkan dengan kelompok gemcitabine-carboplatin. Hal ini bisa disebabkan oleh karena efek antagonis celecoxib terhadap aktivitas sitotoksik carboplatin, celecoxib menghambat CTR1, celecoxib menekan pembelahan PARP dan menghambat aktivitas caspase-3 serta celecoxib mencegah terjadinya DNA platination. celecoxib pada kombinasi gemcitabine-carboplatin, tidak memiliki efek yang signifikan dalam mempengaruhi aktivitas apoptosis sel karsinoma urothelial.*

Kata kunci:*Gemcitabine, carboplatin, celecoxib, karsinoma buli, apoptosis.*

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INTRODUCTION

Bladder carcinoma is the second largest malignancy in the urogenital system after prostate carcinoma in Europe and the United States. Based on gender, bladder carcinoma in men is the fourth most common type of malignancy after prostate, lung, and colorectal carcinoma with an incidence rate of 5-10% (6.6% new cases per year). Whereas in women is the eighth most malignancy with an incidence of 2-3% (around 2.4% new cases per year) from all malignancies.[1-3] Based on Globocan 2008 data, the incidence of bladder carcinoma in Indonesia in men and women per 100.000 population was 4.9-10.6 and 1.2-1.8, respectively.[2] The incidence of bladder carcinoma per 100.000 male population is 10.[1] while the incidence of women is 2.5.[1] Bladder carcinoma can be classified according to clinical parameters of tumors, nodes, and metastases (TNM).[4] Bladder carcinoma can also be distinguished as non-muscle invasive bladder cancer (NMIBC) and muscle-invasive (MIBC) for the therapeutic purpose.[4,5] Histopathologically, bladder carcinoma can be classified into urothelial papilloma, grade 1 (well differentiated), grade 2 (moderately differentiated), and grade 3 (poorly differentiated).[5]

Some therapeutic modalities are definitive and can be selected based on the agreement between doctors and patients. Open surgery (open radical cystectomy) is still the gold standard for the treatment of upper stage T2 infiltrative carcinoma (T2-T4a, N0-Nx, M0), including high-grade superficial carcinoma (T1G3 or CIS). Radical laparoscopic cystectomy is still often debated and compared to open surgery because it has several advantages and disadvantages. For urine diversion, there are four choices such as ileal conduit, continent cutaneous diversion, orthotopic neobladder or ureterosigmoidostomy. Each type of urine diversion will affect the quality of life of the patients, especially those related to sexual function, voiding and digestion.[5-7] The combination platinum-based chemotherapy, cisplatin, and carboplatin which for locally advanced or metastatic bladder carcinoma, have been widely used.

Gemcitabine was the first compound to obtain a license based on antitumor efficacy and on increasing the quality of life in studies of patients with pancreatic carcinoma.[8] Gemcitabine which similar to any other nucleosides analog are hydrophilic and cannot pass through cell membranes by passive diffusion. Gemcitabine enters cells through molecular transport for nucleosides.[9] Gemcitabine had been reported a synergistic effect when combined with cisplatin. Gemcitabine also increased its intracellular concentration with a repetitive mechanism of positive feedback activation.[8] Summarily, gemcitabine anti-tumor activity is achieved through inhibition of DNA replication and inhibition of cell growth by combining gemcitabine into DNA replication and through inhibition of repair mechanisms with DNA chain termination.[10]

Cisplatin chemotherapy is widely used to treat bladder cancer, even though more than 50% of patients with bladder cancer are not ideal for chemotherapy using cisplatin due to a decrease in kidney function.[11] Platinum compounds react with DNA, RNA, proteins, phospholipid membranes, cytoskeletal microfilaments, and molecules containing thiols. The primary cellular targets for platinum complexes are generally considered DNA. Approximately 1% of platinum's total cellular binding to DNA results in intra-strand cross-linking.[12] It has been found that platinum-damaged DNA causes termination of the G2 phase. Because of the cessation of the G2 phase, repair of damaged DNA will not occur so that the cell will stop mitosis and will experience cell death through the process of apoptosis.[12]

The activation of apoptosis could be available through the extrinsic pathway (death-receptor pathway) by the death cells trigger with an extracellular signal which caused ligand bounding with the specific transmembrane receptor such as death receptor (DR). The intrinsic apoptosis pathway (mitochondrial pathway) is activated due to the response of stress conditions including DNA damage and oxidative stress. Stress conditions and damage to cell environment causing the activation of intrinsic apoptosis pathway (mitochondrial pathway). These conditions include DNA damage and oxidative stress which can be detected by B-cell lymphoma 2 (Bcl-2)-homology protein only (BH3-only) which consists of several pro-apoptotic proteins and anti-apoptotic proteins. Pro-apoptotic mediates apoptosis by interfering with membrane integrity either directly on its pores or by binding to mitochondrial proteins, whereas anti-apoptosis will prevent apoptosis by inhibiting aggregation from pro-apoptosis.[13,14]

The antitumor mechanism on the COX-2 selective inhibitor had been unclearly known even it

had been discovered that COX-2 selective inhibitor has the antitumor effect for invasive bladder cancer. The prostaglandin enhancement especially prostaglandin E2 had an essential role to the cell proliferation, Angiogenesis stimulation, and inhibit apoptosis activity.[15] It was previously known that catalysis of prostaglandins was influenced by COX, where inhibition of COX-2 would reduce the catalytic rate in prostaglandin biosynthesis.[16-18] Selective COX-2 inhibitors have the effect of inducing apoptosis and changing angiogenic factors where the effect acts as an antitumor.[18]

Selective COX-2 inhibitors have the effect of inducing apoptosis and changing angiogenic factors where the effect acts as an antitumor. Selective COX-2 inhibitors still require high doses to be achieved as antitumor, if used with a general dose it will inhibit several cell lines.[18] Based on these data this study aims to determine the synergistic effects of gemcitabine-carboplatin and celecoxib in inducing apoptosis in bladder cancer cell lines.

OBJECTIVE

We aimed to compare the apoptosis effect of gemcitabine-carboplatin and gemcitabine-carboplatin-celecoxib on urothelial carcinoma cell line.

MATERIAL & METHODS

The design of this study was an in vitro experimental study using urothelial carcinoma cells lines in the laboratory presented with gemcitabine-carboplatin and celecoxib chemotherapy. The experimental unit in this study was urothelial carcinoma culture cells taken from ATCC 5637 which received carboplatin gemcitabine chemotherapy. In this study using urothelial carcinoma culture cells in vitro. Each research group was quintuplicated for measurement. So that is obtained by each group of 5 well. The tools used in this study were a plate for culture, CO2 incubator, centrifuge tube, centrifuge machine, disposable pipette, micropipette, scissors, syringe, microscope, counting machine, magnetic styrene, Styrofoam container, vortex mixer. Urothelial carcinoma cells were cells originating from bladder ca 5637. This tissue was processed in the medium and repeated centrifugation and passage were carried out. Giving gemcitabine chemotherapy in each cell of urothelial carcinoma 5637. The dose was 0.086 μM gemcitabine and 290 μM carboplatin. Gemcitabine and carboplatin were diluted with distilled water to a dose of 0.086 μM gemcitabine according to 0.0013mg/100ml and carboplatin 290 μM according to 4.3838mg/100ml. The administration of celecoxib to urothelial cell culture cells according to urothelial carcinoma culture cells 5637 was only given celecoxib, Celebrex®, with a dose, 25 μM. Celecoxib was dissolved with distilled water until a dose of 1.1337mg/100ml was obtained in the exposure group. The exposure of celecoxib to urothelial culture cells with carboplatin gemcitabine was given exposure to celecoxib in the treatment group. With a dose of celecoxib, celebrex®, which is 25 μM. Celecoxib was dissolved with distilled water until a dose of 1.1337mg/100ml was obtained in the exposure group.

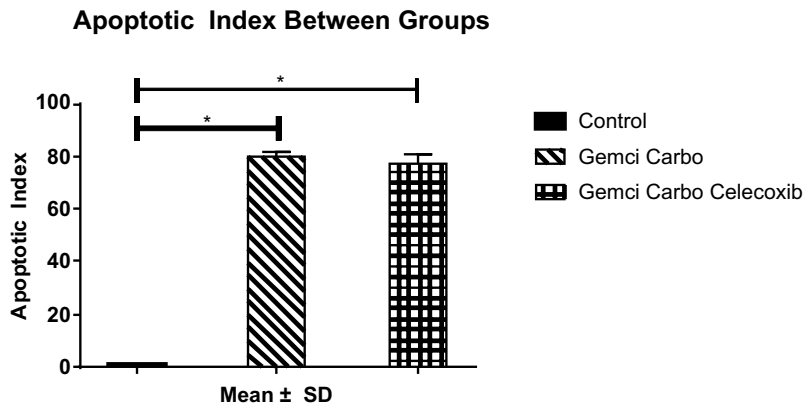
RESULT

Normality of apoptotic index was tested using the Shapiro Wilk test, the apoptotic index data in each group had a normal distribution with a value of $p>0.05$ and the data was not homogeneous with $p<0.05$. Based on the normality test, the data then analyzed using the One-Way ANOVA test to see differences between groups. There were significant differences between groups with a value of $p<0.05$.

Table 1. The comparison value apoptotic index on each group after 24 hours of treatment.

Groups	n	Mean ± SD	Normality Test	Homogeneity Test	p-value
Control	5	0.74 ± 0.36	0.68	0.026**	0.00*
Gemcitabine-carboplatin	5	80.33 ± 1.79	0.88		
Gemcitabine-carboplatin and celecoxib	5	77.49 ± 3.11	0.19		

* $p<0.05$ statistically significant
** $p<0.05$ variance data not homogeneous



* statistically significant

Figure 1. Comparison of the apoptotic index on each group after 24 hours of treatment.

Table 2. Post Hoc Test Games-Howell apoptotic index on each group after 24 hours treatment.

Comparison Between Each Group	Mean Differences	Confidence Interval 95%		p-value
		Lower Bound	Upper Bound	
Control vs gemcitabine-carboplatin	-79.5	-82.40	-76.76	0.00*
Control vs gemcitabine-carboplatin and celecoxib	-76.7	-81.67	-71.80	0.00*
Gemcitabine-carboplatin vs gemcitabine-carboplatin and celecoxib	2.84	-1.99	7.69	0.253

* $p<0.05$ statistically significant

Therefore, the Games-Howell (because the data variance is not homogeneous) post hoc test was done to determine the differences in each group.

Post hoc Games-Howell showed a significant difference in the apoptotic index between control and gemcitabine-carboplatin group and between control and combination of gemcitabine-carboplatin and celecoxib group after 24 hours of treatment (Table 2). The mean apoptotic index of combination gemcitabine-carboplatin and celecoxib group was lower than the gemcitabine-carboplatin group but not statistically significant ($p>0.05$).

DISCUSSION

Apoptosis program or cell death is a physiological response to multicellular organizations in eliminating cells that experience mutase, an immune reaction when cells are damaged by diseases or other toxic substances.[13] Gemcitabine is the first compound to be licensed based on antitumor efficacy and on increasing the quality of life in patients with pancreatic carcinoma. Gemcitabine reported in a study has a synergistic effect when combined with cisplatin by increasing its intracellular concentration with the mechanism of initiating positive feedback activation.[8] Gemcitabine has a good response and efficacy to some solid tumors, including transitional bladder cells, advanced bile carcinoma, and soft tissue sarcoma.[19-22]

Celecoxib is a selective COX-2 inhibitor and has been evaluated as anti-cancer in human trials. Celecoxib 400 mg twice per day for six months reduced the occurrence of colorectal polyps by 28%.[23,24] Combination therapy can also be applied as chemopreventive. The combination of ornithine decarboxylase inhibitors and NSAIDs was found to be more effective than monotherapy in suppressing genetic tumors in FAP. There are several ongoing clinical trials that evaluating the efficacy of selective COX-2 inhibitors such as celecoxib and rofecoxib in preventing sporadic colorectal adenomas in larger population. Separately in another placebo control study comparing the effects of celecoxib and selenomethionine both as monotherapy and a combination of the occurrence of adenomas.[17] But,

in-vitro studies which aims to evaluate synergistic effects of gemcitabine, carboplatin, and celecoxib on the bladder cancer have not yet been studied so far. Based on these data this study aims to determine the synergistic effects of gemcitabine-carboplatin and celecoxib in inducing apoptosis in bladder cancer cell lines.

Cell culture used was human bladder cell carcinoma type 5637. Type 5637 cells were one of the most found subtypes of human bladder cell carcinoma besides T24 type cells. The dosage used in gemcitabine and carboplatin according to the research conducted by Wang et al., based on IC50 is gemcitabine 0.086 μM and carboplatin 290 μM. While the dose of celecoxib was 25 μM. The time of apoptotic observation was carried out at 24 hours.

The MTT is done to determine the dose of celecoxib and the time of evaluation of apoptosis. MTT was conducted at the Universitas Airlangga Stem Cell Research and Development Centre. MTT is useful for knowing the number in percentage of living cells. MTT assessment uses Spectrophotometry to determine Optical Density. From the MTT results, it was found that the number of % of living cells will decrease if the treatment was longer and the dose of celecoxib was greater.

The MTT result was chosen the dose of celecoxib 25 μM chosen and the time of apoptotic observation was chosen at 24 hours. The combination of celecoxib with carboplatin gemcitabine was the administration of gemcitabine-carboplatin chemotherapy with a dose of 0.086 μM gemcitabine and carboplatin 290 μM including the addition of celecoxib at a dose of 25 μM. Celecoxib, celebrex®, is a COX-2 selective inhibitor that has the effect of inducing apoptosis and changing angiogenic factors where the effect acts as an antitumor.[25]

One-way ANOVA test showed that there was a significant difference in the apoptotic index with p-value <0.05. Analysis continued by Games-Howell post hoc test and found that there was a significant difference between the control group and the gemcitabine-carboplatin group and also between the control group and the combination of gemcitabine-carboplatin and celecoxib group. Gemcitabine-carboplatin groups and the combination of gemcitabine-carboplatin and celecoxib group has a higher apoptotic index and differed significantly compared to the control group. Combination of gemcitabine-carboplatin and celecoxib group had a lower apoptotic index compared to the gemcitabine-carboplatin group but not statistically significant (p>0.05)

These results contradict the hypothesis of this study, it means that celecoxib may have a mechanism that can reduce the apoptotic index in the combination of gemcitabine-carboplatin. Some hypothesis that can cause a decrease in apoptosis in this study is: (1) Celecoxib antagonizes the cytotoxic activity of platinum-based chemotherapy (cisplatin) by reducing intracellular cisplatin level.[26] Shi (2014) also reported the same results that celecoxib antagonizes the cytotoxic activity of carboplatin and could inhibit carboplatin-induced apoptosis in human esophageal cancer cells by reducing the accumulation of carboplatin in these cancer cells.[27] (2) Platinum-based chemotherapy is not easily diffuse through cell membranes because of it has high polarity.[28,29] Copper transporter 1 (CTR1) has been shown to mediates cisplatin influx in mammalian cells. In Yu's study, it was found that a combination of cisplatin and celecoxib can decrease the expression of the CTR1 protein, so it was assumed that a decrease in CTR1 could inhibit cisplatin influx which might have an impact on reducing cancer cells apoptosis.[26,27] (3) The antagonistic effect of celecoxib is not related to COX-inhibitor activity but might occur because celecoxib suppresses the cleavage of poly (ADP-ribose) polymerase (PARP) induced by carboplatin, and also inhibits caspase-3 activity that plays a role in the apoptosis process. This process could decrease the carboplatin-induced apoptosis.[27] (4) Another hypothesis is that celecoxib can prevent DNA platination. The way platinum-based chemotherapy works is by binding to DNA and forming DNA platination which can cause cell apoptosis.[30] Because of inhibition of DNA platination, cell apoptosis may also be reduced.[26]

This study has several limitations including: (1) this study is not using pure drugs instead of combination, (2) effective doses in this study have not been known so can influence the result of the study, (3) there is no previous research about administration of gemcitabine-carboplatin and celecoxib combination in urothelial cancer cell lines. Therefore, this study underlies the need for further study using celecoxib to obtain more significant data and to evaluate the efficacy of the therapy, the mechanism of action of celecoxib if combined with another chemotherapy regimens and evaluate the possible side effects.

CONCLUSION

Gemcitabine-carboplatin and a combination of gemcitabine-carboplatin and celecoxib can improve apoptosis in urothelial cancer cell lines. However, the apoptotic index in the combination group of gemcitabine-carboplatin and celecoxib was lower compared to the gemcitabine-carboplatin group, although it was not significantly different. Some hypothesis that may cause this were celecoxib antagonizes the cytotoxic activity of carboplatin, celecoxib inhibits CTR1, celecoxib suppresses the cleavage of PARP also inhibits caspase-3 activity and celecoxib prevents the DNA platination. Further research focusing on interactions and the mechanism of celecoxib against another chemotherapeutic agent need to be done for the selection of more effective chemotherapy regimens.

ACKNOWLEDGEMENT

The Authors acknowledge to Nora Ertanti in Stem Cell Research and Development Center, Faculty of Medicine/Universitas Airlangga Surabaya.

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