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Veterinary Medicine Inter... 17 Jan

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Authors' Feedback Needed : 5599452

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Dari: **Sumanta Nandi** <neil.caringal@hindawi.com>
Date: Sel, 23 Mar 2021 pukul 11.58
Subject: 5599452: Revision requested
To: <herinda.pertiwi@vokasi.unair.ac.id>

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We are pleased to inform you that your manuscript, AIF PROTEIN EXPRESSION AND APOPTOSIS CHANGES WITH GLUTAMINE IN PODOCYTES CELLS EXPOSED CISPLATIN, has been accepted for publication in the journal, Veterinary Medicine International. We will be in touch shortly to request electronic files for your manuscript.

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Maulida

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Material checker

e. [subaranjini.suresh@hindawi.com]

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(Lu et al., 2008; Madrazo-Ibarra and Vaitla, 2020; Mazieres et al., 2013; Miller et al., 2010; Natarajan and Becker, 2012; Nematbakhsh et al., 2017; Newsholme et al., 1986; Pabla and Dong, 2008; Papaliagkas et al., 2007; Shah et al., 2020; World Health Organization, 2020)

Best Regards,
Subaranjini

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Material checker

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4

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Thanks for your email.

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Thank you very much.

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Dear Dr. Maulida,

I am pleased to let you know that your article has been published in its final form in "Veterinary Medicine International."

Himmayatussorofil Maulida, "Apoptosis-Inducing Factor, Protein Expression, and Apoptosis Changes with Glutamine in Podocytes Cells Exposed with Cisplatin," Veterinary Medicine International, vol. 2021, Article ID 5599452, 7 pages, 2021. https://doi.org/10.1155/2021/5599452.

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Draf 22 Apr
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REVISI I

AIF PROTEIN EXPRESSION AND APOPTOSIS CHANGES WITH GLUTAMINE IN PODOCYTES CELLS EXPOSED CISPLATIN

Imam Susilo[1], Himmayatussorofil Maulida[2], Lindawati Alimsardjono[3], Dyah Fauziah[4], Herinda Pertiwi[5]

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[4]Departement of Pathological Anatomy, Faculty of Medicine, Universitas Airlangga, Jalan Mayjen Prof. Dr. Moestopo 47, Surabaya, Indonesia; dyah_fauziah@yahoo.com

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Corresponding authot: Himmayatussorofil Maulida: Medicine Undergraduate Program, Faculty of Medicine, Universitas Airlangga, Jalan Mayjen Prof. Dr. Moestopo 47, Surabaya, Indonesia; 60132 himmayatussorofil.maulida-2018@fk.unair.ac.id;Tel. +62 813-3095-3848

Running title: Glutamine Effect on Apoptosis-inducing factor Expression

Abstract

Cisplatin is a well-known chemotherapeutic drug as one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. However, side effects in normal tissues and organs, like nephrotoxicity induce apoptosis epithelial cells in kidney, limit the use of cisplatin. Glutamine is a substrate for the synthesis of glutathione as antioxidant, and promote HSP70 release, protecting cells from apoptosis induced by different stimuli. In the present study, we investigated the protective effect of glutamine on nephrotoxicity cisplatin in the kidney. Mice were divided into three groups a group of control (P0), a group of intraperitoneal injection of a single dose cisplatin 20mg/kg BW at 7th day (P1), and a group of intravenous glutamine injection 100mg/kg BW at day 1—7 and given an intraperitoneal injection of single dose cisplatin 20mg/kg BW at 7th-day (P2). Measurement of AIF expression and apoptotic cells was carried out by immunohistochemical methods. The number of AIF expressions and apoptotic cells is expressed in Allerd score. AIF expression result: P0: 3.29

±0.79 P1: 5.32 ±0.68 P2: 4.49 ± 0.47. Apoptosis result: P0: 3.04 ±0.70 P1: 5.26 ±0.53 P2: 4.44 ± 0.41. There is decreased expression of AIF on intravenous glutamine administration, followed by a decrease in apoptosis in the podocyte. In conclusion, glutamine administration might represent for the treatment of nephrotoxic induced cisplatin.

Keyword: Cisplatin, Glutamine, AIF Protein, Apoptosis, Podocyte

Introduction

Cancer is a large group of diseases that can occur in almost any organ or tissue of the body when abnormal cells grow uncontrollably beyond normal limits to invade adjoining parts of the body and/or spread to other organs. Cancer is the second leading cause of death globally, with an estimated 9.6 million deaths, or one in six deaths, in 2018. In Indonesia, 348,809 people suffered from cancer, and 207,210 died from cancer in 2018 (WHO, 2020).

Cisplatin (CAS No.15663-27-1, MF-Cl2H6N2Pt; NCF-119875), cisplatinum, also called cis-diamminedichloroplatinum (II), is the first platinum drug approved globally for the treatment of cancer in 1978. Cisplatin is one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. Cisplatin can bind to purine bases in DNA, interfered DNA repair, causes DNA damage, and then induces apoptosis in cancer cells (Achkar et al., 2018; Dasari & Tchounwou, 2015)

Behind the benefits of cisplatin, there are various side effects, one of them is nephrotoxic. Cisplatin nephrotoxicity can present with a variety of symptoms of acute kidney injury (AKI), hypomagnesemia, fanconi-like syndromes, distal renal tubular acidosis, hypokalemia, wasted renal salt, and hyperuricemia. However, the most serious and most common side effect is AKI, which occurs in 20-30% of patients and hypomagnesemia which occurs in 40-100% of patients (Miller et al., 2010).

Cisplatin-induced nephrotoxic can be pathophysiologically classified into four types, tubular toxicity (cell death by apoptosis or necrosis), blood vessel damage (renal vasoconstriction), glomerular injury (damage to the glomerular compartment including capillaries, basement membrane, mesangial cells, podocyte cells, and parietal cells), and interstitial injury (damage from the inflammatory response). (Oh et al., 2014).

The mechanisms of cisplatin-induced nephrotoxicity are complex and involve many cellular processes including oxidative stress, apoptosis, and inflammation. Tsuruya et al. in 2003 observed that renal epithelial cells deficient in TNFR1 and Fas were resistant to cisplatin-induced cell death (Tsuruya et al. 2003). Seth et al. in 2005 identified that cisplatin increases caspase-2 and caspase-3 activity (Seth, et al., 2005). Takeda et al. in 1997 demonstrated that caspase-8 inhibition reduced cisplatin-induced cell death in vitro (Takeda et al., 1997).

Apoptosis-inducing factor (AIF) is a mitochondrial oxidoreductase that plays a role in oxidative phosphorylation and redox control in normal cells. AIF was initially cloned and identified as a caspase-independent mitochondrial effector of apoptotic cell death. AIF is usually confined to the mitochondrial intermembrane space and released in response to death

stimuli, and after the induction of apoptosis, AIF is translocated to the nucleus, where it affects the chromosome condensation and fragmentation. Besides, AIF can induce mitochondria to release apoptogenic-proteins including cytochrome c and caspase-9 (Wang et al., 2019).

Cytoplasmic interactions of apoptogenic AIF. Once released into the cytoplasm, AIF can promote apoptosis by interacting with CYP, in which CypA assists in apoptogenic cytonuclear translocation of AIF. In contrast, Hsp70 maintains AIF in the cytoplasm and, therefore, can delay or prevent initiation of nuclear apoptosis. (Sevrioukova, 2011).

Glutamine is an α-amino acid and is the most abundant free amino acid in the body. Glutamine has two amino groups, namely an α-amino group and a side-chain amide group that is easily hydrolyzed (Cruzat et al., 2018). The function of glutamine within cells is generally separated into four categories: its role in nitrogen transport; the importance of maintaining a cellular redox state; its position as a metabolic intermediary; and its role as an energy source. (Newsholme et al. 1986; Shah, Wang & Ma, 2020).

Glutamine plays an important role in the modulation of HSP expression via the biosynthetic pathway hexosamine (HBP). HSP70 (HSP72 and HSP 73) acts as anti-inflammatory protein based on deactivating NF-κB and attenuates the production of inflammatory mediators. In addition, HSP70 modulates autophagy by regulating the mTOR / Akt pathway and blocking signaling pathways associated with protein degradation (Cruzat et al., 2018).

GSH is an antioxidant that can react directly with ROS, produce oxidized GSH (GSSG), and can also donate electrons for peroxide reduction, which is catalyzed by the enzyme glutathione peroxidase (GPx). Glutamine (via glutamate), cysteine, and glycine are precursor amino acids for the synthesis of GSH. (Cruzat et al., 2018).

Cell damage due to exposure to cisplatin chemotherapy is generally studied in the proximal renal tubule area because there is a process of reabsorption and primary urine secretion in that area. However, kidney cell damage may occur in glomerular visceral epithelial cells (podocyte) as a filtration site in the urinary system, which causes all substances to pass through the filtering process in the glomerulus before going to the proximal tubule and directly exposed to cisplatin. Therefore, research on apoptosis and AIF expression was carried out on glomerular visceral epithelial cells (podocyte).

This study is analyzing the nephroprotective effect of intravenous glutamine on the incidence of apoptosis of the glomerular epithelial cells (podocyte) by examining the expression of AIF which is the initiator of apoptosis in the apoptosis independent caspase. This research is expected to be an alternative problem solving for kidney failure caused by cisplatin chemotherapy modalities.

Material and Methods

Animal and housing

2-3 months old wistar male white mouse weighing 150–200 g were used after one week for proper acclimatization to the animal house conditions (12 h lighting cycle and 29-31°C

temperature) with free access to water and standard rodent chow. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, Airlangga University, Indonesia. Animals were randomly divided into three groups with 10 animals in each group. The first group as the control group (P0). The second group was treated with cisplatin (20 mg/kg I.p) as a positive control (P1). The third group was injected with glutamine (100 mg/kg, i.v.), a gram glutamine suspended in 10 ml solution of ml 0.9% daily for seven consecutive days, and injected with cisplatin (20 mg/kg I.p) on the seventh day to induce nephrotoxicity (P2). All groups received equivalent volumes of the used vehicles. Mouse were sacrificed on the tenth day. The longitudinal section of the left kidney was excised from each animal for immunohistochemical examination.

Glutamine product from Serva-Germany, cisplatin product from Kalbe Farma, POD Apoptosis Detection Kit (11684817910 ROCHE), Anti-AIF Antibody (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880))

Immunohistochemical Examination

Kidney tissue samples were fixed in 10% buffered neutral formalin, embedded in paraffin, cut, and stained with immunohistochemical staining for AIF expression and apoptosis examination using light microscopy.

Immunohistochemical detection of AIF expression was conducted using anti-AIF antibodies (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880)). Immunohistochemical staining was carried out with anti-AIF antibodies in brief steps as follows: Deparaffinated preparations on glass objects; Washed with PBS pH 7.4; Blocking endogenous peroxide with 3% H2O2 for 20 minutes; Blocking unspecific protein with 5% FBS; Incubation with primary antibody (anti-AIF antibody) overnight at 4^0C, Incubation with conjugated anti-mouse biotin for 1 hour at room temperature; Incubation with Strep-Avidin Horse Radish Peroxidase for 40 minutes, drop DAB and incubate for 10 minutes; Counterstaining with Mayer Hematoxylin, the preparation is rinsed with dH2O and aerated; Mounting with a swab and the preparation is covered with a glass cover.

Immunohistochemical detection of apoptosis was processing by Apoptotic Detection Kit POD (11684817910 ROCHE) with the following brief steps: Deparaffinated tissue; Preparations given proteinase K for 15 minutes, and dH2O in a Coplin jar for 2 x 2 minutes; Removal endogenous peroxide with 3% H2O2 for 5 minutes at room temperature; Drop the working strength of tdT enzyme in tissues, incubate at 37oC for 1 hour; Place the preparation in a coplin jar containing the working strength of the stop / wash buffer and incubated for 10 minutes at room temperature; Drop anti-digoxigenin conjugate incubation at room temperature in a damp container for 30 minutes; Staining with substrate peroxidase for 10 minutes at room temperature; Counterstaining with methyl green for 30 seconds at room temperature; Covered with a glass cover.

Statistical analysis

The data are expressed as means ± SEM. Statistical analysis was performed by one-way ANOVA followed by LSD post-analysis test for multiple comparisons with α = 0,05 being considered as statistically significant, and Pearson correlation with α = 0,05 being considered as statistically significant.

Result

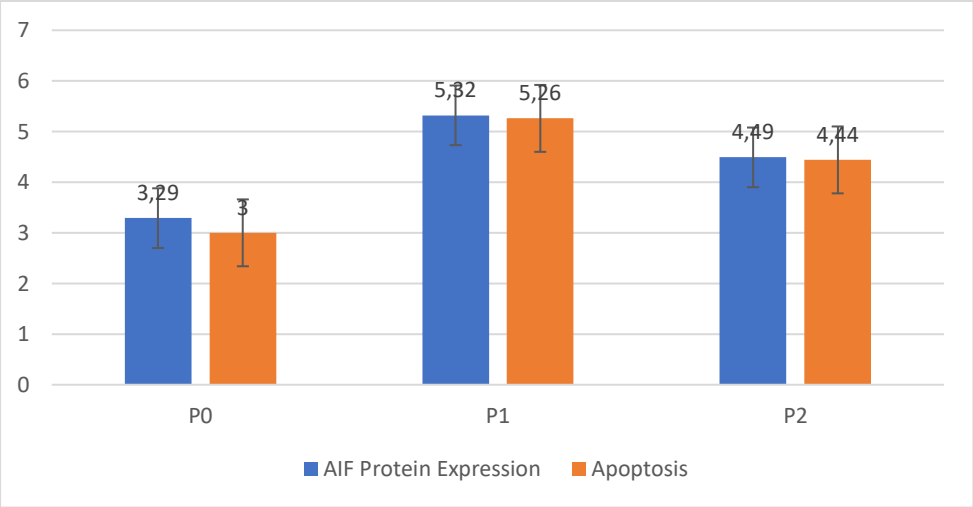

Figure 1. Graph of mean score of AIF protein expression and apoptosis.

The results of the AIF expression and apoptosis study used IHC staining and assessed with the H score shows a significant difference in each group (Figure 1). Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of AIF expression compared to giving cisplatin without glutamine administration (P1). The mean score of AIF expression in P2 with score 4.49 ± 0.47 compared to of P1 with score 5.32 ± 0.68 (Table 1, Table 2 and Table 3).

Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of apoptosis compared with giving cisplatin without glutamine administration (P1). The mean score of apoptosis in P2 with score 4.44 ± 0.41 compared to P1 with score 5.26 ± 0.53 (Table 1).

Discussion

In this study, mice were divided into three groups, each group consisted of 10 male white mice. The negative control group (P0), the positive control group (P1), and the treatment group (P2). P1 had the largest mean AIF expression and apoptosis compared to other groups.

The research data were analyzed using the ANOVA test and if there were differences, it would be followed by a multiple comparison test, LSD. To use the ANOVA test, several

conditions must be fulfilled: the sample comes from independent data, the variance between groups must be homogeneous and the data in each group is normally distributed. The homogeneity test in this study used the Levene test and showed a sig value,> 0.05, which means that the data in this study were homogeneous, for normality test used the Shapiro-Wilk test and showed the sig value > 0.05, which means that the data in this study were normally distributed (Table 2, Table 3, Table 4 and Table 5).

The research data were correlation using Person correlation, condition must be fulfilled, the data must be normal, the normality test used Kolmogorov-Smirnov test and showed the sig. value > 0.5, which means that the data in this study were normal (Table 6 and Table 7).

Apoptosis and AIF excretion in glomerular visceral epithelial cells (podocyte) in all groups in this study. Apoptosis and AIF expression in the positive control groups (P1) and (P2) are apoptotic processes triggered by cisplatin chemotherapy as a substance that has a nephrotoxic effect (Figure 2 and 3). Cisplatin-induced nephrotoxicity can occur from several pathways, including extrinsic, intrinsic apoptosis that can trigger AIF expression, cell regulators, MAPK, inflammation, and ROS. Meanwhile, apoptosis in the negative control group (P0) can be caused by physiological processes that can be experienced by all cells. The influence of external variables that cannot be controlled can also cause apoptosis in the glomerular visceral epithelial cells (podocyte).

The Effect of Glutamine on AIF Protein Expression

ANOVA test results on the AIF expression variable (Table 4), showed different evidence between groups in 1 research variable. With the LSD comparison test between P0 and P1, the mean score of AIF expression P0 = 3.29 ± 0.79 and P1 = 5.32 ± 0.68 (Table 5) had a significant difference from the expression AIF protein in the glomerular visceral epithelial cells (podocyte). The increase in AIF excretion in the positive control group (P1) was caused by exposure to cisplatin, an increase in free radicals and DNA damage which caused the maturation of the P53 gene to induce AIF protein transcription and induce Bcl-2 causing mitochondrial dysfunction to form holes in the mitochondrial membrane, so that the AIF protein could translocate to cytoplasm and nucleus.

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. Apoptosis, which represents the form of cell death performed by caspases, has traditionally been the only form of physiological and programmed cell death. However, recent evidence suggests that programmed cell death (PCD) can occur in the absence of caspase activation at all. Indeed, a large number of caspase-independent models are now defined and a key protein involved in this type of PCD, the apoptosis inducing factor (AIF), has been identified. Cisplatin causes changes in the mitochondrial PTP to cause the pores in the mitochondria to open and allow small molecules such as the AIF protein to escape (Yang et al. 2008)

LSD test results on P1 and P2 with a mean score of P1 = 5.32 ± 0.68 and P2 = 4.49 ± 0.47 (Table 5) had a decreased AIF excretion involved in glomerular visceral epithelial cells (podocytes). The decrease in AIF expression in the treatment group (P2) was due to glutamine, which is the precursor to glutathione in cells, directly binding to free radicals which can prevent mitochondrial dysfunction due to cisplatin administration. In addition, glutamine can increase the expression of Hsp 70 which is an antiapoptotic agent that can prevent various cell death pathways, one of which is by inhibiting the maturation of P53 to induce Bcl-2, preventing Bcl-2 from causing mitochondrial dysfunction, so that translocation of AIF protein from mitochondria can be prevented.

These results are consistent with previous studies conducted by Sabirzhanov et al., 2012, showing that HSP70 modulates apoptosis of the caspase-independent pathway in primary cortical neurons and SH-SY5Y cells through interaction with AIF and by preventing translocation to the nucleus (Sabirzhanov, et al., 2012).

The Effect of Glutamine on Cell Apoptosis

ANOVA test results on the apoptosis variable (Table 4), showed a significant difference between groups in 1 research variable. With the LSD double comparison test between P0 and P, 1 mean apoptosis score of P0 = 3.04 ± 0.70 and P1 = 5.26 ± 0.53 (Table 5) had a significant difference from the number of cells experiencing apoptosis in the glomerular visceral epithelial cells (podocyte). The increase in apoptosis in the positive control group (P1) due to cisplatin exposure triggered an increase in free radicals that can activate various cell death pathways. In addition, cisplatin induces maturation of the p53 gene and induces apoptosis.

These results are consistent with previous studies conducted by Marullo, et al., 2013, showing that cisplatin exposure induces a mitochondrial dependent ROS response that significantly contributes to cell killing by enhancing the cytotoxic effect exerted through the formation of nDNA damage and Yang et al., 2008, which states that translocation of the AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Marullo, et al., 2013; Yang et al., 2008)

LSD test results on P1 and P2 with an average score of P1 = 5.26 ± 0.53 and P2 = 4.44 ± 0.41 (Table 5) had a significant reduction in the number of apoptosis in glomerular visceral epithelial cells (podocytes). The decrease in apoptosis in the treatment group (P2) was due to glutamine which is a precursor to GSH, which is a powerful antioxidant that can bind free radicals triggered by cisplatin administration. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing apoptosis of the caspase-independent pathway and the caspase-dependent pathway. So that apoptosis due to cisplatin can be prevented.

These results are in accordance with the theory described by Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2017, stated that glutamine is a precurso of GSH where GSH is a powerful antioxidant and plays an important role in the metabolism of exogenous and endogenous substances. GSH participates in many cellular reactions. It directly

scavenges free radicals and other reactive oxygen species (hydroxyl radicals, lipid peroxyl radicals, peroxynitrite, and H2O2), and is indirectly linked to enzymatic reactions that can decrease apoptosis. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent through the HBP pathway and can also reduce apoptosis (Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2016).

Correlation of AIF Protein Expression and Apoptosis

The results of the Pearson correlation test on the AIF and apoptosis expression (Table 7 and Table 6) variables showed that the correlation was very strong with the direction of the relationship being directly proportional. Increased AIF expression and increased apoptosis in the positive control group (P1) were strongly associated. The increase in AIF expression caused by cisplatin exposure triggers AIF synthesis and AIF translocation to the nucleus and causes condesation and large-scale chromatin fragmentation which triggers apoptosis.

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Yang et al., 2008).

The decrease in AIF expression and the decrease in apoptosis in the treatment group (P2) were strongly associated. The decrease in AIF expression in the group injected with glutamine prior to cisplatin administration was due to the nephroprotective effect of glutamine as an antioxidant precursor that can bind to antioxidants directly and so it can inhibit / prevent mitochondrial dysfunction which can cause AIF protein to translocate to the cytoplasm and nucleus. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing translocation of AIF to the nucleus, maturation of the P53 gene, and Bcl-2 pro apoptosis. Prevents AIF protein from translocating to the cytoplasm and nucleus and causing large-scale condesation and fragmentation of chromatin, thus preventing apoptosis.

These results are in accordance with previous studies conducted by Ruchalski et, al. 2003, which stated that Hsp 72 inhibits the release of AIF protein from mitochondria which can inhibit apoptosis in renal epithelial cells exposed to metabolic inhibitors (Ruchalski et al. 2003)

CONCLUSION

Based on the research that has been conducted, there is a change in the expression of AIF and apoptotic cells in intravenous glutamine administration on glomerular visceral epithelial cells (podocyte) of male white mouse exposed to cisplatin, the changes that occur in this study are in the form of a decrease. Decreased expression of AIF on intravenous glutamine administration is correlation with a decrease in apoptosis in the glomerular visceral cells (podocyte). Glutamin administration can decrease AIF expression and apoptosis that induced by cisplatin administratio. Glutamine administration might represent for the treatment of nephrotoxic induced cisplatin

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FIGURE AND TABLE

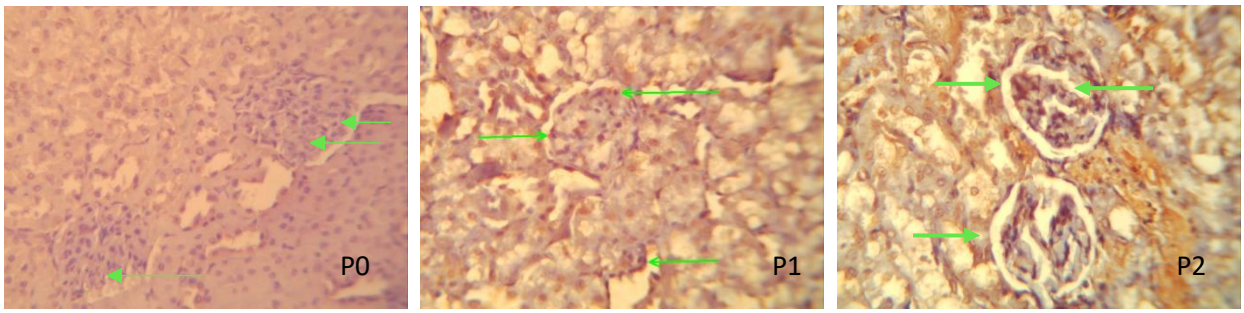

Figure 3. Morphology of the glomerular epithelial cells given anti-AIF antibodies. P0 is control, in which the mouse's kidney is not treated. P1 is a kidney of mouse given cisplatin i.p on the 7th day. P2 is the kidney of mouse injected glutamine i.v 7 days in a row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

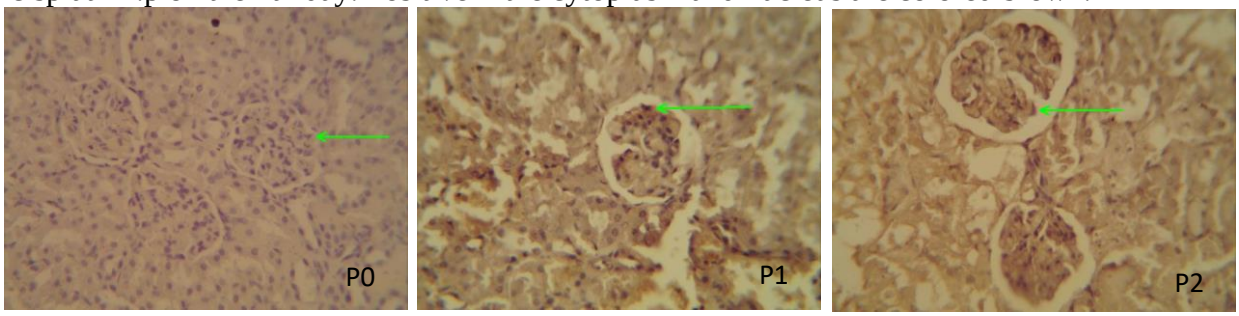

Figure 2. Morphology of the glomerular epithelial cells used Apoptotic Detection Kit. P0 is control, in which the mouse's kidney is not treated. P1 is a kidney of mouse given cisplatin i.p on the 7th day. P2 is the kidney of mouse injected glutamine i.v 7 days in a row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

Variable	AIF protein expression	Apoptotic cells
	Average ± sd	Average ± sd
P0	3.29±0.79	3.04±0.69
P1	5.32±0.68	5.26±0.53
P2	4.49±0.47	4.44±0.41

Table 1. Descriptive statistic of the AIF protein expression and apoptotic cells. P0 is control, in which the rat's kidney is not treated. P1 is a kidney that is given cisplatin injection on the 7th day. P2 is the kidney of rat given intravenous glutamine injection 7 days in a row before being injected intraperitoneal with cisplatin on the 7th day.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
P0	0.702	0.137
P1	0.539	0.189
P2	0.733	0.647

Table 2. The result of test normality Shapiro-Wilk showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
Levene Statistic	0.150	0.448

Table 3. The result of homogeneity variances Levene Statistic showed a sig value,> 0.05, which means that the data in this study were homogeneous.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
ANOVA	0.000	0.000

Table 4. The result of AVONA showed the sig value > 0.05, which means that the data in this study had different results.

Variable	Comparison		Sig.	Interpretation
AIF protein expression	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.009	
Apoptotic cells	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.003	

Table 5. The result of LSD showed the sig value > 0.05, which means that the data in this study had different results in each group.

Variable	AIF protein expression	Apoptotic cells
	Sig. (2-tailed)	Sig. (2-tailed)
Kolmogorov-Smirnov Test	0.200	0.200

Table 6. The result of test normality Kolmogorov-Smirnov showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	Correlation	Sig.	Interpretation
AIF protein expression and Apoptotic cells	0.928	0.00	Correlation is significant and linier

Table 7. The result of Pearson correlation showed the r (Pearson correlation) > 0.349 (positive) which means that the data in this study had correlation and linier. Thus, showed the sig value > 0.05, which means that the data in this study had a significant correlation between AIF protein expression and apoptosis cells.

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REVISI II

AIF PROTEIN EXPRESSION AND APOPTOSIS CHANGES WITH GLUTAMINE IN PODOCYTES CELLS EXPOSED CISPLATIN

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Running title: Glutamine Effect on Apoptosis-inducing factor Expression

Abstract

Cisplatin is a well-known chemotherapeutic drug as one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. However, side effects in normal tissues and organs, like nephrotoxicity induce apoptosis epithelial cells in kidney, limit the use of cisplatin. Glutamine is a substrate for the synthesis of glutathione as antioxidant, and promote HSP70 release, protecting cells from apoptosis induced by different stimuli. In the present study, we investigated the protective effect of glutamine on nephrotoxicity cisplatin in the kidney. Rat were divided into three groups a group of control (P0), a group of intraperitoneal injection of a single dose cisplatin 20mg/kg BW at 7th day (P1), and a group of intravenous glutamine injection 100mg/kg BW at day 1—7 and given an intraperitoneal injection of single dose cisplatin 20mg/kg BW at 7th-day (P2). Measurement of AIF expression and apoptotic cells was carried out by immunohistochemical methods. The number of AIF expressions and apoptotic cells is expressed in Allerd score. AIF expression result: P0: 3.29 ±0.79 P1: 5.32

±0.68 P2: 4.49 ± 0.47. Apoptosis result: P0: 3.04 ±0.70 P1: 5.26 ±0.53 P2: 4.44 ± 0.41. There is decreased expression of AIF on intravenous glutamine administration, followed by a decrease in apoptosis in the podocyte. In conclusion, glutamine administration might represent for the treatment of nephrotoxic induced cisplatin.

Keyword: Cisplatin, Glutamine, AIF Protein, Apoptosis, Podocyte

Introduction

Cancer is a large group of diseases that can occur in almost any organ or tissue of the body when abnormal cells grow uncontrollably beyond normal limits to invade adjoining parts of the body and/or spread to other organs. Cancer is the second leading cause of death globally, with an estimated 9.6 million deaths, or one in six deaths, in 2018. In Indonesia, 348,809 people suffered from cancer, and 207,210 died from cancer in 2018 (WHO, 2020).

Cisplatin (CAS No.15663-27-1, MF-Cl2H6N2Pt; NCF-119875), cisplatinum, also called cis-diamminedichloroplatinum (II), is the first platinum drug approved globally for the treatment of cancer in 1978. Cisplatin is one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. Cisplatin can bind to purine bases in DNA, interfered DNA repair, causes DNA damage, and then induces apoptosis in cancer cells (Achkar et al., 2018; Dasari & Tchounwou, 2015)

Behind the benefits of cisplatin, there are various side effects, one of them is nephrotoxic. Cisplatin nephrotoxicity can present with a variety of symptoms of acute kidney injury (AKI), hypomagnesemia, fanconi-like syndromes, distal renal tubular acidosis, hypokalemia, wasted renal salt, and hyperuricemia. However, the most serious and most common side effect is AKI, which occurs in 20-30% of patients and hypomagnesemia which occurs in 40-100% of patients (Miller et al., 2010).

Cisplatin-induced nephrotoxic can be pathophysiologically classified into four types, tubular toxicity (cell death by apoptosis or necrosis), blood vessel damage (renal vasoconstriction), glomerular injury (damage to the glomerular compartment including capillaries, basement membrane, mesangial cells, podocyte cells, and parietal cells), and interstitial injury (damage from the inflammatory response). (Oh et al., 2014).

The mechanisms of cisplatin-induced nephrotoxicity are complex and involve many cellular processes including oxidative stress, apoptosis, and inflammation. Tsuruya et al. in 2003 observed that renal epithelial cells deficient in TNFR1 and Fas were resistant to cisplatin-induced cell death (Tsuruya et al. 2003). Seth et al. in 2005 identified that cisplatin increases caspase-2 and caspase-3 activity (Seth, et al., 2005). Takeda et al. in 1997 demonstrated that caspase-8 inhibition reduced cisplatin-induced cell death in vitro (Takeda et al., 1997).

Apoptosis-inducing factor (AIF) is a mitochondrial oxidoreductase that plays a role in oxidative phosphorylation and redox control in normal cells. AIF was initially cloned and identified as a caspase-independent mitochondrial effector of apoptotic cell death. AIF is usually confined to the mitochondrial intermembrane space and released in response to death

stimuli, and after the induction of apoptosis, AIF is translocated to the nucleus, where it affects the chromosome condensation and fragmentation. Besides, AIF can induce mitochondria to release apoptogenic-proteins including cytochrome c and caspase-9 (Wang et al., 2019).

Cytoplasmic interactions of apoptogenic AIF. Once released into the cytoplasm, AIF can promote apoptosis by interacting with CYP, in which CypA assists in apoptogenic cytonuclear translocation of AIF. In contrast, Hsp70 maintains AIF in the cytoplasm and, therefore, can delay or prevent initiation of nuclear apoptosis. (Sevrioukova, 2011).

Glutamine is an α-amino acid and is the most abundant free amino acid in the body. Glutamine has two amino groups, namely an α-amino group and a side-chain amide group that is easily hydrolyzed (Cruzat et al., 2018). The function of glutamine within cells is generally separated into four categories: its role in nitrogen transport; the importance of maintaining a cellular redox state; its position as a metabolic intermediary; and its role as an energy source. (Newsholme et al. 1986; Shah, Wang & Ma, 2020).

Glutamine plays an important role in the modulation of HSP expression via the biosynthetic pathway hexosamine (HBP). HSP70 (HSP72 and HSP 73) acts as anti-inflammatory protein based on deactivating NF-κB and attenuates the production of inflammatory mediators. In addition, HSP70 modulates autophagy by regulating the mTOR / Akt pathway and blocking signaling pathways associated with protein degradation (Cruzat et al., 2018).

GSH is an antioxidant that can react directly with ROS, produce oxidized GSH (GSSG), and can also donate electrons for peroxide reduction, which is catalyzed by the enzyme glutathione peroxidase (GPx). Glutamine (via glutamate), cysteine, and glycine are precursor amino acids for the synthesis of GSH. (Cruzat et al., 2018).

Cell damage due to exposure to cisplatin chemotherapy is generally studied in the proximal renal tubule area because there is a process of reabsorption and primary urine secretion in that area. However, kidney cell damage may occur in glomerular visceral epithelial cells (podocyte) as a filtration site in the urinary system, which causes all substances to pass through the filtering process in the glomerulus before going to the proximal tubule and directly exposed to cisplatin. Therefore, research on apoptosis and AIF expression was carried out on glomerular visceral epithelial cells (podocyte).

This study is analyzing the nephroprotective effect of intravenous glutamine on the incidence of apoptosis of the glomerular epithelial cells (podocyte) by examining the expression of AIF which is the initiator of apoptosis in the apoptosis independent caspase. This research is expected to be an alternative problem solving for kidney failure caused by cisplatin chemotherapy modalities.

Materials and Methods

Animal and housing

2-3 months old wistar male rat weighing 150–200 g were used after one week for proper acclimatization to the animal house conditions (12 h lighting cycle and 29-31°C temperature)

with free access to water and standard rodent chow. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, Airlangga University, Indonesia. Animals were randomly divided into three groups with 10 animals in each group. The first group as the control group (P0). The second group was treated with cisplatin (20 mg/kg I.p) as a positive control (P1). The third group was injected with glutamine (100 mg/kg, i.v.), a gram glutamine suspended in 10 ml solution of ml 0.9% daily for seven consecutive days, and injected with cisplatin (20 mg/kg I.p) on the seventh day to induce nephrotoxicity (P2). All groups received equivalent volumes of the used vehicles. Rat were sacrificed on the tenth day. The longitudinal section of the left kidney was excised from each animal for immunohistochemical examination.

Glutamine product from Serva-Germany, cisplatin product from Kalbe Farma, POD Apoptosis Detection Kit (11684817910 ROCHE), Anti-AIF Antibody (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880))

Immunohistochemical Assay

Kidney tissue samples were fixed in 10% buffered neutral formalin, embedded in paraffin, cut, and stained with immunohistochemical staining for AIF expression and apoptosis examination using light microscopy.

Immunohistochemical detection of AIF expression was conducted using anti-AIF antibodies (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880)). Immunohistochemical staining was carried out with anti-AIF antibodies in brief steps as follows: Deparaffinated preparations on glass objects; Washed with PBS pH 7.4; Blocking endogenous peroxide with 3% H2O2 for 20 minutes; Blocking unspecific protein with 5% FBS; Incubation with primary antibody (anti-AIF antibody) overnight at 4^0C, Incubation with conjugated anti-mouse biotin for 1 hour at room temperature; Incubation with Strep-Avidin Horse Radish Peroxidase for 40 minutes, drop DAB and incubate for 10 minutes; Counterstaining with Mayer Hematoxylin, the preparation is rinsed with dH2O and aerated; Mounting with a swab and the preparation is covered with a glass cover.

Immunohistochemical detection of apoptosis was processing by Apoptotic Detection Kit POD (11684817910 ROCHE) with the following brief steps: Deparaffinated tissue; Preparations given proteinase K for 15 minutes, and dH2O in a Coplin jar for 2 x 2 minutes; Removal endogenous peroxide with 3% H2O2 for 5 minutes at room temperature; Drop the working strength of tdT enzyme in tissues, incubate at 37oC for 1 hour; Place the preparation in a coplin jar containing the working strength of the stop / wash buffer and incubated for 10 minutes at room temperature; Drop anti-digoxigenin conjugate incubation at room temperature in a damp container for 30 minutes; Staining with substrate peroxidase for 10 minutes at room temperature; Counterstaining with methyl green for 30 seconds at room temperature; Covered with a glass cover.

Statistical analysis

The data are expressed as means ± SEM. Statistical analysis was performed by one-way ANOVA followed by LSD post-analysis test for multiple comparisons with α = 0,05 being considered as statistically significant, and Pearson correlation with α = 0,05 being considered as statistically significant.

Result

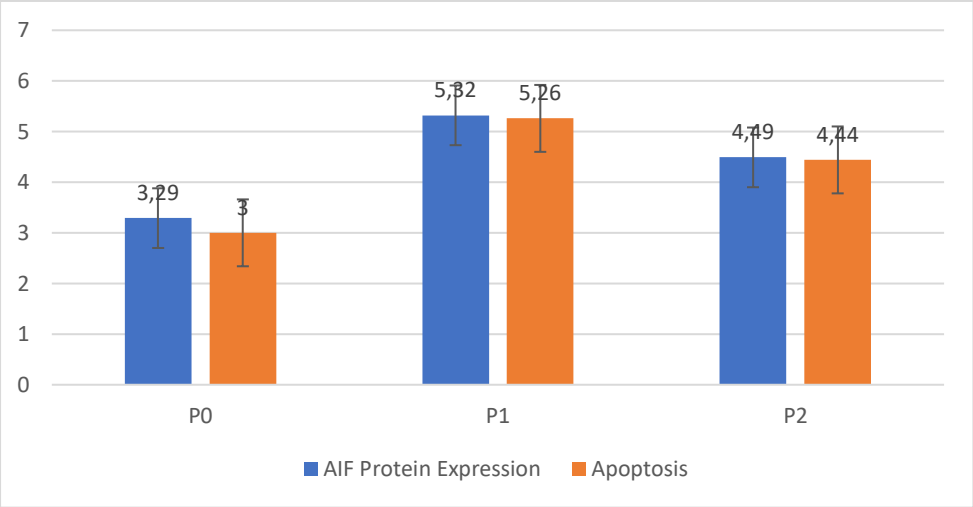

Figure 4. Graph of mean score of AIF protein expression and apoptosis.

The results of the AIF expression and apoptosis study used IHC staining and assessed with the H score shows a significant difference in each group (Figure 1). Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of AIF expression compared to giving cisplatin without glutamine administration (P1). The mean score of AIF expression in P2 with score 4.49 ± 0.47 compared to of P1 with score 5.32 ± 0.68 (Table 1, Table 2, and Table 3).

Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of apoptosis compared with giving cisplatin without glutamine administration (P1). The mean score of apoptosis in P2 with score 4.44 ± 0.41 compared to P1 with score 5.26 ± 0.53 (Table 1).

Discussion

In this study, rats were divided into three groups, each group consisted of 10 male white rats. The negative control group (P0), the positive control group (P1), and the treatment group (P2). P1 had the largest mean AIF expression and apoptosis compared to other groups.

The research data were analyzed using the ANOVA test and if there were differences, it would be followed by a multiple comparison test, LSD. To use the ANOVA test, several

conditions must be fulfilled: the sample comes from independent data, the variance between groups must be homogeneous and the data in each group is normally distributed. The homogeneity test in this study used the Levene test and showed a sig value,> 0.05, which means that the data in this study were homogeneous, for normality test used the Shapiro-Wilk test and showed the sig value > 0.05, which means that the data in this study were normally distributed.

The research data were correlation using Person correlation, condition must be fulfilled, the data must be normal, the normality test used Kolmogorov-Smirnov test and showed the sig. value > 0.5, which means that the data in this study were normal.

Apoptosis and AIF excretion in glomerular visceral epithelial cells (podocyte) in all groups were occurred in this study. Apoptosis and AIF expression in the positive control groups (P1) and (P2) are apoptotic processes triggered by cisplatin chemotherapy as a substance that has a nephrotoxic effect (Figure 2 and Figure 3). Cisplatin-induced nephrotoxicity can occur from several pathways, including extrinsic, intrinsic apoptosis that can trigger AIF expression, cell regulators, MAPK, inflammation, and ROS. Meanwhile, apoptosis in the negative control group (P0) can be caused by physiological processes that can be experienced by all cells. The influence of external variables that cannot be controlled can also cause apoptosis in the glomerular visceral epithelial cells (podocyte).

The Effect of Glutamine on AIF Expression

ANOVA test results on the AIF expression variable (Table 4), showed different evidence between groups in 1 research variable. With the LSD comparison test between P0 and P1, the mean score of AIF expression P0 = 3.29 ± 0.79 and P1 = 5.32 ± 0.68 (Table 5 and Table 6) had a significant difference from the expression AIF protein in the glomerular visceral epithelial cells (podocyte). The increase in AIF excretion in the positive control group (P1) was caused by exposure to cisplatin, an increase in free radicals and DNA damage which caused the maturation of the P53 gene to induce AIF protein transcription and induce Bcl-2 causing mitochondrial dysfunction to form holes in the mitochondrial membrane, so that the AIF protein could translocate to cytoplasm and nucleus.

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. Apoptosis, which represents the form of cell death performed by caspases, has traditionally been the only form of physiological and programmed cell death. However, recent evidence suggests that programmed cell death (PCD) can occur in the absence of caspase activation at all. Indeed, a large number of caspase-independent models are now defined and a key protein involved in this type of PCD, the apoptosis inducing factor (AIF), has been identified. Cisplatin causes changes in the mitochondrial PTP to cause the pores in the mitochondria to open and allow small molecules such as the AIF protein to escape (Yang et al. 2008)

LSD test results on P1 and P2 with a mean score of P1 = 5.32 ± 0.68 and P2 = 4.49 ± 0.47 (Table 5) had a decreased AIF excretion involved in glomerular visceral epithelial cells

(podocytes). The decrease in AIF expression in the treatment group (P2) was due to glutamine, which is the precursor to glutathione in cells, directly binding to free radicals which can prevent mitochondrial dysfunction due to cisplatin administration. In addition, glutamine can increase the expression of Hsp 70 which is an antiapoptotic agent that can prevent various cell death pathways, one of which is by inhibiting the maturation of P53 to induce Bcl-2, preventing Bcl-2 from causing mitochondrial dysfunction, so that translocation of AIF protein from mitochondria can be prevented.

These results are consistent with previous studies conducted by Sabirzhanov et al., 2012, showing that HSP70 modulates apoptosis of the caspase-independent pathway in primary cortical neurons and SH-SY5Y cells through interaction with AIF and by preventing translocation to the nucleus (Sabirzhanov, et al., 2012).

Effect of Glutamine on Cell Apoptosis

ANOVA test results on the apoptosis variable (Table 4), showed a significant difference between groups in 1 research variable. With the LSD double comparison test between P0 and P, 1 mean apoptosis score of P0 = 3.04 ± 0.70 and P1 = 5.26 ± 0.53 (Table 5) had a significant difference from the number of cells experiencing apoptosis in the glomerular visceral epithelial cells (podocyte). The increase in apoptosis in the positive control group (P1) due to cisplatin exposure triggered an increase in free radicals that can activate various cell death pathways. In addition, cisplatin induces maturation of the p53 gene and induces apoptosis.

These results are consistent with previous studies conducted by Marullo, et al., 2013, showing that cisplatin exposure induces a mitochondrial dependent ROS response that significantly contributes to cell killing by enhancing the cytotoxic effect exerted through the formation of nDNA damage and Yang et al., 2008, which states that translocation of the AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Marullo, et al., 2013; Yang et al., 2008)

LSD test results on P1 and P2 with an average score of P1 = 5.26 ± 0.53 and P2 = 4.44 ± 0.41 (Table 5) had a significant reduction in the number of apoptosis in glomerular visceral epithelial cells (podocytes). The decrease in apoptosis in the treatment group (P2) was due to glutamine which is a precursor to GSH, which is a powerful antioxidant that can bind free radicals triggered by cisplatin administration. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing apoptosis of the caspase-independent pathway and the caspase-dependent pathway. So that apoptosis due to cisplatin can be prevented.

These results are in accordance with the theory described by Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2017, stated that glutamine is a precurso of GSH where GSH is a powerful antioxidant and plays an important role in the metabolism of exogenous and endogenous substances. GSH participates in many cellular reactions. It directly scavenges free radicals and other reactive oxygen species (hydroxyl radicals, lipid peroxyl radicals, peroxynitrite, and H2O2), and is indirectly linked to enzymatic reactions that can

decrease apoptosis. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent through the HBP pathway and can also reduce apoptosis (Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2016).

Correlation of AIF Protein Expression and Apoptosis

The results of the Pearson correlation test on the AIF and apoptosis expression (Table 7) variables showed that the correlation was very strong with the direction of the relationship being directly proportional. Increased AIF expression and increased apoptosis in the positive control group (P1) were strongly associated. The increase in AIF expression caused by cisplatin exposure triggers AIF synthesis and AIF translocation to the nucleus and causes condesation and large-scale chromatin fragmentation which triggers apoptosis.

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Yang et al., 2008).

The decrease in AIF expression and the decrease in apoptosis in the treatment group (P2) were strongly associated. The decrease in AIF expression in the group injected with glutamine prior to cisplatin administration was due to the nephroprotective effect of glutamine as an antioxidant precursor that can bind to antioxidants directly and so it can inhibit / prevent mitochondrial dysfunction which can cause AIF protein to translocate to the cytoplasm and nucleus. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing translocation of AIF to the nucleus, maturation of the P53 gene, and Bcl-2 pro apoptosis. Prevents AIF protein from translocating to the cytoplasm and nucleus and causing large-scale condesation and fragmentation of chromatin, thus preventing apoptosis.

These results are in accordance with previous studies conducted by Ruchalski et, al. 2003, which stated that Hsp 72 inhibits the release of AIF protein from mitochondria which can inhibit apoptosis in renal epithelial cells exposed to metabolic inhibitors (Ruchalski et al. 2003)

Conclussion

Based on the research that has been conducted, there is a change in the expression of AIF and apoptotic cells in intravenous glutamine administration on glomerular visceral epithelial cells (podocyte) of male white rat exposed to cisplatin, the changes that occur in this study are in the form of a decrease. Decreased expression of AIF on intravenous glutamine administration is correlation with a decrease in apoptosis in the glomerular visceral cells (podocyte). Glutamin administration can decrease AIF expression and apoptosis that induced by cisplatin administratio. Glutamine administration might represent for the treatment of nephrotoxic induced cisplatin

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FIGURE AND TABLE

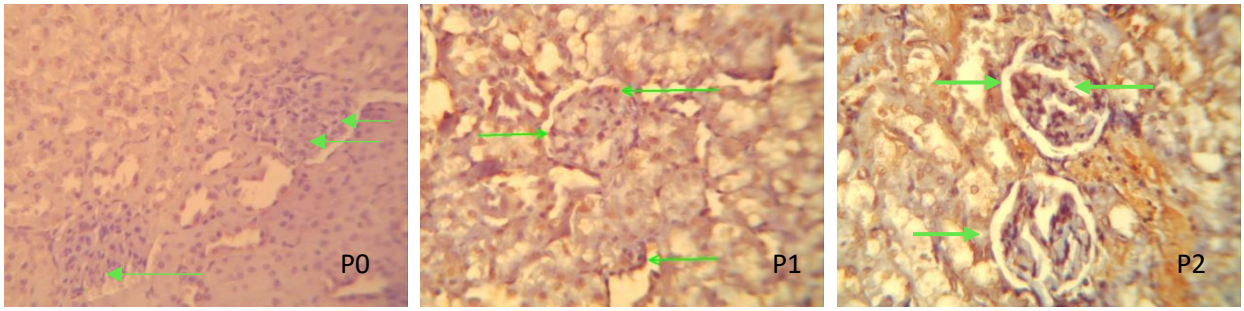

Figure 6. Morphology of the glomerular epithelial cells given anti-AIF antibodies. P0 is control, in which the rat's kidney is not treated. P1 is a kidney that is given cisplatin i.p on the 7th day. P2 is the kidney of rat injected glutamine i.v 7 days in a row row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

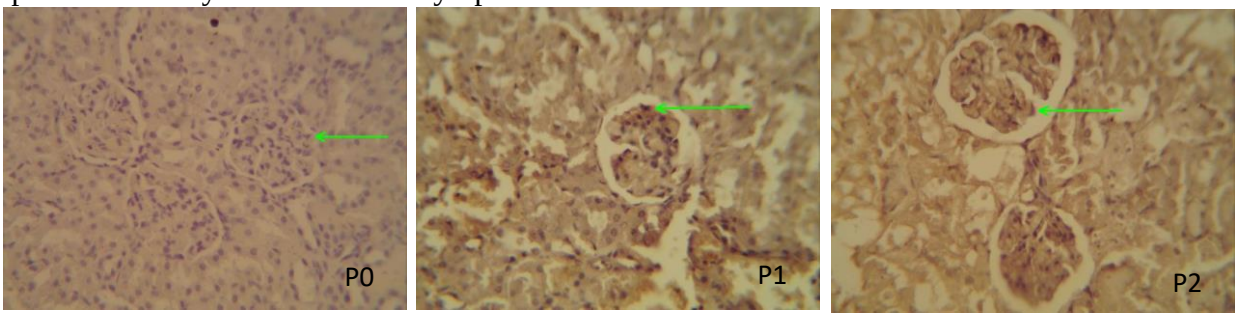

Figure 5. Morphology of the glomerular epithelial cells used Apoptotic Detection Kit. P0 is control, in which the rat's kidney is not treated. P1 is a kidney that is given cisplatin I.p on the 7th day. P2 is the kidney of rat injected glutamine i.v 7 days in a r row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

Variable	AIF protein expression	Apoptotic cells
	Average ± sd	Average ± sd
P0	3.29±0.79	3.04±0.69
P1	5.32±0.68	5.26±0.53
P2	4.49±0.47	4.44±0.41

Table 8. Descriptive statistic of the AIF protein expression and apoptotic cells. P0 is control, in which the rat's kidney is not treated. P1 is a kidney that is given cisplatin injection on the 7th day. P2 is the kidney of rat given intravenous glutamine injection 7 days in a row before being injected intraperitoneal with cisplatin on the 7th day.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
P0	0.702	0.137
P1	0.539	0.189
P2	0.733	0.647

Table 9. The result of test normality Shapiro-Wilk showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
Levene Statistic	0.150	0.448

Table 10. The result of homogeneity variances Levene Statistic showed a sig value,> 0.05, which means that the data in this study were homogeneous.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
ANOVA	0.000	0.000

Table 11. The result of AVONA showed the sig value > 0.05, which means that the data in this study had different results.

Variable	Comparison		Sig.	Interpretation
AIF protein expression	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.009	
Apoptotic cells	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.003	

Table 12. The result of LSD showed the sig value > 0.05, which means that the data in this study had different results in each group.

Variable	AIF protein expression	Apoptotic cells
	Sig. (2-tailed)	Sig. (2-tailed)
Kolmogorov-Smirnov Test	0.200	0.200

Table 13. The result of test normality Kolmogorov-Smirnov showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	Correlation	Sig.	Interpretation
AIF protein expression and Apoptotic cells	0.928	0.00	Correlation is significant and linier

Table 14. The result of Pearson correlation showed the r (Pearson correlation) > 0.349 (positive) which means that the data in this study had correlation and linier. Thus, showed the sig value > 0.05, which means that the data in this study had a significant correlation between AIF protein expression and apoptosis cells.

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REVISI III

AIF PROTEIN EXPRESSION AND APOPTOSIS CHANGES WITH GLUTAMINE IN PODOCYTES CELLS EXPOSED CISPLATIN

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Running title: Glutamine Effect on Apoptosis-inducing factor Expression

Abstract

Cisplatin is a well-known chemotherapeutic drug as one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. However, side effects in normal tissues and organs, like nephrotoxicity induce apoptosis epithelial cells in kidney, limit the use of cisplatin. Glutamine is a substrate for the synthesis of glutathione as antioxidant, and promote HSP70 release, protecting cells from apoptosis induced by different stimuli. In the present study, we investigated the protective effect of glutamine on nephrotoxicity cisplatin in the kidney. Mice were divided into three groups a group of control (P0), a group of intraperitoneal injection of a single dose cisplatin 20mg/kg BW at 7th day (P1), and a group of intravenous glutamine injection 100mg/kg BW at day 1—7 and given an intraperitoneal injection of single dose cisplatin 20mg/kg BW at 7th-day (P2). Measurement of AIF expression and apoptotic cells was carried out by immunohistochemical methods. The number of AIF expressions and apoptotic cells is expressed in Allerd score. AIF expression result: P0: 3.29

±0.79 P1: 5.32 ±0.68 P2: 4.49 ± 0.47. Apoptosis result: P0: 3.04 ±0.70 P1: 5.26 ±0.53 P2: 4.44 ± 0.41. There is decreased expression of AIF on intravenous glutamine administration, followed by a decrease in apoptosis in the podocyte. In conclusion, glutamine administration might represent for the treatment of nephrotoxic induced cisplatin.

Keyword: Cisplatin, Glutamine, AIF Protein, Apoptosis, Podocyte

Introduction

Cancer is a large group of diseases that can occur in almost any organ or tissue of the body when abnormal cells grow uncontrollably beyond normal limits to invade adjoining parts of the body and/or spread to other organs. Cancer is the second leading cause of death globally, with an estimated 9.6 million deaths, or one in six deaths, in 2018. In Indonesia, 348,809 people suffered from cancer, and 207,210 died from cancer in 2018 (WHO, 2020).

Cisplatin (CAS No.15663-27-1, MF-Cl2H6N2Pt; NCF-119875), cisplatinum, also called cis-diamminedichloroplatinum (II), is the first platinum drug approved globally for the treatment of cancer in 1978. Cisplatin is one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. Cisplatin can bind to purine bases in DNA, interfered DNA repair, causes DNA damage, and then induces apoptosis in cancer cells (Achkar et al., 2018; Dasari & Tchounwou, 2015)

Behind the benefits of cisplatin, there are various side effects, one of them is nephrotoxic. Cisplatin nephrotoxicity can present with a variety of symptoms of acute kidney injury (AKI), hypomagnesemia, fanconi-like syndromes, distal renal tubular acidosis, hypokalemia, wasted renal salt, and hyperuricemia. However, the most serious and most common side effect is AKI, which occurs in 20-30% of patients and hypomagnesemia which occurs in 40-100% of patients (Miller et al., 2010).

Cisplatin-induced nephrotoxic can be pathophysiologically classified into four types, tubular toxicity (cell death by apoptosis or necrosis), blood vessel damage (renal vasoconstriction), glomerular injury (damage to the glomerular compartment including capillaries, basement membrane, mesangial cells, podocyte cells, and parietal cells), and interstitial injury (damage from the inflammatory response). (Oh et al., 2014).

The mechanisms of cisplatin-induced nephrotoxicity are complex and involve many cellular processes including oxidative stress, apoptosis, and inflammation. Tsuruya et al. in 2003 observed that renal epithelial cells deficient in TNFR1 and Fas were resistant to cisplatin-induced cell death (Tsuruya et al. 2003). Seth et al. in 2005 identified that cisplatin increases caspase-2 and caspase-3 activity (Seth, et al., 2005). Takeda et al. in 1997 demonstrated that caspase-8 inhibition reduced cisplatin-induced cell death in vitro (Takeda et al., 1997).

Apoptosis-inducing factor (AIF) is a mitochondrial oxidoreductase that plays a role in oxidative phosphorylation and redox control in normal cells. AIF was initially cloned and identified as a caspase-independent mitochondrial effector of apoptotic cell death. AIF is usually confined to the mitochondrial intermembrane space and released in response to death

stimuli, and after the induction of apoptosis, AIF is translocated to the nucleus, where it affects the chromosome condensation and fragmentation. Besides, AIF can induce mitochondria to release apoptogenic-proteins including cytochrome c and caspase-9 (Wang et al., 2019).

Cytoplasmic interactions of apoptogenic AIF. Once released into the cytoplasm, AIF can promote apoptosis by interacting with CYP, in which CypA assists in apoptogenic cytonuclear translocation of AIF. In contrast, Hsp70 maintains AIF in the cytoplasm and, therefore, can delay or prevent initiation of nuclear apoptosis. (Sevrioukova, 2011).

Glutamine is an α-amino acid and is the most abundant free amino acid in the body. Glutamine has two amino groups, namely an α-amino group and a side-chain amide group that is easily hydrolyzed (Cruzat et al., 2018). The function of glutamine within cells is generally separated into four categories: its role in nitrogen transport; the importance of maintaining a cellular redox state; its position as a metabolic intermediary; and its role as an energy source. (Newsholme et al. 1986; Shah, Wang & Ma, 2020).

Glutamine plays an important role in the modulation of HSP expression via the biosynthetic pathway hexosamine (HBP). HSP70 (HSP72 and HSP 73) acts as anti-inflammatory protein based on deactivating NF-κB and attenuates the production of inflammatory mediators. In addition, HSP70 modulates autophagy by regulating the mTOR / Akt pathway and blocking signaling pathways associated with protein degradation (Cruzat et al., 2018).

GSH is an antioxidant that can react directly with ROS, produce oxidized GSH (GSSG), and can also donate electrons for peroxide reduction, which is catalyzed by the enzyme glutathione peroxidase (GPx). Glutamine (via glutamate), cysteine, and glycine are precursor amino acids for the synthesis of GSH. (Cruzat et al., 2018).

Cell damage due to exposure to cisplatin chemotherapy is generally studied in the proximal renal tubule area because there is a process of reabsorption and primary urine secretion in that area. However, kidney cell damage may occur in glomerular visceral epithelial cells (podocyte) as a filtration site in the urinary system, which causes all substances to pass through the filtering process in the glomerulus before going to the proximal tubule and directly exposed to cisplatin. Therefore, research on apoptosis and AIF expression was carried out on glomerular visceral epithelial cells (podocyte).

This study is analyzing the nephroprotective effect of intravenous glutamine on the incidence of apoptosis of the glomerular epithelial cells (podocyte) by examining the expression of AIF which is the initiator of apoptosis in the apoptosis independent caspase. This research is expected to be an alternative problem solving for kidney failure caused by cisplatin chemotherapy modalities.

Material and Methods

Animal and housing

2-3 months old wistar male white mouse weighing 150–200 g were used after one week for proper acclimatization to the animal house conditions (12 h lighting cycle and 29-31°C

temperature) with free access to water and standard rodent chow. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, Airlangga University, Indonesia. Animals were randomly divided into three groups with 10 animals in each group. The first group as the control group (P0). The second group was treated with cisplatin (20 mg/kg I.p) as a positive control (P1). The third group was injected with glutamine (100 mg/kg, i.v.), a gram glutamine suspended in 10 ml solution of ml 0.9% daily for seven consecutive days, and injected with cisplatin (20 mg/kg I.p) on the seventh day to induce nephrotoxicity (P2). All groups received equivalent volumes of the used vehicles. Mouse were sacrificed on the tenth day. The longitudinal section of the left kidney was excised from each animal for immunohistochemical examination.

Glutamine product from Serva-Germany, cisplatin product from Kalbe Farma, POD Apoptosis Detection Kit (11684817910 ROCHE), Anti-AIF Antibody (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880))

Immunohistochemical Examination

Kidney tissue samples were fixed in 10% buffered neutral formalin, embedded in paraffin, cut, and stained with immunohistochemical staining for AIF expression and apoptosis examination using light microscopy.

Immunohistochemical detection of AIF expression was conducted using anti-AIF antibodies (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880)). Immunohistochemical staining was carried out with anti-AIF antibodies in brief steps as follows: Deparaffinated preparations on glass objects; Washed with PBS pH 7.4; Blocking endogenous peroxide with 3% H2O2 for 20 minutes; Blocking unspecific protein with 5% FBS; Incubation with primary antibody (anti-AIF antibody) overnight at 4^0C, Incubation with conjugated anti-mouse biotin for 1 hour at room temperature; Incubation with Strep-Avidin Horse Radish Peroxidase for 40 minutes, drop DAB and incubate for 10 minutes; Counterstaining with Mayer Hematoxylin, the preparation is rinsed with dH2O and aerated; Mounting with a swab and the preparation is covered with a glass cover.

Immunohistochemical detection of apoptosis was processing by Apoptotic Detection Kit POD (11684817910 ROCHE) with the following brief steps: Deparaffinated tissue; Preparations given proteinase K for 15 minutes, and dH2O in a Coplin jar for 2 x 2 minutes; Removal endogenous peroxide with 3% H2O2 for 5 minutes at room temperature; Drop the working strength of tdT enzyme in tissues, incubate at 37oC for 1 hour; Place the preparation in a coplin jar containing the working strength of the stop / wash buffer and incubated for 10 minutes at room temperature; Drop anti-digoxigenin conjugate incubation at room temperature in a damp container for 30 minutes; Staining with substrate peroxidase for 10 minutes at room temperature; Counterstaining with methyl green for 30 seconds at room temperature; Covered with a glass cover.

Statistical analysis

The data are expressed as means ± SEM. Statistical analysis was performed by one-way ANOVA followed by LSD post-analysis test for multiple comparisons with α = 0,05 being considered as statistically significant, and Pearson correlation with α = 0,05 being considered as statistically significant.

Result

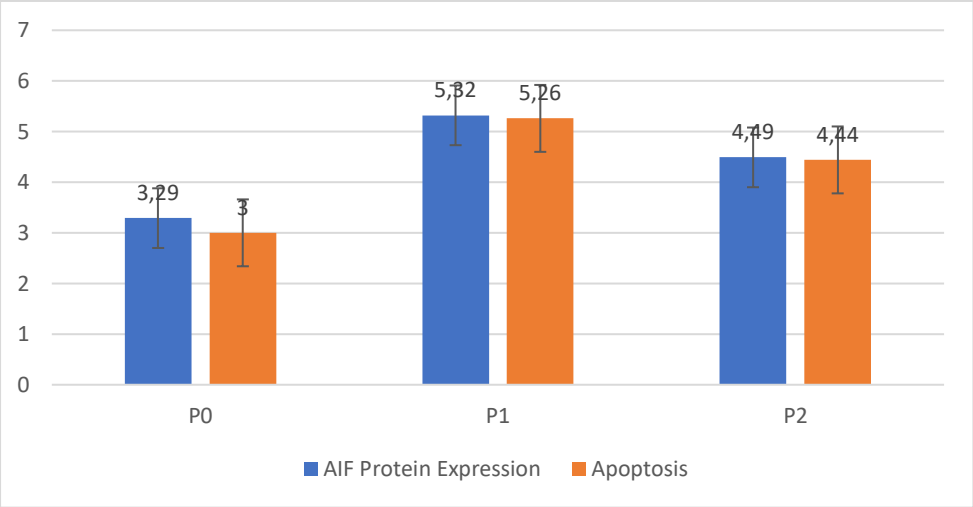

Figure 7. Graph of mean score of AIF protein expression and apoptosis.

The results of the AIF expression and apoptosis study used IHC staining and assessed with the H score shows a significant difference in each group (Figure 1). Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of AIF expression compared to giving cisplatin without glutamine administration (P1). The mean score of AIF expression in P2 with score 4.49 ± 0.47 compared to of P1 with score 5.32 ± 0.68 (Table 1, Table 2 and Table 3).

Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of apoptosis compared with giving cisplatin without glutamine administration (P1). The mean score of apoptosis in P2 with score 4.44 ± 0.41 compared to P1 with score 5.26 ± 0.53 (Table 1).

Discussion

In this study, mice were divided into three groups, each group consisted of 10 male white mice. The negative control group (P0), the positive control group (P1), and the treatment group (P2). P1 had the largest mean AIF expression and apoptosis compared to other groups.

The research data were analyzed using the ANOVA test and if there were differences, it would be followed by a multiple comparison test, LSD. To use the ANOVA test, several

conditions must be fulfilled: the sample comes from independent data, the variance between groups must be homogeneous and the data in each group is normally distributed. The homogeneity test in this study used the Levene test and showed a sig value,> 0.05, which means that the data in this study were homogeneous, for normality test used the Shapiro-Wilk test and showed the sig value > 0.05, which means that the data in this study were normally distributed (Table 2, Table 3, Table 4 and Table 5).

The research data were correlation using Person correlation, condition must be fulfilled, the data must be normal, the normality test used Kolmogorov-Smirnov test and showed the sig. value > 0.5, which means that the data in this study were normal (Table 6 and Table 7).

Apoptosis and AIF excretion in glomerular visceral epithelial cells (podocyte) in all groups in this study. Apoptosis and AIF expression in the positive control groups (P1) and (P2) are apoptotic processes triggered by cisplatin chemotherapy as a substance that has a nephrotoxic effect (Figure 2 and 3). Cisplatin-induced nephrotoxicity can occur from several pathways, including extrinsic, intrinsic apoptosis that can trigger AIF expression, cell regulators, MAPK, inflammation, and ROS. Meanwhile, apoptosis in the negative control group (P0) can be caused by physiological processes that can be experienced by all cells. The influence of external variables that cannot be controlled can also cause apoptosis in the glomerular visceral epithelial cells (podocyte).

The Effect of Glutamine on AIF Protein Expression

ANOVA test results on the AIF expression variable (Table 4), showed different evidence between groups in 1 research variable. With the LSD comparison test between P0 and P1, the mean score of AIF expression P0 = 3.29 ± 0.79 and P1 = 5.32 ± 0.68 (Table 5) had a significant difference from the expression AIF protein in the glomerular visceral epithelial cells (podocyte). The increase in AIF excretion in the positive control group (P1) was caused by exposure to cisplatin, an increase in free radicals and DNA damage which caused the maturation of the P53 gene to induce AIF protein transcription and induce Bcl-2 causing mitochondrial dysfunction to form holes in the mitochondrial membrane, so that the AIF protein could translocate to cytoplasm and nucleus.

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. Apoptosis, which represents the form of cell death performed by caspases, has traditionally been the only form of physiological and programmed cell death. However, recent evidence suggests that programmed cell death (PCD) can occur in the absence of caspase activation at all. Indeed, a large number of caspase-independent models are now defined and a key protein involved in this type of PCD, the apoptosis inducing factor (AIF), has been identified. Cisplatin causes changes in the mitochondrial PTP to cause the pores in the mitochondria to open and allow small molecules such as the AIF protein to escape (Yang et al. 2008)

LSD test results on P1 and P2 with a mean score of P1 = 5.32 ± 0.68 and P2 = 4.49 ± 0.47 (Table 5) had a decreased AIF excretion involved in glomerular visceral epithelial cells (podocytes). The decrease in AIF expression in the treatment group (P2) was due to glutamine, which is the precursor to glutathione in cells, directly binding to free radicals which can prevent mitochondrial dysfunction due to cisplatin administration. In addition, glutamine can increase the expression of Hsp 70 which is an antiapoptotic agent that can prevent various cell death pathways, one of which is by inhibiting the maturation of P53 to induce Bcl-2, preventing Bcl-2 from causing mitochondrial dysfunction, so that translocation of AIF protein from mitochondria can be prevented.

These results are consistent with previous studies conducted by Sabirzhanov et al., 2012, showing that HSP70 modulates apoptosis of the caspase-independent pathway in primary cortical neurons and SH-SY5Y cells through interaction with AIF and by preventing translocation to the nucleus (Sabirzhanov, et al., 2012).

The Effect of Glutamine on Cell Apoptosis

ANOVA test results on the apoptosis variable (Table 4), showed a significant difference between groups in 1 research variable. With the LSD double comparison test between P0 and P, 1 mean apoptosis score of P0 = 3.04 ± 0.70 and P1 = 5.26 ± 0.53 (Table 5) had a significant difference from the number of cells experiencing apoptosis in the glomerular visceral epithelial cells (podocyte). The increase in apoptosis in the positive control group (P1) due to cisplatin exposure triggered an increase in free radicals that can activate various cell death pathways. In addition, cisplatin induces maturation of the p53 gene and induces apoptosis.

These results are consistent with previous studies conducted by Marullo, et al., 2013, showing that cisplatin exposure induces a mitochondrial dependent ROS response that significantly contributes to cell killing by enhancing the cytotoxic effect exerted through the formation of nDNA damage and Yang et al., 2008, which states that translocation of the AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Marullo, et al., 2013; Yang et al., 2008)

LSD test results on P1 and P2 with an average score of P1 = 5.26 ± 0.53 and P2 = 4.44 ± 0.41 (Table 5) had a significant reduction in the number of apoptosis in glomerular visceral epithelial cells (podocytes). The decrease in apoptosis in the treatment group (P2) was due to glutamine which is a precursor to GSH, which is a powerful antioxidant that can bind free radicals triggered by cisplatin administration. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing apoptosis of the caspase-independent pathway and the caspase-dependent pathway. So that apoptosis due to cisplatin can be prevented.

These results are in accordance with the theory described by Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2017, stated that glutamine is a precurso of GSH where GSH is a powerful antioxidant and plays an important role in the metabolism of exogenous and endogenous substances. GSH participates in many cellular reactions. It directly

scavenges free radicals and other reactive oxygen species (hydroxyl radicals, lipid peroxyl radicals, peroxynitrite, and H2O2), and is indirectly linked to enzymatic reactions that can decrease apoptosis. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent through the HBP pathway and can also reduce apoptosis (Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2016).

Correlation of AIF Protein Expression and Apoptosis

The results of the Pearson correlation test on the AIF and apoptosis expression (Table 7 and Table 6) variables showed that the correlation was very strong with the direction of the relationship being directly proportional. Increased AIF expression and increased apoptosis in the positive control group (P1) were strongly associated. The increase in AIF expression caused by cisplatin exposure triggers AIF synthesis and AIF translocation to the nucleus and causes condesation and large-scale chromatin fragmentation which triggers apoptosis.

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Yang et al., 2008).

The decrease in AIF expression and the decrease in apoptosis in the treatment group (P2) were strongly associated. The decrease in AIF expression in the group injected with glutamine prior to cisplatin administration was due to the nephroprotective effect of glutamine as an antioxidant precursor that can bind to antioxidants directly and so it can inhibit / prevent mitochondrial dysfunction which can cause AIF protein to translocate to the cytoplasm and nucleus. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing translocation of AIF to the nucleus, maturation of the P53 gene, and Bcl-2 pro apoptosis. Prevents AIF protein from translocating to the cytoplasm and nucleus and causing large-scale condesation and fragmentation of chromatin, thus preventing apoptosis.

These results are in accordance with previous studies conducted by Ruchalski et, al. 2003, which stated that Hsp 72 inhibits the release of AIF protein from mitochondria which can inhibit apoptosis in renal epithelial cells exposed to metabolic inhibitors (Ruchalski et al. 2003)

CONCLUSION

Based on the research that has been conducted, there is a change in the expression of AIF and apoptotic cells in intravenous glutamine administration on glomerular visceral epithelial cells (podocyte) of male white mouse exposed to cisplatin, the changes that occur in this study are in the form of a decrease. Decreased expression of AIF on intravenous glutamine administration is correlation with a decrease in apoptosis in the glomerular visceral cells (podocyte). Glutamin administration can decrease AIF expression and apoptosis that induced by cisplatin administratio. Glutamine administration might represent for the treatment of nephrotoxic induced cisplatin

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FIGURE AND TABLE

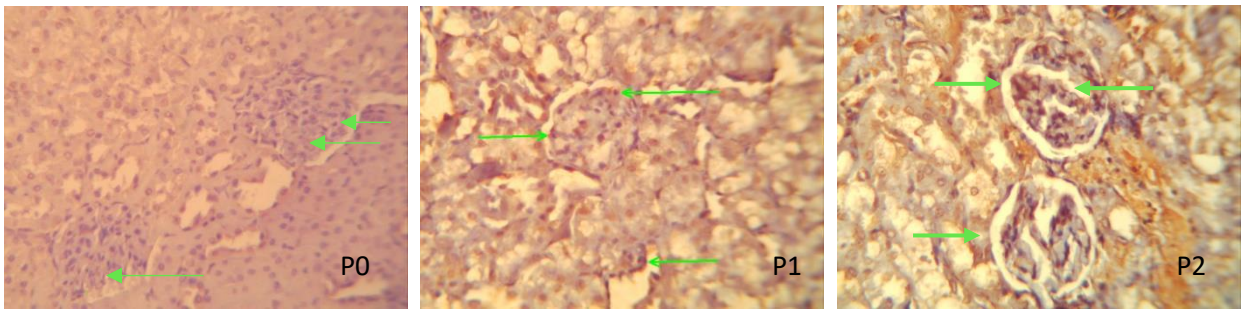

Figure 9. Morphology of the glomerular epithelial cells given anti-AIF antibodies. P0 is control, in which the mouse's kidney is not treated. P1 is a kidney of mouse given cisplatin i.p on the 7th day. P2 is the kidney of mouse injected glutamine i.v 7 days in a row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

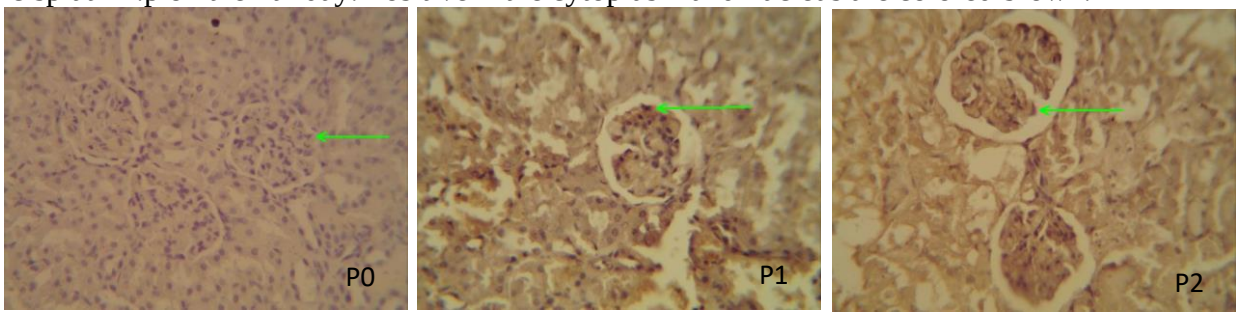

Figure 8. Morphology of the glomerular epithelial cells used Apoptotic Detection Kit. P0 is control, in which the mouse's kidney is not treated. P1 is a kidney of mouse given cisplatin i.p on the 7th day. P2 is the kidney of mouse injected glutamine i.v 7 days in a row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

Variable	AIF protein expression	Apoptotic cells
	Average ± sd	Average ± sd
P0	3.29±0.79	3.04±0.69
P1	5.32±0.68	5.26±0.53
P2	4.49±0.47	4.44±0.41

Table 15. Descriptive statistic of the AIF protein expression and apoptotic cells. P0 is control, in which the rat's kidney is not treated. P1 is a kidney that is given cisplatin injection on the 7th day. P2 is the kidney of rat given intravenous glutamine injection 7 days in a row before being injected intraperitoneal with cisplatin on the 7th day.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
P0	0.702	0.137
P1	0.539	0.189
P2	0.733	0.647

Table 16. The result of test normality Shapiro-Wilk showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
Levene Statistic	0.150	0.448

Table 17. The result of homogeneity variances Levene Statistic showed a sig value,> 0.05, which means that the data in this study were homogeneous.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
ANOVA	0.000	0.000

Table 18. The result of AVONA showed the sig value > 0.05, which means that the data in this study had different results.

Variable	Comparison		Sig.	Interpretation
AIF protein expression	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.009	
Apoptotic cells	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.003	

Table 19. The result of LSD showed the sig value > 0.05, which means that the data in this study had different results in each group.

Variable	AIF protein expression	Apoptotic cells
	Sig. (2-tailed)	Sig. (2-tailed)
Kolmogorov-Smirnov Test	0.200	0.200

Table 20. The result of test normality Kolmogorov-Smirnov showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	Correlation	Sig.	Interpretation
AIF protein expression and Apoptotic cells	0.928	0.00	Correlation is significant and linier

Table 21. The result of Pearson correlation showed the r (Pearson correlation) > 0.349 (positive) which means that the data in this study had correlation and linier. Thus, showed the sig value > 0.05, which means that the data in this study had a significant correlation between AIF protein expression and apoptosis cells.

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REVISI IV

APOPTOSIS-INDUCING FACTOR PROTEIN EXPRESSION AND APOPTOSIS CHANGES WITH GLUTAMINE IN PODOCYTES CELLS EXPOSED WITH CISPLATIN

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Running title: Glutamine Effect on Apoptosis-inducing factor Expression

BAB 1Abstract

Cisplatin is a well-known chemotherapeutic drug as one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. However, side effects in normal tissues and organs, like nephrotoxicity induce apoptosis epithelial cells in kidney, limit the use of cisplatin. Glutamine is a substrate for the synthesis of glutathione as antioxidant, and promote HSP70 release, protecting cells from apoptosis induced by different stimuli. In the present study, we investigated the protective effect of glutamine on nephrotoxicity cisplatin in the kidney. Mice were divided into three groups a group of control (P0), a group of intraperitoneal injection of a single dose cisplatin 20mg/kg BW at 7th day (P1), and a group of intravenous glutamine injection 100mg/kg BW at day 1—7 and given an intraperitoneal injection of single dose cisplatin 20mg/kg BW at 7th-day (P2). Measurement of AIF expression and apoptotic cells was carried out by immunohistochemical methods. The number of AIF expressions and apoptotic cells is expressed in Allerd score. AIF expression result: P0: 3.29 ±0.79 P1: 5.32 ±0.68 P2: 4.49 ± 0.47. Apoptosis result: P0: 3.04 ±0.70 P1: 5.26 ±0.53 P2: 4.44 ± 0.41. There is decreased expression of AIF on intravenous

glutamine administration, followed by a decrease in apoptosis in the podocyte. In conclusion, glutamine administration might represent for the treatment of nephrotoxic induced cisplatin.

Keyword: Cisplatin, Glutamine, AIF Protein, Apoptosis, Podocyte

BAB 2Introduction

Cisplatin (CAS No.15663-27-1, MF-Cl2H6N2Pt; NCF-119875), cisplatinum, also called cis-diamminedichloroplatinum (II), is the first platinum drug approved globally for the treatment of cancer in 1978. Cisplatin is one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. Cisplatin can bind to purine bases in

DNA, interfered DNA repair, causes DNA damage, and then induces apoptosis in cancer cells

(Achkar et al., 2018; Dasari & Tchounwou, 2015). Behind the benefits of cisplatin, there are various side effects, one of them is nephrotoxic. The mechanisms of cisplatin-induced nephrotoxicity are complex and involve many cellular processes including oxidative stress, apoptosis, and inflammation. Tsuruya et al. in 2003 observed that renal epithelial cells deficient in TNFR1 and Fas were resistant to cisplatininduced cell death (Tsuruya et al. 2003). Seth et al. in 2005 identified that cisplatin increases caspase-2 and caspase-3 activity (Seth, et al., 2005). Takeda et al. in 1997 demonstrated that caspase-8 inhibition reduced cisplatin-induced cell death in vitro (Takeda et al., 1997).

Apoptosis-inducing factor (AIF) is a mitochondrial oxidoreductase that plays a role in oxidative phosphorylation and redox control in normal cells. AIF was initially cloned and identified as a caspase-independent mitochondrial effector of apoptotic cell death. AIF is usually confined to the mitochondrial intermembrane space and released in response to death stimuli. In the cytoplasm, AIF can promote apoptosis by interacting with CYP, in which CypA assists in apoptogenic cytonuclear translocation of AIF. In contrast, Hsp70 maintains AIF in the cytoplasm and, therefore, can delay or prevent initiation of nuclear apoptosis. In the nucleus, AIF affects the chromosome condensation and fragmentation. Besides, AIF can induce mitochondria to release apoptogenic-proteins including cytochrome c and caspase-9 (Sevrioukova, 2011; Wang et al., 2019).

Glutamine is an α-amino acid and is the most abundant free amino acid in the body. (Cruzat et al., 2018). Glutamine plays an important role in the modulation of HSP expression via the biosynthetic pathway hexosamine (HBP). HSP70 (HSP72 and HSP 73) acts as antiapoptosis protein (Evans, Pinto & Wischmeyer, 2009). Glutamine (via glutamate), cysteine, and glycine are precursor amino acids for the synthesis of GSH. GSH is an antioxidant that can react directly with ROS, produce oxidized GSH (GSSG) (Cruzat et al., 2018).

Cell damage due to exposure to cisplatin chemotherapy is generally studied in the proximal renal tubule area because there is a process of reabsorption and primary urine secretion in that area. However, kidney cell damage may occur in glomerular visceral epithelial cells (podocyte) as a filtration site in the urinary system, which causes all substances to pass through the filtering process in the glomerulus before going to the proximal tubule and directly exposed to cisplatin. Therefore, research on apoptosis and AIF expression was carried out on glomerular visceral epithelial cells (podocyte). This study is analyzing the nephroprotective effect of intravenous glutamine on the incidence of apoptosis of the glomerular epithelial cells (podocyte) by examining the expression of AIF which is the initiator of apoptosis in the apoptosis independent caspase. This research is expected to be an alternative problem solving for kidney failure caused by cisplatin chemotherapy modalities.

BAB 3Material and Methods

BAB 4Animal and housing

2-3 months old wistar male white mouse weighing 150–200 g were used after one week for proper acclimatization to the animal house conditions (12 h lighting cycle and 29-31°C temperature) with free access to water and standard rodent chow. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, Airlangga University, Indonesia. Animals were randomly divided into three groups with 10 animals in each group. The first group as the control group (P0). The second group was treated with cisplatin (20 mg/kg I.p) as a positive control (P1). The third group was injected with glutamine (100 mg/kg, i.v.), a gram glutamine suspended in 10 ml solution of ml 0.9% daily for seven consecutive days, and injected with cisplatin (20 mg/kg I.p) on the seventh day to induce nephrotoxicity (P2). All groups received equivalent volumes of the used vehicles. Mouse were sacrificed on the tenth day. The longitudinal section of the left kidney was excised from each animal for immunohistochemical examination.

Glutamine product from Serva-Germany, cisplatin product from Kalbe Farma, POD Apoptosis Detection Kit (11684817910 ROCHE), Anti-AIF Antibody (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880))

BAB 5Immunohistochemical Examination

Kidney tissue samples were fixed in 10% buffered neutral formalin, embedded in paraffin, cut, and stained with immunohistochemical staining for AIF expression and apoptosis examination using light microscopy.

Immunohistochemical detection of AIF expression was conducted using anti-AIF antibodies (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880)). Immunohistochemical staining was carried out with anti-AIF antibodies in brief steps as follows: Deparaffinated preparations on glass objects; Washed with PBS pH 7.4; Blocking endogenous peroxide with 3% H2O2 for 20 minutes; Blocking unspecific protein with 5% FBS; Incubation with primary antibody (anti-AIF antibody) overnight at 4^0C, Incubation with conjugated anti-mouse biotin for 1 hour at room temperature; Incubation with Strep-Avidin Horse Radish Peroxidase for 40 minutes, drop DAB and incubate for 10 minutes; Counterstaining with Mayer Hematoxylin, the preparation is rinsed with dH2O and aerated; Mounting with a swab and the preparation is covered with a glass cover.

Immunohistochemical detection of apoptosis was processing by Apoptotic Detection Kit POD (11684817910 ROCHE) with the following brief steps: Deparaffinated tissue;

Preparations given proteinase K for 15 minutes, and dH2O in a Coplin jar for 2 x 2 minutes; Removal endogenous peroxide with 3% H2O2 for 5 minutes at room temperature; Drop the working strength of tdT enzyme in tissues, incubate at 37oC for 1 hour; Place the preparation in a coplin jar containing the working strength of the stop / wash buffer and incubated for 10 minutes at room temperature; Drop anti-digoxigenin conjugate incubation at room temperature in a damp container for 30 minutes; Staining with substrate peroxidase for 10 minutes at room temperature; Counterstaining with methyl green for 30 seconds at room temperature; Covered with a glass cover.

BAB 6Statistical analysis

The data are expressed as means ± SEM. Statistical analysis was performed by one-way ANOVA followed by LSD post-analysis test for multiple comparisons with α = 0,05 being considered as statistically significant, and Pearson correlation with α = 0,05 being considered as statistically significant.

BAB 7Result

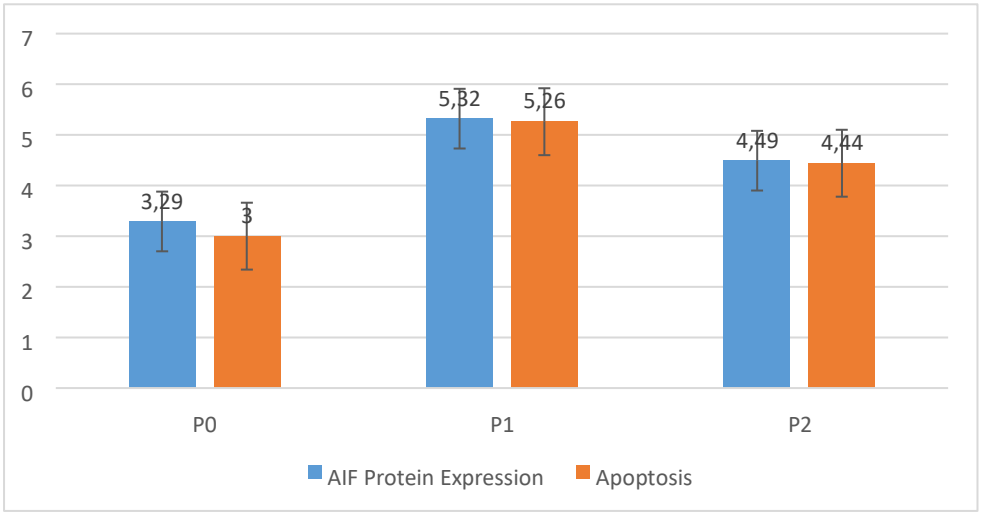

Figure 1. Graph of mean score of AIF protein expression and apoptosis.

The results of the AIF expression and apoptosis study used IHC staining and assessed with the H score shows a significant difference in each group (Figure 1). Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of AIF expression compared to giving cisplatin without glutamine administration (P1). The mean score of AIF expression in P2 with score 4.49 ± 0.47 compared to of P1 with score 5.32 ± 0.68 (Table 1, Table 2 and Table 3).

Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of AIF expression compared to giving cisplatin without glutamine administration (P1) (Table 1, Table 2, Table 3, table 4 and Table 5).

ANOVA test results on the AIF expression variable (Table 4), showed different evidence between groups in 1 research variable. With the LSD comparison test between P0 and P1, the mean score of AIF expression P0 = 3.29 ± 0.79 and P1 = 5.32 ± 0.68 (Table 1 and Table 5) had a significant difference from the expression AIF protein in the glomerular visceral epithelial cells (podocyte). LSD test results on P1 and P2 with a mean score of P1 = 5.32 ± 0.68 and P2 = 4.49 ± 0.47 (Table 5) had a decreased AIF excretion involved in glomerular visceral epithelial cells (podocytes).

Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of apoptosis compared with giving cisplatin without glutamine administration (P1) (Table 1, Table 2, Table 3, table 4 and Table 5).

ANOVA test results on the apoptosis variable (Table 4), showed a significant difference between groups in 1 research variable. With the LSD double comparison test between P0 and P, 1 mean

apoptosis score of P0 = 3.04 ± 0.70 and P1 = 5.26 ± 0.53 (Table 1 and Table 5) had a significant difference from the number of cells experiencing apoptosis in the glomerular visceral epithelial cells (podocyte). LSD test results on P1 and P2 with an average score of P1 = 5.26 ± 0.53 and P2 = 4.44 ± 0.41 (Table 5) had a significant reduction in the number of apoptosis in glomerular visceral epithelial cells (podocytes).

The results of the Pearson correlation test on the AIF and apoptosis expression (Table 7 and Table 6) variables showed that the correlation was very strong with the direction of the relationship being directly proportional.

BAB 8Discussion

In this study, mice were divided into three groups, each group consisted of 10 male white mice. The negative control group (P0), the positive control group (P1), and the treatment group (P2). P1 had the largest mean AIF expression and apoptosis compared to other groups.

The research data were analyzed using the ANOVA test and if there were differences, it would be followed by a multiple comparison test, LSD. To use the ANOVA test, several conditions must be fulfilled: the sample comes from independent data, the variance between groups must be homogeneous and the data in each group is normally distributed. The homogeneity test in this study used the Levene test and showed a sig value,> 0.05, which means that the data in this study were homogeneous, for normality test used the Shapiro-Wilk test and showed the sig value > 0.05, which means that the data in this study were normally distributed (Table 2, Table 3, Table 4 and Table 5).

The research data were correlation using Person correlation, condition must be fulfilled, the data must be normal, the normality test used Kolmogorov-Smirnov test and showed the sig. value > 0.5, which means that the data in this study were normal (Table 6 and Table 7).

Apoptosis and AIF excretion in glomerular visceral epithelial cells (podocyte) in all groups in this study. Apoptosis and AIF expression in the positive control groups (P1) and (P2) are apoptotic processes triggered by cisplatin chemotherapy as a substance that has a nephrotoxic effect (Figure 3 and figure 4). Cisplatin-induced nephrotoxicity can occur from several pathways, including extrinsic, intrinsic apoptosis that can trigger AIF expression, cell regulators, MAPK, inflammation, and ROS. Meanwhile, apoptosis in the negative control group (P0) can be caused by physiological processes that can be experienced by all cells. The influence of external variables that cannot be controlled can also cause apoptosis in the glomerular visceral epithelial cells (podocyte).

The increase in AIF excretion in the positive control group (P1) was caused by exposure to cisplatin, an increase in free radicals and DNA damage which caused the maturation of the P53 gene to induce AIF protein transcription and induce Bcl-2 causing mitochondrial dysfunction to form holes in the mitochondrial membrane, so that the AIF protein could translocate to cytoplasm and nucleus (Figure 2).

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. Apoptosis, which represents the form of cell death performed by caspases, has traditionally been the only form of physiological and programmed cell death. However, recent evidence suggests that programmed cell death (PCD) can occur in the absence of caspase activation at all. Indeed, a large number of caspaseindependent models are

now defined and a key protein involved in this type of PCD, the apoptosis inducing factor (AIF), has been identified. Cisplatin causes changes in the mitochondrial PTP to cause the pores in the mitochondria to open and allow small molecules such as the AIF protein to escape (Yang et al. 2008)

The decrease in AIF expression in the treatment group (P2) was due to glutamine, which is the precursor to glutathione in cells, directly binding to free radicals which can prevent mitochondrial dysfunction due to cisplatin administration. In addition, glutamine can increase the expression of Hsp 70 which is an antiapoptotic agent that can prevent various cell death pathways, one of which is by inhibiting the maturation of P53 to induce Bcl-2, preventing Bcl2 from causing mitochondrial dysfunction, so that translocation of AIF protein from mitochondria can be prevented (Figure 2).

These results are consistent with previous studies conducted by Sabirzhanov et al., 2012, showing that HSP70 modulates apoptosis of the caspase-independent pathway in primary cortical neurons and SH-SY5Y cells through interaction with AIF and by preventing translocation to the nucleus (Sabirzhanov, et al., 2012).

The increase in apoptosis in the positive control group (P1) due to cisplatin exposure triggered an increase in free radicals that can activate various cell death pathways. In addition, cisplatin induces maturation of the p53 gene and induces apoptosis (Figure 2).

These results are consistent with previous studies conducted by Marullo, et al., 2013, showing that cisplatin exposure induces a mitochondrial dependent ROS response that significantly contributes to cell killing by enhancing the cytotoxic effect exerted through the formation of nDNA damage and Yang et al., 2008, which states that translocation of the AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Marullo, et al., 2013; Yang et al., 2008)

The decrease in apoptosis in the treatment group (P2) was due to glutamine which is a precursor to GSH, which is a powerful antioxidant that can bind free radicals triggered by cisplatin administration. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing apoptosis of the caspase-independent pathway and the caspase-dependent pathway. So that apoptosis due to cisplatin can be prevented (Figure 2).

These results are in accordance with the theory described by Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2017, stated that glutamine is a precurso of GSH where GSH is a powerful antioxidant and plays an important role in the metabolism of exogenous and endogenous substances. GSH participates in many cellular reactions. It directly scavenges free radicals and other reactive oxygen species (hydroxyl radicals, lipid peroxyl radicals, peroxynitrite, and H2O2), and is indirectly linked to enzymatic reactions that can decrease apoptosis. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent through the HBP pathway and can also reduce apoptosis (Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2016).

Increased AIF expression and increased apoptosis in the positive control group (P1) were strongly associated. The increase in AIF expression caused by cisplatin exposure triggers AIF synthesis and AIF translocation to the nucleus and causes condesation and large-scale chromatin fragmentation which triggers apoptosis (Figure 2).

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Yang et al., 2008).

The decrease in AIF expression and the decrease in apoptosis in the treatment group (P2) were strongly associated. The decrease in AIF expression in the group injected with glutamine prior to cisplatin administration was due to the nephroprotective effect of glutamine as an antioxidant precursor that can bind to antioxidants directly and so it can inhibit / prevent mitochondrial dysfunction which can cause AIF protein to translocate to the cytoplasm and nucleus. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing translocation of AIF to the nucleus, maturation of the P53 gene, and Bcl-2 pro apoptosis. Prevents AIF protein from translocating to the cytoplasm and nucleus and causing large-scale condesation and fragmentation of chromatin, thus preventing apoptosis (Figure 2).

These results are in accordance with previous studies conducted by Ruchalski et, al. 2003, which stated that Hsp 72 inhibits the release of AIF protein from mitochondria which can inhibit apoptosis in renal epithelial cells exposed to metabolic inhibitors (Ruchalski et al. 2003)

BAB 9CONCLUSION

Based on the research that has been conducted, there is a change in the expression of AIF and apoptotic cells in intravenous glutamine administration on glomerular visceral epithelial cells (podocyte) of male white mouse exposed to cisplatin, the changes that occur in this study are in the form of a decrease. Decreased expression of AIF on intravenous glutamine administration is correlation with a decrease in apoptosis in the glomerular visceral cells (podocyte). Glutamin administration can decrease AIF expression and apoptosis that induced by cisplatin administratio. Glutamine administration might represent for the treatment of nephrotoxic induced cisplatin

BAB 10ACKNOWLEDGEMENT

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BAB 11FIGURE AND TABLE

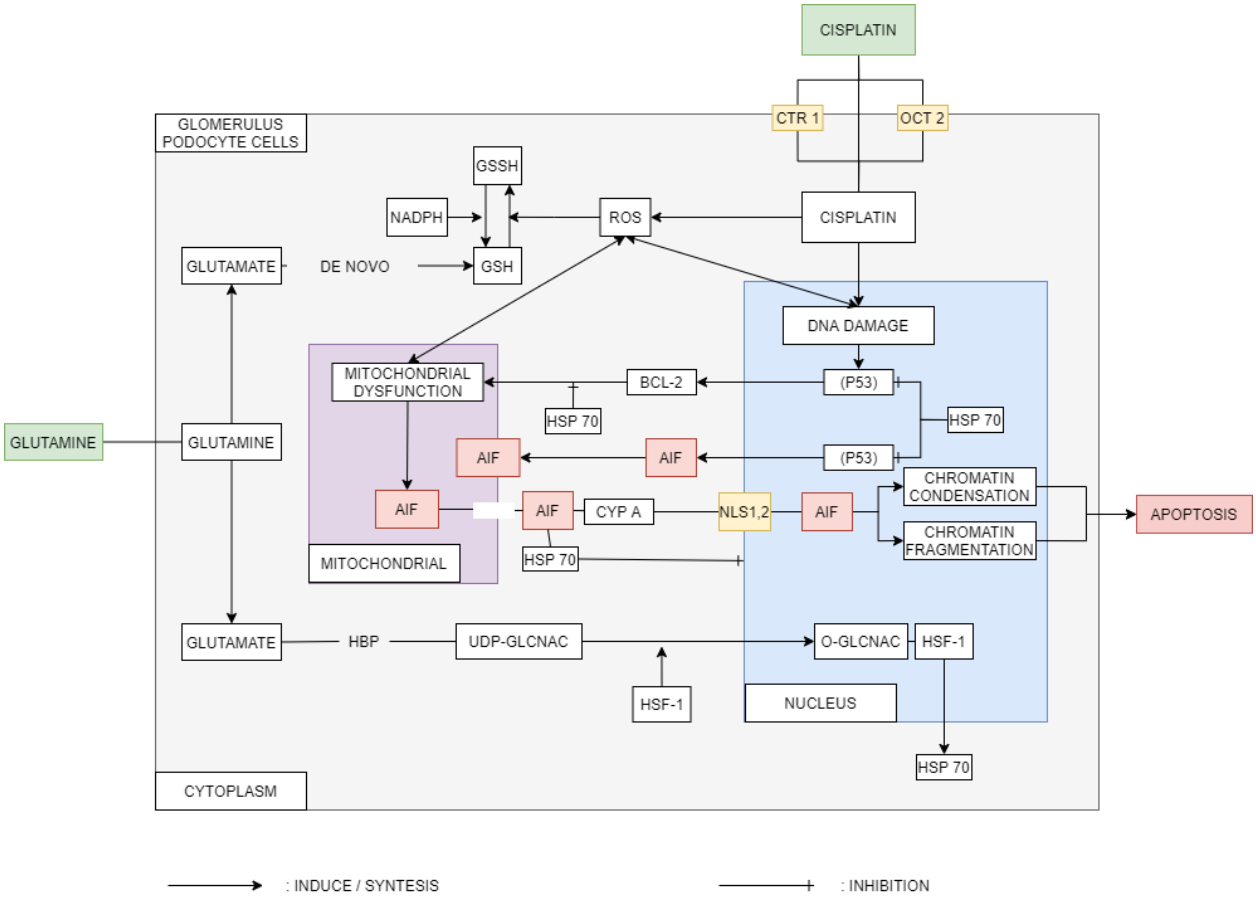

Figure 2. Conceptual framework

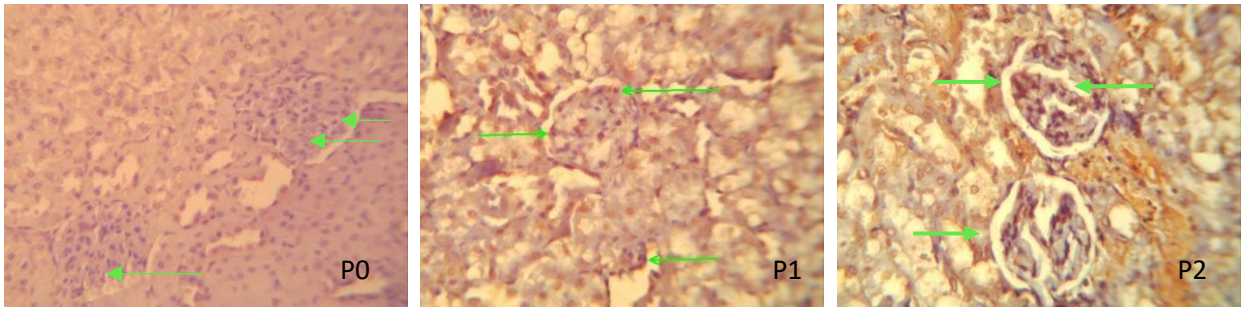

Figure 3. Morphology of the glomerular epithelial cells given anti -AIF antibodies. P0 is control, in which the mouse's kidney is not treated. P1 is a kidney of mouse given cisplatin i.p on the 7th day. P2 is the kidney of mouse injected glutamine i.v 7 days in a row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

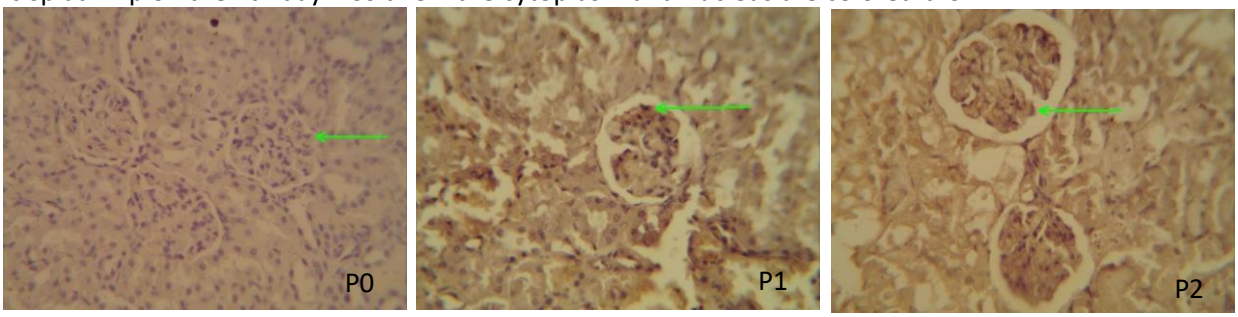

Figure 4. Morphology of the glomerular epithelial cells used Apoptotic Detection Kit. P0 is control, in which the mouse's kidney is not treated. P1 is a kidney of mouse given cisplatin i.p on the 7th day. P2 is the kidney of mouse injected glutamine i.v 7 days in a row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

Variable	AIF protein expression	Apoptotic cells
	Average ± sd	Average ± sd
P0	3.29±0.79	3.04±0.69
P1	5.32±0.68	5.26±0.53
P2	4.49±0.47	4.44±0.41

Table 1. Descriptive statistic of the AIF protein expression and apoptotic cells. P0 is control, in which the rat's kidney is not treated. P1 is a kidney that is given cisplatin injection on the 7th day. P2 is the kidney of rat given intravenous glutamine injection 7 days in a row before being injected intraperitoneal with cisplatin on the 7th day.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
P0	0.702	0.137
P1	0.539	0.189
P2	0.733	0.647

Table 2. The result of test normality Shapiro -Wilk showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
Levene Statistic	0.150	0.448

Table 3. The result of homogeneity variances Levene Statistic showed a sig value,> 0.05, which means that the data in this study were homogeneous .

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
ANOVA	0.000	0.000

Table 4. The result of AVONA showed the sig value > 0.05, which means that the data in this study had different results .

Variable	Comparison		Sig.	Interpretation
AIF protein expression	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.009	
Apoptotic cells	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.003	

Table 5. The result of LSD showed the sig value > 0.05, which means that the data in this study had different results in each group.

Variable	AIF protein expression	Apoptotic cells
	Sig. (2-tailed)	Sig. (2-tailed)
Kolmogorov-Smirnov Test	0.200	0.200

Table 6. The result of test normality Kolmogorov -Smirnov showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	Correlation	Sig.	Interpretation
AIF protein expression and Apoptotic cells	0.928	0.00	Correlation is significant and linier

Table7. The result of Pearson correlation showed the r (Pearson correlation) > 0.349 (positive) which means that the data in this study had correlation and linier. Thus, showed the sig value > 0.05, which means that the data in this study had a significant correlation between AIF protein expression and apoptosis cells.

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REVISI V

APOPTOSIS-INDUCING FACTOR PROTEIN EXPRESSION AND APOPTOSIS CHANGES WITH GLUTAMINE IN PODOCYTES CELLS EXPOSED WITH CISPLATIN

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Running title: Glutamine Effect on Apoptosis-inducing factor Expression

BAB 12Abstract

Cisplatin is a well-known chemotherapeutic drug as one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. However, side effects in normal tissues and organs, like nephrotoxicity induce apoptosis epithelial cells in kidney, limit the use of cisplatin. Glutamine is a substrate for the synthesis of glutathione as antioxidant, and promote HSP70 release, protecting cells from apoptosis induced by different stimuli. In the present study, we investigated the protective effect of glutamine on nephrotoxicity cisplatin in the kidney. Mice were divided into three groups a group of control (P0), a group of intraperitoneal injection of a single dose cisplatin 20mg/kg BW at 7th day (P1), and a group of intravenous glutamine injection 100mg/kg BW at day 1—7 and given an intraperitoneal injection of single dose cisplatin 20mg/kg BW at 7th-day (P2). Measurement of AIF expression and apoptotic cells was carried out by immunohistochemical methods. The number of AIF expressions and apoptotic cells is expressed in Allerd score. AIF expression result: P0: 3.29 ±0.79 P1: 5.32 ±0.68 P2: 4.49 ± 0.47. Apoptosis result: P0: 3.04 ±0.70 P1: 5.26 ±0.53 P2: 4.44 ± 0.41. There is decreased expression of AIF on intravenous

glutamine administration, followed by a decrease in apoptosis in the podocyte. In conclusion, glutamine administration might represent for the treatment of nephrotoxic induced cisplatin.

Keyword: Cisplatin, Glutamine, AIF Protein, Apoptosis, Podocyte

BAB 13Introduction

Cisplatin (CAS No.15663-27-1, MF-Cl2H6N2Pt; NCF-119875), cisplatinum, also called cis-diamminedichloroplatinum (II), is the first platinum drug approved globally for the treatment of cancer in 1978. Cisplatin is one of the most effective anticancer agents and is widely used for the treatment of several types of tumors. Cisplatin can bind to purine bases in

DNA, interfered DNA repair, causes DNA damage, and then induces apoptosis in cancer cells

(Achkar et al., 2018; Browning et al., 2017; Dasari & Tchounwou, 2015). Behind the benefits of cisplatin, there are various side effects, one of them is nephrotoxic. The mechanisms of cisplatin-induced nephrotoxicity are complex and involve many cellular processes including oxidative stress, apoptosis, and inflammation (Miller et al., 2010; Oh et al., 2014; Pabla & Dong, 2008). Tsuruya et al. in 2003 observed that renal epithelial cells deficient in TNFR1 and Fas were resistant to cisplatininduced cell death (Tsuruya et al. 2003). Seth et al. in 2005 identified that cisplatin increases caspase-2 and caspase-3 activity (Seth, et al., 2005). Takeda et al. in 1997 demonstrated that caspase-8 inhibition reduced cisplatin-induced cell death in vitro (Takeda et al., 1997). Faubel et al. in 2007, determined that the expression of IL-1β, IL-18, CX3CL1, and IL-6 in renal tissue was increased by presenting cisplatin, in mice (Faubel et al., 2007).

Apoptosis-inducing factor (AIF) is a mitochondrial oxidoreductase that plays a role in oxidative phosphorylation and redox control in normal cells. AIF was initially cloned and identified as a caspase-independent mitochondrial effector of apoptotic cell death. AIF is usually confined to the mitochondrial intermembrane space and released in response to death stimuli. In the cytoplasm, AIF can promote apoptosis by interacting with CYP, in which CypA assists in apoptogenic cytonuclear translocation of AIF. In contrast, Hsp70 maintains AIF in the cytoplasm and, therefore, can delay or prevent initiation of nuclear apoptosis. In the nucleus, AIF affects the chromosome condensation and fragmentation. Besides, AIF can induce mitochondria to release apoptogenic-proteins including cytochrome c and caspase-9 (Natarajan and Becker, 2012; Sevrioukova, 2011; Wang et al., 2019).

Glutamine is an α-amino acid and is the most abundant free amino acid in the body. (Cruzat et al., 2018). Glutamine plays an important role in the modulation of HSP expression via the biosynthetic pathway hexosamine (HBP). HSP70 (HSP72 and HSP 73) acts as antiapoptosis protein (Evans, Pinto & Wischmeyer, 2009; Nematbakhsh et al., 2017). Glutamine (via glutamate), cysteine, and glycine are precursor amino acids for the synthesis of GSH. GSH is an antioxidant that can react directly with ROS, produce oxidized GSH (GSSG) (Cruzat et al., 2018).

Cell damage due to exposure to cisplatin chemotherapy is generally studied in the proximal renal tubule area because there is a process of reabsorption and primary urine secretion in that area (Hall, 2013). However, kidney cell damage may occur in glomerular visceral epithelial cells (podocyte) as a filtration site in the urinary system, which causes all substances to pass through the filtering process in the glomerulus before going to the proximal tubule and directly exposed to cisplatin (Liapis et al., 2013). Therefore, research on apoptosis and AIF expression was carried out on glomerular visceral epithelial cells (podocyte). This study is analyzing the nephroprotective effect of intravenous glutamine on the incidence of apoptosis of the glomerular epithelial cells

(podocyte) by examining the expression of AIF which is the initiator of apoptosis in the apoptosis independent caspase. This research is expected to be an alternative problem solving for kidney failure caused by cisplatin chemotherapy modalities.

BAB 14Material and Methods

BAB 15Animal and housing

2-3 months old wistar male white mouse weighing 150–200 g were used after one week for proper acclimatization to the animal house conditions (12 h lighting cycle and 29-31°C temperature) with free access to water and standard rodent chow. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, Airlangga University, Indonesia. Animals were randomly divided into three groups with 10 animals in each group. The first group as the control group (P0). The second group was treated with cisplatin (20 mg/kg I.p) as a positive control (P1). The third group was injected with glutamine (100 mg/kg, i.v.), a gram glutamine suspended in 10 ml solution of ml 0.9% daily for seven consecutive days, and injected with cisplatin (20 mg/kg I.p) on the seventh day to induce nephrotoxicity (P2). All groups received equivalent volumes of the used vehicles. Mouse were sacrificed on the tenth day. The longitudinal section of the left kidney was excised from each animal for immunohistochemical examination.

Glutamine product from Serva-Germany, cisplatin product from Kalbe Farma, POD Apoptosis Detection Kit (11684817910 ROCHE), Anti-AIF Antibody (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880))

BAB 16Immunohistochemical Examination

Kidney tissue samples were fixed in 10% buffered neutral formalin, embedded in paraffin, cut, and stained with immunohistochemical staining for AIF expression and apoptosis examination using light microscopy.

Immunohistochemical detection of AIF expression was conducted using anti-AIF antibodies (AIF Monoclonal Antibody (Thermo Fisher Scientific Cat. # MA5-15880)). Immunohistochemical staining was carried out with anti-AIF antibodies in brief steps as follows: Deparaffinated preparations on glass objects; Washed with PBS pH 7.4; Blocking endogenous peroxide with 3% H2O2 for 20 minutes; Blocking unspecific protein with 5% FBS; Incubation with primary antibody (anti-AIF antibody) overnight at 4^0C, Incubation with conjugated anti-mouse biotin for 1 hour at room temperature; Incubation with Strep-Avidin Horse Radish Peroxidase for 40 minutes, drop DAB and incubate for 10 minutes; Counterstaining with Mayer Hematoxylin, the preparation is rinsed with dH2O and aerated; Mounting with a swab and the preparation is covered with a glass cover.

Immunohistochemical detection of apoptosis was processing by Apoptotic Detection Kit POD (11684817910 ROCHE) with the following brief steps: Deparaffinated tissue;

Preparations given proteinase K for 15 minutes, and dH2O in a Coplin jar for 2 x 2 minutes; Removal endogenous peroxide with 3% H2O2 for 5 minutes at room temperature; Drop the working strength of tdT enzyme in tissues, incubate at 37oC for 1 hour; Place the preparation in a coplin jar containing the working strength of the stop / wash buffer and incubated for 10 minutes at room temperature; Drop anti-digoxigenin conjugate incubation at room temperature in a damp

container for 30 minutes; Staining with substrate peroxidase for 10 minutes at room temperature; Counterstaining with methyl green for 30 seconds at room temperature; Covered with a glass cover.

The TUNEL reaction has a preference for labeling DNA chain termination in apoptosis so that it can be distinguished from DNA damage in necrosis (Bjorkerud & Bjorkerud, 1996). The specificity of the TUNEL reaction ranges from 70-80% and the sensitivity ranges from 61-90% (Kelly et al., 2003)

BAB 17Statistical analysis

The results of the AIF expression and apoptosis study used IHC staining and assessed with the H score shows a significant difference in each group. The formula for calculating the H score is as follows TS (total score) = PS (proportion score) + IS (intensity score) (Mazieres et al., 2013). The data are expressed as means ± SEM. Statistical analysis was performed by one-way ANOVA followed by LSD post-analysis test for multiple comparisons with α = 0,05 being considered as statistically significant, and Pearson correlation with α = 0,05 being considered as statistically significant.

BAB 18Result

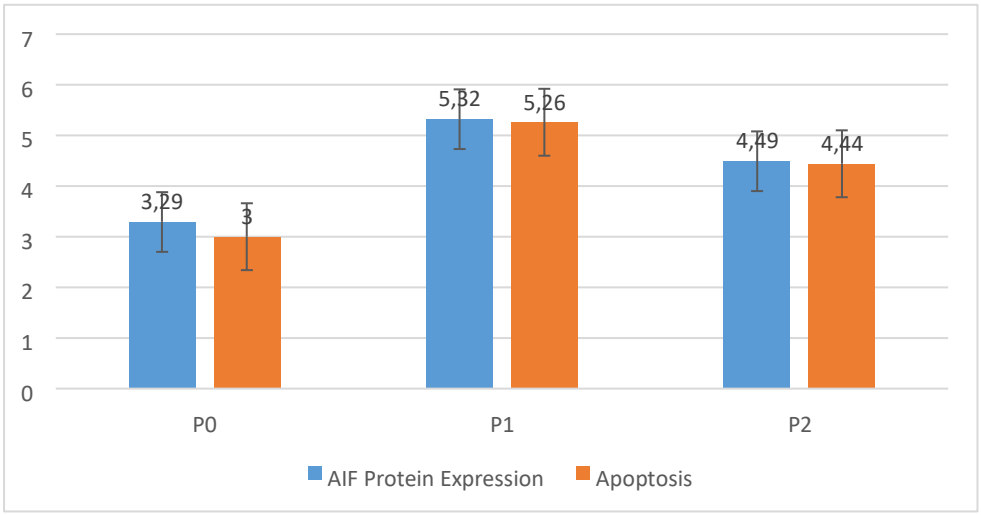

Figure 1. Graph of mean score of AIF protein expression and apoptosis.

The results of the AIF expression and apoptosis study used IHC staining and assessed with the H score shows a significant difference in each group (Figure 1). Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of AIF expression compared to giving cisplatin without glutamine administration (P1). The mean score of AIF expression in P2 with score 4.49 ± 0.47 compared to of P1 with score 5.32 ± 0.68 (Table 1, Table 2 and Table 3).

Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of AIF expression compared to giving cisplatin without glutamine administration (P1) (Table 1, Table 2, Table 3, table 4 and Table 5).

ANOVA test results on the AIF expression variable (Table 4), showed different evidence between groups in 1 research variable. With the LSD comparison test between P0 and P1, the mean score of AIF expression P0 = 3.29 ± 0.79 and P1 = 5.32 ± 0.68 (Table 1 and Table 5) had a significant

difference from the expression AIF protein in the glomerular visceral epithelial cells (podocyte). LSD test results on P1 and P2 with a mean score of P1 = 5.32 ± 0.68 and P2 = 4.49 ± 0.47 (Table 5) had a decreased AIF excretion involved in glomerular visceral epithelial cells (podocytes).

Administration of glutamine i.v before cisplatin i.p administration (P2) significantly decreased the amount of apoptosis compared with giving cisplatin without glutamine administration (P1) (Table 1, Table 2, Table 3, table 4 and Table 5).

ANOVA test results on the apoptosis variable (Table 4), showed a significant difference between groups in 1 research variable. With the LSD double comparison test between P0 and P, 1 mean apoptosis score of P0 = 3.04 ± 0.70 and P1 = 5.26 ± 0.53 (Table 1 and Table 5) had a significant difference from the number of cells experiencing apoptosis in the glomerular visceral epithelial cells (podocyte). LSD test results on P1 and P2 with an average score of P1 = 5.26 ± 0.53 and P2 = 4.44 ± 0.41 (Table 5) had a significant reduction in the number of apoptosis in glomerular visceral epithelial cells (podocytes).

The results of the Pearson correlation test on the AIF and apoptosis expression (Table 7 and Table 6) variables showed that the correlation was very strong with the direction of the relationship being directly proportional.

BAB 19Discussion

In this study, mice were divided into three groups, each group consisted of 10 male white mice. The negative control group (P0), the positive control group (P1), and the treatment group (P2). P1 had the largest mean AIF expression and apoptosis compared to other groups.

The research data were analyzed using the ANOVA test and if there were differences, it would be followed by a multiple comparison test, LSD. To use the ANOVA test, several conditions must be fulfilled: the sample comes from independent data, the variance between groups must be homogeneous and the data in each group is normally distributed. The homogeneity test in this study used the Levene test and showed a sig value,> 0.05, which means that the data in this study were homogeneous, for normality test used the Shapiro-Wilk test and showed the sig value > 0.05, which means that the data in this study were normally distributed (Table 2, Table 3, Table 4 and Table 5).

The research data were correlation using Person correlation, condition must be fulfilled, the data must be normal, the normality test used Kolmogorov-Smirnov test and showed the sig. value > 0.5, which means that the data in this study were normal (Table 6 and Table 7).

Apoptosis and AIF excretion in glomerular visceral epithelial cells (podocyte) in all groups in this study. Apoptosis and AIF expression in the positive control groups (P1) and (P2) are apoptotic processes triggered by cisplatin chemotherapy as a substance that has a nephrotoxic effect (Figure 3 and figure 4). Cisplatin-induced nephrotoxicity can occur from several pathways, including extrinsic, intrinsic apoptosis that can trigger AIF expression, cell regulators, MAPK, inflammation, and ROS. Meanwhile, apoptosis in the negative control group (P0) can be caused by physiological processes that can be experienced by all cells. The influence of external variables that cannot be controlled can also cause apoptosis in the glomerular visceral epithelial cells (podocyte).

The increase in AIF excretion in the positive control group (P1) was caused by exposure to cisplatin, an increase in free radicals and DNA damage which caused the maturation of the P53

gene to induce AIF protein transcription and induce Bcl-2 causing mitochondrial dysfunction to form holes in the mitochondrial membrane, so that the AIF protein could translocate to cytoplasm and nucleus (Figure 2).

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. Apoptosis, which represents the form of cell death performed by caspases, has traditionally been the only form of physiological and programmed cell death. However, recent evidence suggests that programmed cell death (PCD) can occur in the absence of caspase activation at all. Indeed, a large number of caspaseindependent models are now defined and a key protein involved in this type of PCD, the apoptosis inducing factor (AIF), has been identified. Cisplatin causes changes in the mitochondrial PTP to cause the pores in the mitochondria to open and allow small molecules such as the AIF protein to escape (Yang et al. 2008)

The decrease in AIF expression in the treatment group (P2) was due to glutamine, which is the precursor to glutathione in cells, directly binding to free radicals which can prevent mitochondrial dysfunction due to cisplatin administration. In addition, glutamine can increase the expression of Hsp 70 which is an antiapoptotic agent that can prevent various cell death pathways, one of which is by inhibiting the maturation of P53 to induce Bcl-2, preventing Bcl2 from causing mitochondrial dysfunction, so that translocation of AIF protein from mitochondria can be prevented (Figure 2).

These results are consistent with previous studies conducted by Sabirzhanov et al., 2012, showing that HSP70 modulates apoptosis of the caspase-independent pathway in primary cortical neurons and SH-SY5Y cells through interaction with AIF and by preventing translocation to the nucleus (Sabirzhanov, et al., 2012).

The increase in apoptosis in the positive control group (P1) due to cisplatin exposure triggered an increase in free radicals that can activate various cell death pathways. In addition, cisplatin induces maturation of the p53 gene and induces apoptosis (Figure 2).

These results are consistent with previous studies conducted by Marullo, et al., 2013, showing that cisplatin exposure induces a mitochondrial dependent ROS response that significantly contributes to cell killing by enhancing the cytotoxic effect exerted through the formation of nDNA damage and Yang et al., 2008, which states that translocation of the AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Marullo, et al., 2013; Yang et al., 2008)

The decrease in apoptosis in the treatment group (P2) was due to glutamine which is a precursor to GSH, which is a powerful antioxidant that can bind free radicals triggered by cisplatin administration. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing apoptosis of the caspase-independent pathway and the caspase-dependent pathway. So that apoptosis due to cisplatin can be prevented (Figure 2).

These results are in accordance with the theory described by Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2017, stated that glutamine is a precurso of GSH where GSH is a powerful antioxidant and plays an important role in the metabolism of exogenous and endogenous substances. GSH participates in many cellular reactions. It directly scavenges free radicals and other reactive oxygen species (hydroxyl radicals, lipid peroxyl radicals, peroxynitrite, and H2O2), and is indirectly linked to enzymatic reactions that can decrease apoptosis. In addition,

glutamine can increase the expression of Hsp70 which is an antiapoptotic agent through the HBP pathway and can also reduce apoptosis (Wu, et al., 2004; Circu & Aw, 2008; Evans, Pinto & Wischmeyer, 2009; Yang et al., 2016).

Increased AIF expression and increased apoptosis in the positive control group (P1) were strongly associated. The increase in AIF expression caused by cisplatin exposure triggers AIF synthesis and AIF translocation to the nucleus and causes condesation and large-scale chromatin fragmentation which triggers apoptosis (Figure 2).

These results are consistent with previous studies conducted by Yang et al., 2008, which stated that translocation of AIF protein from mitochondria can be induced by cisplatin in chemosensitive ovarian cancer cells and causes apoptosis. (Yang et al., 2008).

The decrease in AIF expression and the decrease in apoptosis in the treatment group (P2) were strongly associated. The decrease in AIF expression in the group injected with glutamine prior to cisplatin administration was due to the nephroprotective effect of glutamine as an antioxidant precursor that can bind to antioxidants directly and so it can inhibit / prevent mitochondrial dysfunction which can cause AIF protein to translocate to the cytoplasm and nucleus. In addition, glutamine can increase the expression of Hsp70 which is an antiapoptotic agent by preventing translocation of AIF to the nucleus, maturation of the P53 gene, and Bcl-2 pro apoptosis. Prevents AIF protein from translocating to the cytoplasm and nucleus and causing large-scale condesation and fragmentation of chromatin, thus preventing apoptosis (Figure 2).

These results are in accordance with previous studies conducted by Mao et, al. 2003, which stated that Hsp 72 inhibits the release of AIF protein from mitochondria which can inhibit apoptosis in renal epithelial cells exposed to metabolic inhibitors (Mao et al., 2003)

BAB 20CONCLUSION

Based on the research that has been conducted, there is a change in the expression of AIF and apoptotic cells in intravenous glutamine administration on glomerular visceral epithelial cells (podocyte) of male white mouse exposed to cisplatin, the changes that occur in this study are in the form of a decrease. Decreased expression of AIF on intravenous glutamine administration is correlation with a decrease in apoptosis in the glomerular visceral cells (podocyte). Glutamin administration can decrease AIF expression and apoptosis that induced by cisplatin administratio. Glutamine administration might represent for the treatment of nephrotoxic induced cisplatin

BAB 21ACKNOWLEDGEMENT

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BAB 22FIGURE AND TABLE

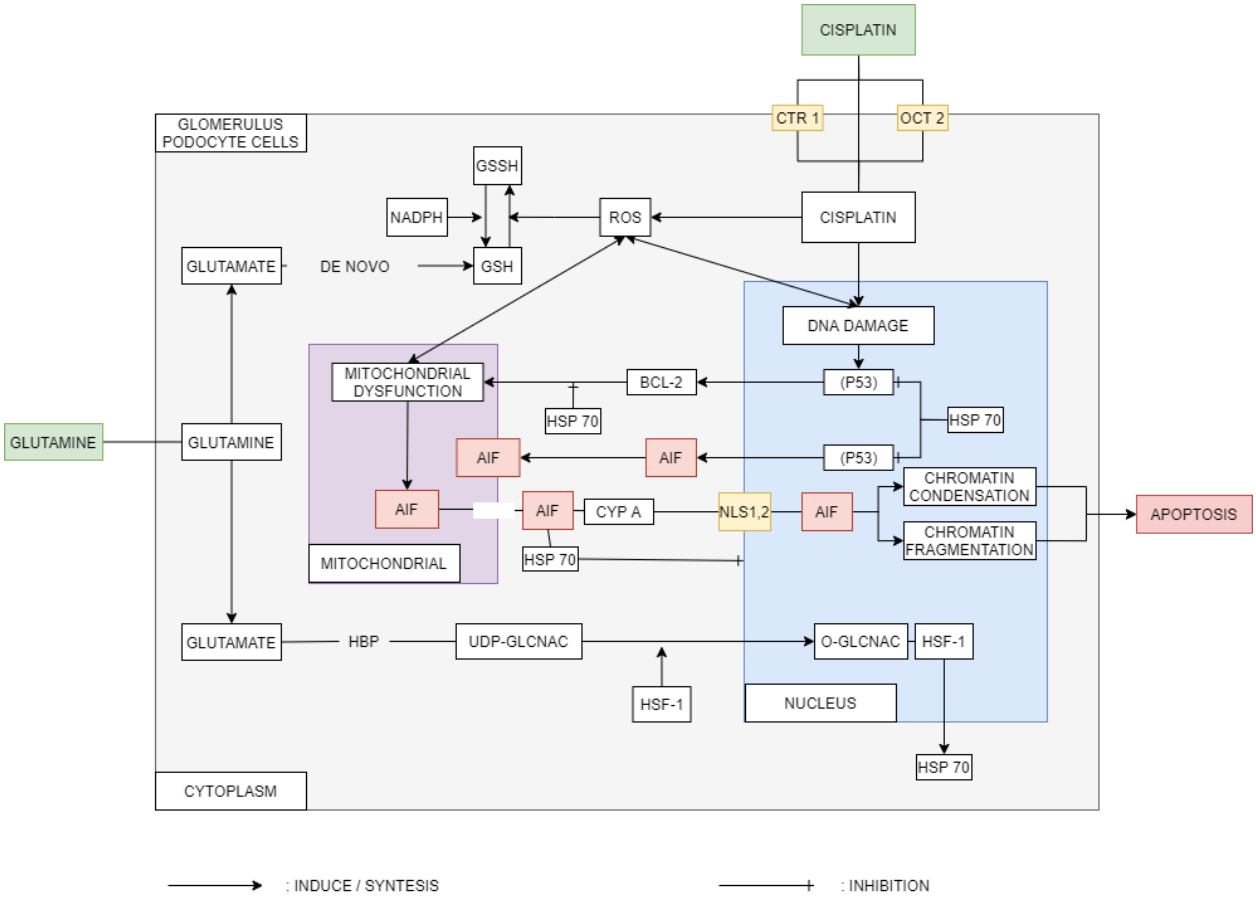

Figure 2. Conceptual framework

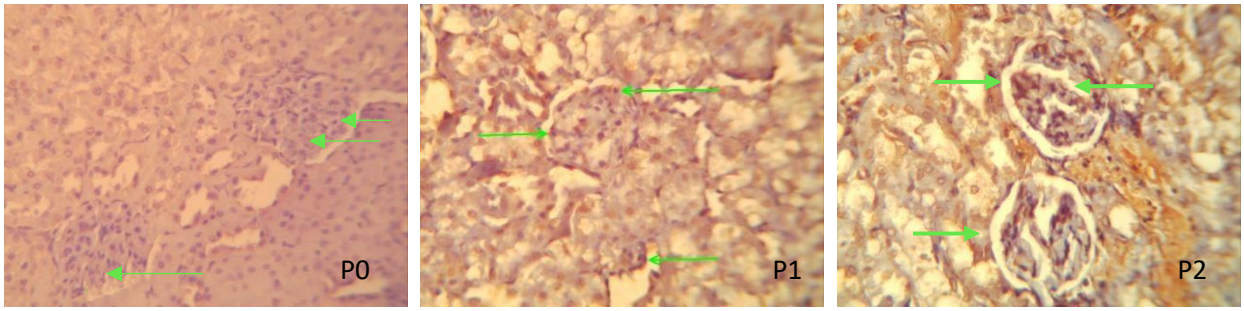

Figure 3. Morphology of the glomerular epithelial cells given anti -AIF antibodies. P0 is control, in which the mouse's kidney is not treated. P1 is a kidney of mouse given cisplatin i.p on the 7th day. P2 is the kidney of mouse injected glutamine i.v 7 days in a row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

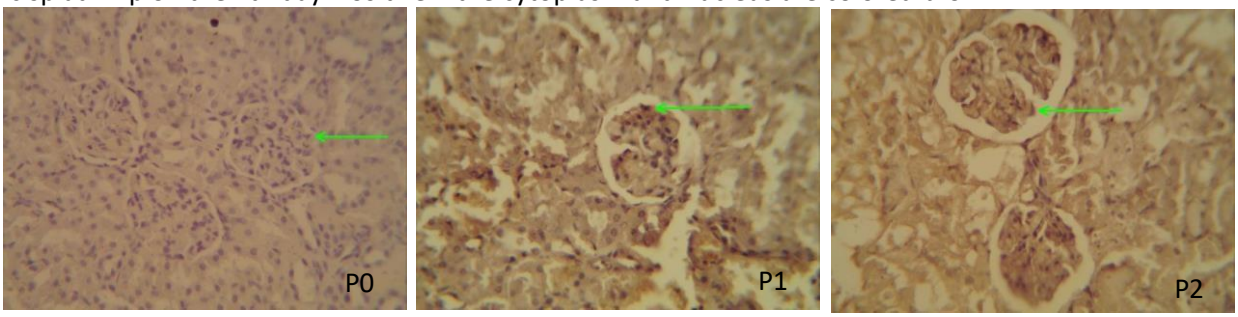

Figure 4. Morphology of the glomerular epithelial cells used Apoptotic Detection Kit. P0 is control, in which the mouse's kidney is not treated. P1 is a kidney of mouse given cisplatin i.p on the 7th day. P2 is the kidney of mouse injected glutamine i.v 7 days in a row before being injected with cisplatin i.p on the 7th day. Positive if the cytoplasm and nucleus are colored brown.

Variable	AIF protein expression	Apoptotic cells
	Average ± sd	Average ± sd
P0	3.29±0.79	3.04±0.69
P1	5.32±0.68	5.26±0.53
P2	4.49±0.47	4.44±0.41

Table 1. Descriptive statistic of the AIF protein expression and apoptotic cells. P0 is control, in which the rat's kidney is not treated. P1 is a kidney that is given cisplatin injection on the 7th day. P2 is the kidney of rat given intravenous glutamine injection 7 days in a row before being injected intraperitoneal with cisplatin on the 7th day.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
P0	0.702	0.137
P1	0.539	0.189
P2	0.733	0.647

Table 2. The result of test normality Shapiro -Wilk showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
Levene Statistic	0.150	0.448

Table 3. The result of homogeneity variances Levene Statistic showed a sig value,> 0.05, which means that the data in this study were homogeneous .

Variable	AIF protein expression	Apoptotic cells
	Sig.	Sig.
ANOVA	0.000	0.000

Table 4. The result of AVONA showed the sig value > 0.05, which means that the data in this study had different results .

Variable	Comparison		Sig.	Interpretation
AIF protein expression	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.009	
Apoptotic cells	P0	P1	0.000	Obtained difference
		P2	0.000	
	P1	P2	0.003	

Table 5. The result of LSD showed the sig value > 0.05, which means that the data in this study had different results in each group.

Variable	AIF protein expression	Apoptotic cells
	Sig. (2-tailed)	Sig. (2-tailed)
Kolmogorov-Smirnov Test	0.200	0.200

Table 6. The result of test normality Kolmogorov -Smirnov showed the sig value > 0.05, which means that the data in this study were normally distributed.

Variable	Correlation	Sig.	Interpretation
AIF protein expression and Apoptotic cells	0.928	0.00	Correlation is significant and linier

Table7. The result of Pearson correlation showed the r (Pearson correlation) > 0.349 (positive) which means that the data in this study had correlation and linier. Thus, showed the sig value > 0.05, which means that the data in this study had a significant correlation between AIF protein expression and apoptosis cells.

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