International Journal of
NASOPHARYNGEAL CARCINOMA

Journal homepage: https://talenta.usu.ac.id/IJNPC

Effects of Cisplatin on Superoxide Anion, Sod, Apoptosis and Cytoplasmic p21Expression in C666-1 Cell Lines Undifferentiated Nasopharyngeal Cancer

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Abstract

Introduction: ROS, a mechanism in CIS pathogenesis, causes toxicity when cisplatin is administered. Superoxide anion (O2-), a radical anion, part of ROS that is initially triggered after a part of oxygen enters a living cell. The main principle of this enzyme is to work as a protection against oxygen toxicity and plays a role as body catalyst. The p21 has a main function in the regulation of cell cycle progression. It is able to regulate cell proliferation through its association with PCNA and DNA polymerase accessory factor as a part of cell regulation and apoptosis.
Objective: To study the effects of cisplatin on the increase or decrease of O2-, SOD, proliferation (MTT), apoptosis and cytoplasmic p21 expression in C666-1 cell lines.
Methods: An experimental study with a post control group design and cisplatin in group dosages of 7.86 µg/mL, 15.36 µg/mL and 30.72 µg/mL.
Results: The mean difference of O2- in C666-1 cell lines between cisplatin groups was not significant (P value 0.871, P>0.05). The mean difference of SOD in C666-1 cell lines was highly significant (P value 0.001, P<0.05) showing a significant increase of SOD. The mean difference of C666-1 cell lines proliferation (MTT) between cisplatin groups was not significant (P value 0.094, P>0.05). The mean difference of C666-1 cell lines apoptosis between cisplatin groups was not significant (P value 0.104, P>0.05). Cytoplasmic p21 highest expression was found at the 30.72 dosage obtained from 24 hours observation.
Conclusion: Increase of SOD after a high dose of cisplatin administration, due to the balance between ROS production and detoxification process caused by antioxidants (SOD), will affect cancer cells to proliferate and survive. Increase of apoptosis at the highest dose found in this study, showed that apoptosis was induced by cisplatin. Therefore, antioxidant administration may be considered for nasopharyngeal cancer patients.

Article Info

Article history:
Received: 10th June 2021
Received in revised form: 16th June 2021
Accepted: 17th June 2021

Keywords:
Cisplatin, superoxide anion, C 666-1 cell lines, apoptosis.

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1. INTRODUCTION

Cisplatin (CIS) is a highly effective drug acting as an antineoplastic agent with DNA alkylation that is used in various solid tumors. Cisplatin is a chemotherapy medication, derived from platinol, used to treat a number of cancers, including sarcoma, carcinoma (such as small cell lung carcinoma and ovarium carcinoma), lymphoma and germinal cell tumors. Platinol is the first unit from the platinum class that contains anticancer benefits, also includes carboplatin and oxaliplatin. Platinum complex reacts in vivo, by binding and causing a cross reaction of the DNA, that will finally trigger apoptosis (programmed cell death) [1,2,3].

Reactive oxygen species (ROS) is a mechanism known in the pathogenesis of CIS, which causes toxicity when cisplatin is administered. Cisplatin not only plays a role in DNA damage, but also causes side effects even when administered at a low dose, causing it to trigger programmed cell death (apoptosis) [4]. Oxidative stress is a condition where the number of free radicals inside the the body exceeds its capacity to neutralize, which causes to imbalance between ROS/RNS (Reactive Nitrogen Species) production and antioxidants [5, 6]. In general, oxidative stress is caused by: 1) decrease in antioxidants, 2) increase in ROS/RNS production.

Superoxide anion is a radical anion (O_2-) that belongs to a group of ROS, initially triggered after a part of oxygen enters a living cell. Superoxide anion is produced because of several factors, which are: 1) UV ray exposure, 2) smoking, 3) environmental pollution, 4) radiation or oxidation from xanthin oxidase or NADPH oxidase. The ability of O_2-is to attack cellular components and cause damage to components of fat, protein and DNA. This is the cause of several diseases, including cancer, atherosclerosis, rheumatoid arthritis and damage to the liver and nervous system.

Superoxide dismutase (SOD) is an enzyme that is widely distributed in all aerobic cells. The working mechanism of this enzyme is to protect against oxygen toxicity and play a role in as a catalyst in changing superoxide radicals (O_2-). There are two forms of SOD in the cells. The first form is found in the cytoplasm containing Cu and Zn (Cu/ZnSOD). SOD protects aerobic organisms in fighting unwanted effects caused by superoxide. This enzyme is found in all aerobic tissues, especially I the mitochondria and cytosol [7].

The p21 is a tumor suppressor with a weight of 21Kda, with a main function in the regulation of cell cycle progression. This action is achieved by blocking the bond between CDK and cyclin. P21 is also known as cyclin-dependent kinase 1, that has a role in the cellular process including cell cycle regulation, apoptosis and autophagy. As a cyclin-dependent kinase 1, it also can directly bind and block the activity of cyclin-CDK2, CDK1 and CDK4/6 complexes, leading in cell cycle blockage at resting phase. The bond between p21 and CDK is strengthened by the cyclin associated with CDK. P21 has a vital role as a cell cycle progress checkpoint, to prevent replication and decrease the amount of DNA damage in its sub-cells [8].

2. MATERIALS AND METHODS

This is a laboratory experimental study with a post control group design. This study was conducted in the Biochemistry Laboratory and Pharmacology Laboratory, Faculty of Medicine, Brawijaya University,

Malang City, after an ethical approval was signed. Laboratory experiment conducted in this study used C666-1 cell lines (undifferentiated nasopharyngeal carcinoma) provided by American Type Culture Collection (ATCC). To achieve a certain cisplatin dose, every group was tested three times under Lc50 [9]. According to a study cisplatin dose used in an in vitro study, using Hela cells, was 1-50 µM; achieving a toxic dose at 13 µM [10]. Those results were tested on C666-1 cell lines by observing the O_2-value (ABCAM Kit) and SOD activity (SOD Assay Kit (STA 340). Immunohistochemistry (monoclonal human antibody) examination was performed on C666-1 cell lines by calculating the amount of staining. Thep21/WAF-1 (SCYTEK TM) reagen was used to analyze p21 expression by the intensity of brown staining. Proliferation rate was analyzed by measuring MTT (cell Quanti-MTT TM(3-(4,5-dimethylthiazole-2-yl)-2,5-siphenyltetrazolium bromide)) while flow cytometry was used to calculate apoptosis. (Apoptosis Kit (FTIC Anexin V Apoptosis Detection Kit)) using Image-based Cytometer.

Data analysis performed to differentiate dependent variables in more than two unpaired data groups (or data from different subjects) was with one-way ANOVA test. This study was to explore the differences of superoxide anion, SOD, cell proliferation (MTT) and apoptosis in C666-1 cell lines and experimented group after administration of cisplatin dosages of 7.86 µg/mL; 15.36 µg/mL and 30.72 µg/mL.

3. RESULTS

This study reported the effects of administration of different cisplatin dosages on superoxide anion, SOD, proliferation and apoptosis, analyzed based on F test using one-way ANOVA, as shown in the corresponding table.

Table 1. Mean difference of superoxide anion (O_2^-) in C666-1 cell lines between cisplatin dose groups of 7.86 µg/mL; 15.36 µg/mL and 30.72 µg/mL

Study Groups	Mean of superoxide anion	Difference of superoxide anion	p-value
Dose 7.86 vs dose 15.36	20.591 vs 22.242	↑ 1.6513	0.941
Dose 7.86 vs dose 30.72	20.591 vs 23.176	↑ 2.5847	0.863
Dose 15.36 vs dose 30.72	22.242 vs 23.176	↑ 0.9333	0.980
F test			**0.141**
P value			**0.871**

There was no significant difference (P > 0.05) found between cisplatin dose groups 7.86 µg/mL and 15.36 µg/mL (P = 0.941; P > 0.05), between cisplatin dose groups 7.86 µg/mL and 30.72 µg/mL (P = 0.863; P > 0.05), and between cisplatin dose groups 15.36 µg/mL and 30.72 µg/mL (P = 0.980; P > 0.05).

Table 2. Mean difference of SOD in C666-1 cell lines between cisplatin dose groups of 7.86 µg/mL; 15.36 µg/mL and 30.72 µg/mL

Study Groups	Mean of SOD	Difference of SOD	p-value
Dose 7.86 vs dose 15.36	0.233 vs 0.804	↑ 0.5717	0.001
Dose 7.86 vs dose 30.72	0.233 vs 0.839	↑ 0.6060	0.001
Dose 15.36 vs dose 30.72	0.804 vs 0.839	↑ 0.0343	0.442
F Test			**336.179**
P value			**0.001**

There was a highly significant difference (P < 0,01) between cisplatin dose groups 7.86 µg/mland15.36 µg/mL (P = 0.001; P < 0,01)and between cisplatin dose groups 7.86 µg/mLand30.72 µg/mL (P = 0,001; P< 0,01), but there was no significant difference found between cisplatin dose groups 15.36 µg/mL and30.72 µg/mL (P = 0.442; P > 0.05).

Table 3. Mean difference of proliferation (MTT)in C666-1 cell lines between cisplatin dose groups 7.86 µg/mL; 15.36 µg/mL and 30.72 µg/mL

Study Group	Mean of MTT	Difference of MTT	p-value
Dose 7.86 vs dose 15.36	76.459 vs 37.035	↓ 39.4233	0.151
Dose 7.86 vs dose 30.72	76.459 vs 32.650	↓ 43.8090	0.111
Dose 15.36 vs dose 30.72	37.035 vs 32.650	↓ 4.3857	0.968
Uji F			**3.604**
P value			**0.094**

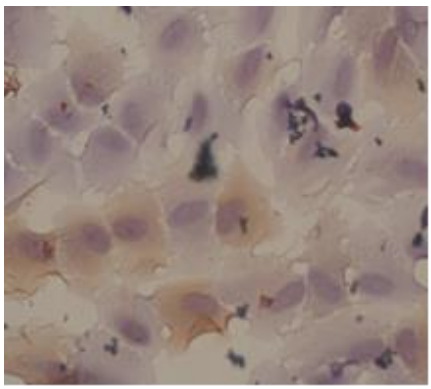
There was no significant difference (P > 0.05) found between cisplatin dose groups 7.86 µg/mLand15.36 µg/mL (P = 0.151; P > 0.05), between cisplatin dose groups 7.86 µg/mLand30.72 µg/mL (P = 0.111; P > 0.05), and between cisplatin dose groups 15.36 µg/mL and30.72 µg/mL (P = 0.968; P> 0.05).

Table 4. Mean difference of apoptosis in C666-1 cell lines between cisplatin dose groups 7.86 µg/mL; 15.36 µg/mL and 30.72 µg/mL

Study Groups	Mean Apoptosis	Difference of Apoptosis	p-value
Dose 7.86 vs dose 15.36	20.387 vs 7243	↓ 13.1433	0.099
Dose 7.86 vs dose 30.72	20.387 vs 11.047	↓ 9.3400	0.249
Dose 15.36 vs dose 30.72	7.243 vs 11.047	↑ 3.8033	0.755
Uji F			**3.379**
P value			**0.104**

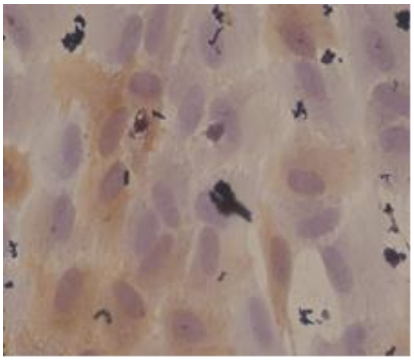
There was no significant difference (P > 0.05) found between cisplatin dose groups 7.86 µ/mLand15.36 µg/mL (P = 0.099; P > 0.05), between cisplatin dose groups 7.86 µg/mLand30.72 µg/mL (P = 0.249; P > 0.05), and between cisplatin dose groups 15.36 µg/mL and30.72 µg/mL (P = 0.755; P > 0.05).

After cisplatin administration dose of 7.86 µ/mL, there was nuclear p21 expression that started to release to the cytoplasm (24 hours).



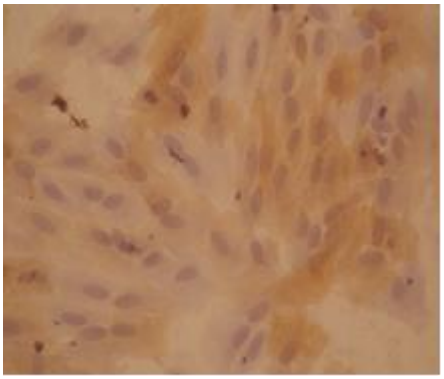
Picture 1. p21 expression results after cisplatin administration dose of 7.86 µg/mL

After cisplatin administration dose of 15.36 µ/mL, there was nuclear p21 expression that was released to the cytoplasm (brown staining partially filling the field of view) (24 hours).



Picture 2. p21 expression results after cisplatin administration dose of 15.36 µg/mL

After cisplatin administration dose of 30.72 µ/mL, there was nuclear p21 overexpression that was released to the cytoplasm (brown staining almost filling the field of view) (24 hours).



Picture 3. p21 expression results after cisplatin administration dose of 30.72 µg/mL

4. DISCUSSION

Research data obtained after cisplatin administration in different dosages will increase superoxide anion, even though there was no significant difference statistically. Cisplatin will increase ROS, which will cause damage to fat cells and lead to failure of mitochondrial function [11, 12]. In the past few years, several studies have tried to explain the relationship between cisplatin and the increase of ROS. ROS, in a smaller scale, plays a role in the apoptotic pathway, where the increase of ROS will change the mitochondrial potential membrane and destruct the cell's respiratory chain, acting as the first trigger for apoptosis.

Superoxide radicals play a main role in the cells as shown in table A, where it increases after cisplatin administration in different dosages. SOD also plays a role as a main element in. maintaining oxidant and antioxidant balance. SOD also functions in protecting aerobic organisms in fighting off side effects of superoxide. This enzyme can be found in all aerobic tissues, especially in the mitochondria and cytosol. SOD as an antioxidant enzyme (catalase [CAT], glutathion reductase [GSH-R], GSH peroxidase [GSH-PX], glutathione-S-transferase and glutamyl cysteine synthetase) has a cytoprotective effect in fighting cisplatin side effects in in-vitro models, as performed in this study.

There was a decrease of proliferation in C666-1 cell lines, as shown in this study, even though it was statistically insignificant, this can be explained by the ability of cisplatin to block proliferation of cancer cells. In other studies, there appears to be other proliferation pathwaysin C666-1 cell lines that causes MAPK(Mitogen Activated Protein Kinase) to promote cell growth and proliferation [13]. The p21 that was observed in this study was seen released from the nucleus, causing itunable to act as a tumor suppressor (shown as the brown staining in immunohistochemistry examination). The role of p21 after cisplatin administration in C666-1 cell lines, correlated with the function of p53 is still unclear. The role of cell cycle already began since the sensitization of cisplatin through mediation from down regulation in p53 pathway to p21 (WAF1(Wild-type 53 activated fragment-1). The increase sensitivity of cisplatin was shown by the loss of contribution of p21 at the G2/M resting phase. This condition will result in premature cell mitosis and those cell lines will die before becoming mature.

There was an increase and decrease of apoptosis in C666-1 cell lines after administration of cisplatin in different dosages even though it was statistically insignificant. The increase of apoptosis in C666-1 cell lines happened after the administration of cisplatin at30.72 µg/mL, compared to smaller dosages (7.86 µg/mL and 15.36 µg/mL), where decrease of apoptosis was found. This is similar to a previous study where cisplatin induces cell death through apoptosis and cause defect in the apoptotic pathway. There are 2 main mechanisms of cell apoptosis : 1) Extrinsic pathway, by bonding with tumor necrosis factor-α (TNFα) receptor super family, followed by procaspase 8 through intrinsic pathway molecule, thedeath-inducing signaling complex (DISC); 2) Intrinsic pathway, by initiating cell stress, for example where cytochrome-c is released from the mitochondria, causing activation of procaspase 9 through interactions with apoptosis promoting activating factor-1 (APAF-1) and formation of an active apoptosome complex. Bcl2 protein family regulates pathways where apoptosis is induced by DNA damage by releasing mitochondrial cytochrome-c in response to DNA damage [14]. Results obtained from this study may become the foundation of cisplatin dosage administration in cancer patients (nasopharyngeal carcinoma patients), where the maximum dosage will affect the cancer cell growth. Cisplatin will induce genotoxic stress in several biomolecular pathways related to apoptosis..

5. CONCLUSION

Cisplatin administration in different dosages will increase superoxide anion even though it was statistically insignificant. Cisplatin will increase ROS, as the cause of fat cell damage and failure to the mitochondrial function [15].

There was an increase of SOD after administration of a high dose of cisplatin where a balance of ROS production and detoxification process by antioxidants (SOD) occurred.

Cisplatin administration in different dosages will block cancer cell proliferation (C666-1 cell lines) studied that there are other pathways related to MAPK that promotes cell proliferation.

Increase of apoptosis in C666-1 cell lines occurred after cisplatin administration at the dose of 30.72 µg/ml in 24 hours. This is similar to a previous study where cisplatin induced cell death and caused defect in the apoptotic pathway.

After cisplatin administration at the dose of 30.72 µ/mL, there was overexpression of nuclear p21 that was released to the cytoplasm (the brown staining filling the field of view) (after 24 hours) This shows that p21 was released from the nucleus to the cytoplasm indicating failure of cell checkpoint.

This study revealed the effect of cisplatin agent in different dosages experimented in vitro in C666-1 cell lines. Cisplatin is an antineoplastic drug, acting as an alkylating agent, serving as an effective chemotherapy drug for nasopharyngeal carcinoma up to this date. One of the focal points of this study is the characteristic of cisplatin experimented on C666-1 cell lines. The purpose of this study is to prove the effect and role of cisplatin on the increase of superoxide anion and SOD even though balance was not yet achieved (needs further studies), the decrease of cell proliferation and the increase of apoptosis of NPC cells (C666-1 cell lines) will eventually give an exact image of cisplatin administration in NPC patients.

Administration of antioxidants in the use of cisplatin may be considered to maintain the homeostasis function in healthy cells between oxidants and antioxidants after cisplatin administration in NPC patients.Using the antioxidants agent is highly recommended according to this study.

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