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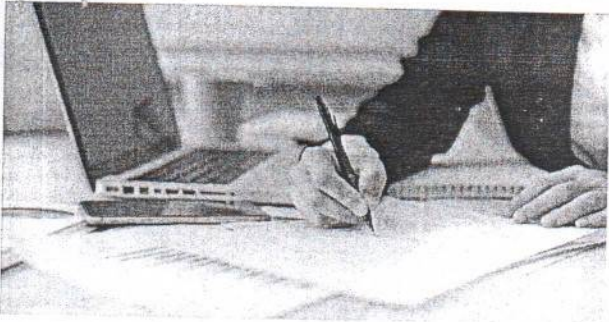
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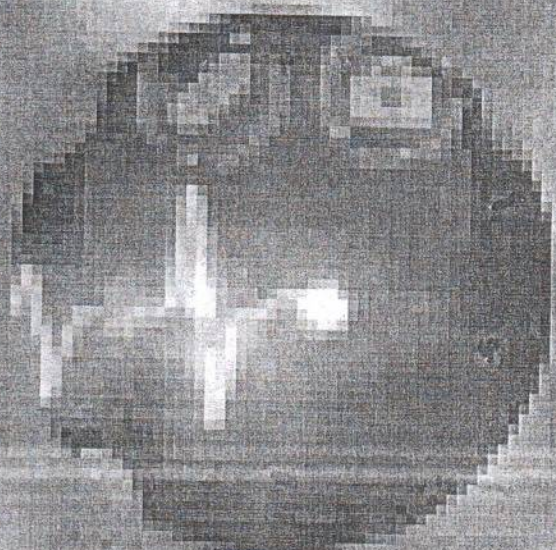
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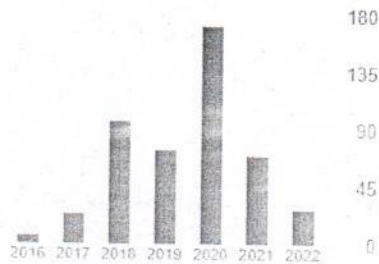
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The effect of intravenous glutamine on the protein expression changes, caspase-12 and apoptosis in renal tubulus russ male, rattusnovergicusstrain wistar, exposed by cisplastin

Yunitati Sutandio

Department of Anatomical Pathology, Faculty of Medicine Universitas Airlangga - Dr. Soetomo Academic General Hospital, 60131 Surabaya, Indonesia

Imam Susilo

Department of Anatomical Pathology, Faculty of Medicine Universitas Airlangga - Dr. Soetomo Academic General Hospital, 60131 Surabaya, Indonesia
Corresponding author email: imam-susilo@fk.unair.ac.id

Endang Joewarini

Department of Anatomical Pathology, Faculty of Medicine Universitas Airlangga - Dr. Soetomo Academic General Hospital, 60131 Surabaya, Indonesia

Abstract--Cisplatin efficacy is dependent on dose. The cisplatin-induced apoptosis pathway can go through the ER stress pathway which will activate Bax and Bak (proapoptosis) causing calcium homeostasis disorders to occur. Glutamine protective effect on caspase-12 expression on the prevention of apoptotic events in renal proximal tubular epithelial cells is unknown. This further study analyzes the effects of intravenous glutamine on caspase- 12 expression in the apoptosis of renal proximal tubular cells of male white rats exposed to cisplatin. The control group (P0) consisted of 10 individuals, group P1 on day 7 was given a single dose of 20mg / kg of intraperitoneal cisplatin injection, group P2 on day 1-7 was given intravenous glutamine injection of 100mg / kg on day 7 also given a single intraperitoneal cisplatin injection 20mg / kg. The number of cells expressing caspase-12 and the number of apoptotic cells was observed after 72 hours. Caspase-12 expression and apoptosis data were collected for processing and analysis. There is a significant effect ($p < 0.0001$) in giving intravenous glutamine on caspase-12 expression in renal proximal tubular cell apoptosis. Glutamine can inhibit the

expression of caspase-12, and the incidence of proximal tubular cell apoptosis were exposed by cisplatin.

Keywords--glutamin, cisplatin, caspase-12, apoptosis.

Introduction

Lately, the incidence of cancer as one type of non-communicable diseases is increasing. According to WHO the number of cancer patients in the world increased by about 7 million people each year, and two-thirds of them are in developing countries. If not controlled, an estimated 26 million people will suffer from cancer and 17 million died of cancer in 2030 (the Indonesian Cancer Foundation, 2013). Based on estimates GLOBOCAN 2012, about 14.1 million new cancer cases and 8.2 million cancer deaths that occurred in 2012 compared to 2008 approximately 12.7 million new cancer cases and 7.6 million cancer deaths that occurred worldwide (Ferlay et al., 2013).

Cisplatin is a potent tumor drug. The combination of cisplatin-based chemotherapy is used as front-line therapy in the treatment of various types of malignancies in humans. The efficacy of cisplatin dose-dependent means that at higher doses also greater antineoplastic effect but a side effect of nephrotoxicity also increased significantly and these often prevent the use of higher doses of cisplatin to maximize the effect neoplastic (Hanigan & Devarajan, 2003).

Nowadays, it is recognized that the prevalence of the use of cisplatin nephrotoxicity at high enough to occur in about one-third of patients undergoing cisplatin therapy (Miller et al., 2010). The most serious cisplatin nephrotoxicity is acute kidney injury (AKI) occurs in 20-30% of patients with nephrotoxic because cisplatin therapy, most often occurs 10 days after the use of cisplatin (Pabla & Dong, 2008). In vitro, the low concentrations of cisplatin causes the cell to undergo apoptosis while at high concentrations the cell will undergo necrosis (Hong et al., 2012).

Most natural materials and various food components have been evaluated as a potential chemoprotective agent against cisplatin one is glutamine (Mora et al., 2003). Glutamine is a free amino acid most commonly found in plasma and body. Glutamine plays a role for the synthesis of glutathione. Glutathione is a powerful antioxidant that is an important factor in the metabolism of drugs and endogenous substances (Mates et al., 2002). Glutamine is a non essential amino acid and is found in large amounts in skeletal muscle (Wernerman,2008). Some researchers found in their recent study that glutamine has potential as an inducer of HSP (Heat Shock Protein). The correlation between glutamine and HSP was first reported by Sanders & Kon (1991) in their study. The addition of glutamine under thermal stress, can increase the expression of HSP 10 times (Sanders & Kon, 1991).

Research Wischmeyer et al (2001) in mice with multiple organs endotoxemia prove that the administration of intravenous glutamine can increase the expression of HSP-70 (Wischmeyer et al., 2001). In critically ill patients parenteral

nutrition glutamine can increase the expression of HSP-70 (Ziegler et al., 2005). Research Zhang et al (2009) showed that a single dose intravenous administration of glutamine can alleviate ischaemia-reperfusion injury (IRI) rat kidneys in the first 24 hours, and mechanisms that may be associated with increased expression of HSP-70 (Zhang et al., 2009).

HSP is a group of proteins essential for cell survival with the condition of stress and apoptosis (Wischmeyer et al., 2001). Heat shock proteins (HSP) protect cells by preventing apoptosis and acts as a molecular chaperone to improve the stress associated with the "misfolding" of proteins (Vermeulen et al., 2010). Apoptosis plays an important role in survival by maintaining homeostasis in multicellular organisms as well as in the management of various diseases, due to malfunctioning of apoptosis pathway can lead to several diseases in humans such as k anchor, neurodegenerative, and some types of autoimmune disorders (Rastogi et al., 2009). The incidence of apoptosis in cisplatin occurs through the use of three apoptotic pathways involved among others: the first track is centered on the intrinsic mitochondrial pathway, the second track is the extrinsic pathway mediated by death receptors, and the third path is the path of the endoplasmic reticulum (ER) stress (Kumar et al., 2015).

The third apoptosis pathway is ER stress pathway activates Bax and Bak (proapoptosis) disturbances in calcium homeostasis resulting in calcium release occurs activation of calpain enzyme that can cleave and activate caspase-12 further induces caspase-9 activate caspase-3 causes the cell to undergo apoptosis (Scorrano et al., 2003; Boyce & Yuan., 2006). ER stress can be caused by the accumulation of protein misfolding that results in the activation of caspases that play a major role in the process of apoptosis (Lai et al., 2006 ; Li et al., 2006). Caspase-12 as the initiator caspase apoptotic pathway ER stress. Prokaspase-12 lies predominantly in the cytoplasmic side of the endoplasmic reticulum and is expressed at high levels in the kidney, particularly in renal proximal tubular epithelial cells. Mice deficient caspase-12 will be resistant to apoptosis through the ER stress (Nakagawa et al., 2000).

Method

This study is a laboratory experimental design was "The Randomized Post Test Only Control Group Design" with 30 male rats sample divided into 3 groups randomly. Each group consisted of 10 animals, namely, the control group (P0), the group P1 on the 7th day given intraperitoneal injection of a single dose of cisplatin 20mg / KgBW (Tsuruya *et al.*, 2003), P2 group on days 1-7 glutamine dose intravenous injections of 100mg / KgBW once daily (Zhang et al., 2009) and on the 7th day was also given intraperitoneal injection of a single dose of cisplatin 20mg / KgBW. After 72 hours of injection of cisplatin, processed rat kidney tissue immunohistochemistry, to observe the renal proximal tubular cells expressing caspase-12, and the number of cells undergoing apoptosis.

Animals used in this study was obtained from the Faculty of Veterinary Medicine, University of Airlangga, with the inclusion criteria is *Rattus norvegicus* healthy male Wistar strain, age 2-3 months, weight 150-200 grams. The sample size in this study is determined by the formula Federer (1991), with the correction of

drop-out (0.1), So, the total sample required is 30 male rats. The treatment of the animal for 7 days, then examined kidney preparations on the 10th day in the form of the number of cells expressing caspase-12, and the number of cells undergoing apoptosis in renal proximal tubular cells male rats, with a light microscope 400X. Renal proximal tubular cells (marked by the tubular lumen that cilia / brush border), each sample is counted in 10 visual field (HPF). The length of time to give Intravenous Glutamine Injection (IGIV) is 7 days. based on previous studies, glutamine HSP will increase after 7 days of IGIV administration (Ziegler et al., 2005).

Observation time on day 10 or 72 hours after intraperitoneal injection of cisplatin (on day 7) due to apoptosis in renal proximal tubular cells was evident after 72 hours of administration of cisplatin (Pabla& Dong, 2008). Processed mouse kidney tissue immunohistochemistry, to observe the number of renal proximal tubular cells expressing caspase-12, and the number of apoptotic cells. Data expression of caspase-12 and apoptosis were collected to test the normality by the Shapiro-Wilk ($\alpha = 0.05$), followed by the Leaven homogeneity test ($\alpha = 0.05$). The test results obtained data were normally distributed ($p > 0.05$) and homogeneous ($p > 0.05$) followed by a different test ANOVA ($\alpha = 0.05$), when there are differences continued by Least Significant Difference (LSD) ($\alpha = 0.05$).

Discussion

The results of this study appear to have changed in the epithelial cells of the proximal renal tubules after 72 hours of observation. Whereas in the treatment group that received glutamine injection, changes in the proximal renal tubular epithelial cells were minimal (Figure 1).

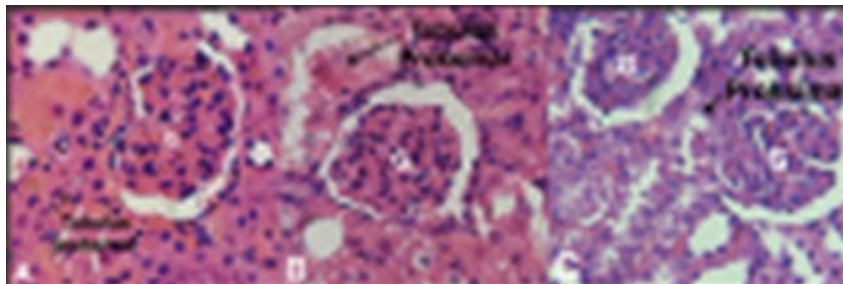


Figure 1. Comparative figures in renal proximal tubular cells (A: The control group (P0), B: groups given cisplatin (P1), C: The group that was given glutamine and cisplatin (P2) (HE, Olympus BX-41-400X))

Table 1

Results of data normality test apoptosis and caspase-12 with the Shapiro-Wilk

Grup	n	P value	
		Apoptosis	Caspase-12
P0	10	0,365	0,549
P1	10	0,878	0,539
P2	10	0,211	0,851

Based on Table 1, the data distribution apoptosis and caspase-12 is normal ($p > 0.05$). The next test of homogeneity with Leaven test shown in Table 2 and Table 3 in the following table below:

Table 2
Results of homogeneity of variances test caspase-12

Levene Statistic	df1	df2	Sig.
1.951	2	27	162

Table 3
Results of homogeneity of variances test apoptosis

Levene Statistic	df1	df2	Sig
032	2	27	968

Based on Table 2 and Table 3 data distribution and apoptosis caspase-12 is homogeneous ($p > 0.05$). Because the data were normally distributed and homogeneous do different test Anova. The results of immunohistochemical examination of kidney tissue with primary antibody caspase-12, found their expression changes caspase-12 in each group (Table 4) and (Figure 2).

Table 4
Anova results of different test data is caspase-12 between groups

Grup	N	The average \pm standard deviation	P value
P0	10	38,90 \pm 5,666 ^a	< 0,0001
P1	10	159,50 \pm 9,721 ^b	
P2	10	79,60 \pm 4,835 ^c	

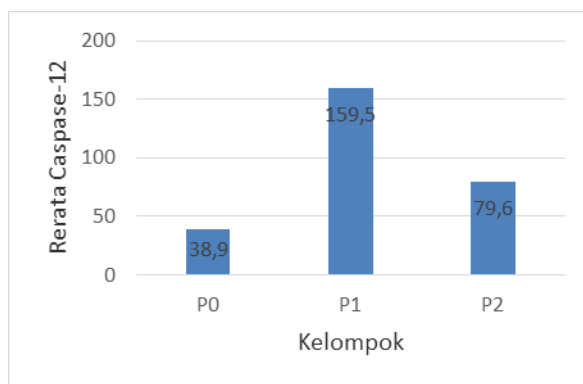


Figure 2. The bar chart is the average expression of caspase-12 in renal proximal tubular cells between groups

Based on Table 4 and Figure 2 shows that the control group (P0) showed the average expression of caspase-12 amounted to 38.90 ± 5.666 . Intraperitoneal cisplatin injections (group P1) showed the average expression of caspase-12

higher to 159.50 ± 9.721 after 72 hours compared to the control group (P0). Glutamine intravenous injections followed intraperitoneal injection of cisplatin (group P2) showed the average expression of caspase-12 lower to 79.60 ± 4.835 after 72 hours than in group P1. There are a significant differences on Anova test results depending on the expression of caspase-12 ($p < 0.0001$) between groups.

Because there are differences in the expression of LSD continued caspase-12 and also found a significant difference ($p < 0.0001$) between P0 and P1, between P0 and P2, between P1 and P2. So the group P2 by administering intravenous injection of glutamine no influence on the expression of caspase-12 was significantly ($p < 0.0001$). It was concluded that intravenous administration of glutamine inhibits the expression of caspase-12 in renal proximal tubular cells. Here is the histological expression of caspase-12 with Immunohistochemistry staining, using Olympus BX-41 microscope magnification of 400x (Figure 3).

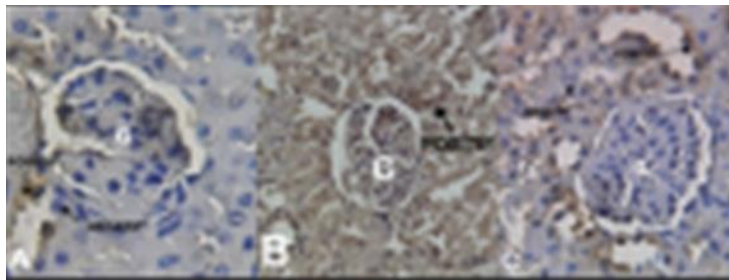


Figure 3. Comparative overview of caspase-12 expression in renal proximal tubular cells. A is a picture in the control group (P0); B is a picture in the group given injections of cisplatin (P1); C is a picture in the group given injections of glutamine and cisplatin (P2); G is the glomerulus. (Immunohistochemistry; Olympus BX-41-400x)

The results of the examination of kidney tissue by apoptosis Detection Kit In Situ Cell Death Detection Kit, POD, found the change in the number of cells undergoing apoptosis in each group (Table 5) and (Figure 4)

Table 5
Anova different test results between groups of data apoptosis

Grup	N	The average ± standard deviation	P value
P0	10	$41,00 \pm$ $4,944^a$	< 0,0001
P1	10	$190,30 \pm$ $4,877^b$	
P2	10	$89,20 \pm$ $4,894^c$	

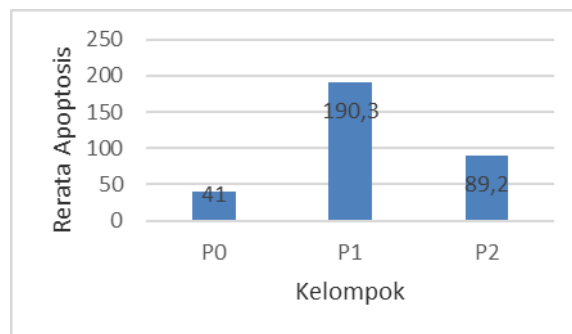


Figure 4. The bar chart shows the average apoptosis of the proximal renal tubular cells between groups

Based on Table 5 and Figure 4 shows that the control group (P0) shows the mean number of apoptotic cells amounted to 41.00 ± 4.944 . Giving injection of cisplatin (group P1) showed higher mean cell apoptosis becomes 190.30 ± 4.877 after 72 hours compared to the control group (P0). Glutamine intravenous injections followed intraperitoneal injection of cisplatin (group P2) shows the average of apoptotic cell lower to 89.20 ± 4.894 after 72 hours than in group P1. Anova different test results on the incidence of apoptosis there is a significant difference ($p < 0.0001$) between groups. Because there were differences in the incidence of advanced apoptosis, LSD found a significant difference ($p < 0.0001$) between P0 and P1, between P0 and P2, between P1 and P2. So the group P2 by administering intravenous injection of glutamine no effect on the incidence of apoptosis was significantly ($p < 0.0001$). It was concluded that administration of Intravenous glutamine will inhibit the incidence of apoptosis in renal proximal tubular cells.

The following histological renal proximal tubular cell apoptosis with Detection Kit In Situ Cell Death Detection Kit, POD, using Olympus BX-41 microscope 400x magnification (Figure 5).

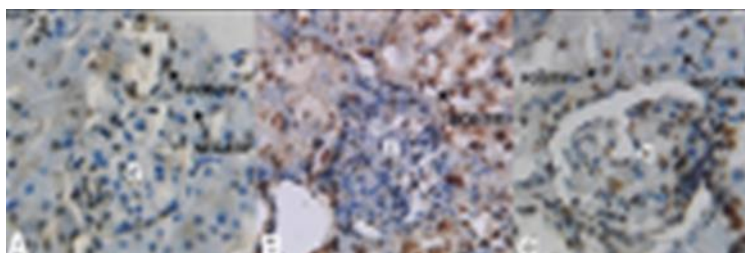


Figure 5. The comparison of proximal renal tubular cell apoptosis between groups. A is a picture in the control group (P0); B is a picture in the group given injections of cisplatin (Group P1); C is a picture in the group given injections of glutamine and cisplatin (Group P2); G is glomerulus. (Immunohistochemistry; Olympus BX-41-400x)

Glutamine is a non essential amino acid and is found in large amounts in skeletal muscle (Wernerman, 2008). Glutamine is one of the precursors for the synthesis of glutathione. Glutathione is the major endogenous antioxidant in mammalian

cells that protects cells from oxidative injury and has antiapoptotic effects (Mates *et al.*, 2006). Glutamine plays a role in the regulation of some specific processes in the cell, including apoptosis. The availability of glutamine is strongly associated with the induction of apoptosis, working as nutrients, signaling molecules, act directly or indirectly on the programmed cell death pathway (Curi *et al.*, 2007). When the patient is critically ill, there is a dramatic drop in plasma and muscle glutamine. In plasma, glutamine levels tend to decrease over several weeks and are associated with increased mortality in patients admitted to the ICU (Intensive Care Unit). The decrease in glutamine is mainly caused by the high consumption of glutamine by rapidly dividing cells such as enterocytes, bone marrow cells and lymphocytes. Therefore, glutamine is needed for critically ill patients because its availability depends on external input (Vermeulen *et al.*, 2010).

Glutamine showed greater benefit when given in higher doses and given for more than 5 days to patients staying in the ICU. The results of clinical trials prove that giving glutamine within the first 24-48 hours after entering the ICU either via enteral or parenteral is the key to healing patients with optimal results. It has been shown to reduce mortality and infection in critically ill patients (Kim & Wischmeyer, 2013).

The addition of intravenous glutamine was able to increase the expression of heat shock protein (HSP) tenfold under thermal stress. Glutamine can induce the expression of heat shock protein in high amounts in almost all organs, one of which is the kidney (Zhang *et al.*, 2009). HSP protects cells by preventing apoptosis and acts as a molecular chaperone to increase stress associated with protein misfolding (Vermeulen *et al.*, 2010). Recent research has revealed that glutamine can trigger HSP and can reduce cell injury under various cellular stress conditions (Pheng *et al.*, 2013).

Currently, cisplatin-based combination chemotherapy is being used as a frontline therapy in the treatment of various types of malignancies in humans. The dose-dependent efficacy of cisplatin means that higher doses have a greater antineoplastic effect. However, the side effects of nephrotoxicity also increased significantly and this often prevents the use of higher doses of cisplatin to maximize the antineoplastic effect (Hanigan & Devarajan, 2003). Reported nephrotoxicity from clinical trials occurred early in the use of cisplatin chemotherapy. It is now known that the prevalence of cisplatin nephrotoxicity is quite high in about one third of patients undergoing cisplatin therapy. Clinically, cisplatin nephrotoxicity is often seen after 10 days of cisplatin use (Miller *et al.*, 2010). *In vitro*, low cisplatin concentrations cause cells to experience apoptosis while at high concentrations cells will experience necrosis (Hong *et al.*, 2012).

One of them is the apoptotic pathway through the endoplasmic reticulum (ER) stress (Kumar *et al.*, 2015). The initiator caspases in this ER pathway is caspase-12, which is localized to the cytosolic side of the ER. Procaspase-12 found in high enough concentrations in the kidneys, especially in renal proximal tubular epithelial cells (Liu & Baliga, 2005). The initiator caspases in this ER pathway is caspase-12, which is localized to the cytosolic side of the ER. Procaspase-12 found in high enough concentrations in the kidneys, especially in renal proximal tubular epithelial cells (Scorrano *et al.*, 2003; Boyce & Yuan, 2006; Shiraiishi *et*

al., 2006). Apoptosis ER stress pathways may also occur due to accumulation of protein misfolding that results in the activation of caspases play a major role in the process of apoptosis (Lai et al., 2006; Li et al., 2006; Wu & Kaufman, 2006).

Observations in vitro was recently expanded with cisplatin nephrotoxicity observations in a mouse model, shows the involvement of ER stress and related signals such as caspase-12. ER-related proteins involved in the injury is Ca²⁺ + cisplatin-independent phospholipase A2 (ER-iPLA2) (Pabla & Dong, 2008). Apoptosis can occur due to pathological accumulation of protein misfolding caused by extrinsic factors, one of which is damage caused by free radicals (Kumar et al., 2015). Results of research on embryonic fibroblasts deficient mouse BAK and BAX in the ER lumen will eventually prevent apoptosis (Scorrano et al., 2003).

This study was conducted on experimental animal treatment that were divided into 3 groups, namely the control group (P0), the P1 group was the group that received intraperitoneal cisplatin injection, and the P2 group was the group that received intraperitoneal cisplatin injection and intravenous injection. glutamine. Treatment response was observed within 72 hours after treatment, because apoptosis was proven after 72 hours of cisplatin injection (Tsuruya et al., 2003).

The role of glutamine on the expression of caspase-12 in renal proximal tubular epithelial cells caused by exposure to cisplatin

Based on Table 4 and Figure 2 can be argued that the injections of cisplatin on group P1 showed the average expression of caspase-12 higher to 159.50 ± 9.721 after 72 hours when compared with the control group (P0). The results are compatible with research Liu & Baliga (2005) that proved the injections of cisplatin in rat renal tubular epithelial cell division will occur procaspase-12 and will be activated into caspase-12 which localized in the cytosol ER as initiator caspases apoptosis pathway ER stress. Based on these results, caspase-12 activation occurred in the proximal renal tubular cells due to cisplatin (Liu & Baliga, 2005).

The results are compatible with research Zhang et al (2009) that showed the addition of intravenous glutamine can increase the expression of heat shock protein (HSP) ten times under thermal stress. Glutamine can induce the expression of heat shock proteins in high amounts in almost all organs and of one of them is kidneys (Zhang et al., 2009). The results of research by Martinez et al (2010) on fibroblast cells from mouse embryos that were deficient in the enzyme kalpain caused a decrease in caspase-12 activation that plays a role in cell apoptosis through ER stress (Martinez et al., 2010). Based on the results of the analysis, the inhibition of the release of the enzyme kalpain which will cause inhibition of caspase-12 expression can further prevent apoptosis in the proximal tubular renal epithelial cells.

In this study, administration of glutamine injection before injection of cisplatin on group P2 showed the average expression of caspase-12 lower to 79.60 ± 4.835 after 72 hours when compared with the group P1. Thus, for group P2, the addition of intravenous injection of glutamine had no significant effect on

caspase-12 expression ($p < 0.0001$). It can be concluded that observation after 72 hours of intravenous glutamine administration can inhibit caspase-12 expression in the proximal renal tubular cells.

The role of glutamine on apoptosis of renal proximal tubular epithelial cells caused by exposure to cisplatin

Based on Table 5 and Figure 3 it can be argued that the injections of cisplatin on group P1 showed the average cell apoptosis higher to $190.30 \pm 4,877$ after 72 hours when compared with the control group (P0). The results of this study are compatible with the results of Hong et al (2012) which proved cisplatin resulted in apoptosis through the ER stress in proximal tubular cells of kidney causes activation of Bax and Bak. Releasing calcium lead to impaired calcium homeostasis when release the enzyme calpain activation of caspase-12 in cytosolic ER. Furthermore, caspase-12 exit from the cytosol ER, and accumulates in the cell nucleus that will induce the incidence of apoptosis in renal proximal tubular epithelial cells, which is a mechanism of nephrotoxicity of cisplatin (Hong et al., 2012).

In this study, administration of intravenous glutamine before injection of cisplatin on group P2 showed the average number of apoptotic cell lower to 89.20 ± 4.894 after 72 hours when compared with the group P1. The addition of intravenous glutamine is able to increase the expression of heat shock protein (HSP) ten times under thermal stress. Glutamine can induce the expression of heat shock proteins in high amounts in almost all organs and one of them is kidneys (Zhang et al., 2009). HSP protect cells by preventing apoptosis and act as molecular chaperons to improve the stress associated with protein misfolding (Vermeulen et al., 2010). Recent research revealed that glutamine may induce HSP, can reduce cellular injury in a variety of cellular stress conditions (Pheng et al., 2013).

The results of this study are consistent with the results of research Scorrano et al (2003) on fibroblast cell mouse embryos that lacked BAK and BAX in the ER lumen will eventually prevent apoptosis (Scorrano et al., 2003). Based on research data above, it can be argued that as a result of cisplatin apoptosis occurs through increased expression of caspase-12, which will induce the incidence of apoptosis in renal proximal tubular epithelial cells. So, it can be concluded that the observation 72 hours after administration of intravenous glutamine inhibit the occurrence of apoptosis in renal proximal tubular epithelial cells was significantly ($p < 0.0001$) between groups and is directly proportional to the effect of glutamine that inhibits the expression of caspase-12.

Conclusion

Based on the results of this study, concluded that there is the effect of intravenous glutamine on the expression of caspase-12 in the incidence of apoptosis of renal proximal tubular epithelial cells that were exposed cisplatin. So, the supply of intravenous glutamine may inhibit the expression of caspase-12, and the incidence of apoptosis of renal proximal tubular epithelial cells of male rats exposed cisplatin.

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