ORIGINAL RESEARCH REPORT

The Effect of Intravenous Glutamine Administration in Lowering AIF Expression in Renal Tubular Cells

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ABSTRACT

Background: Cisplatin, a widely used chemotherapy that is effective for many forms of cancer, has nephrotoxic qualities that can reduce renal function. Due to its side effects, the usage of cisplatin is limited by its dose. Glutamine, an amino acid, possesses properties that can counteract the nephrotoxicity of cisplatin. **Objective:** This study aimed to analyze the effect of intravenous glutamine administration on the decrease of AIF expression and apoptosis in intraglomerular mesangial cells of cisplatin-exposed rats. **Material and Method:** Samples (n) consisting of 30 rats were divided into 3 groups, with each group containing 10 rats. Group P0 received no treatment, group P1 received an injection of cisplatin on the 7th day, and group P2 received glutamine on the 1st–7th day and an injection of cisplatin on the 7th day. After 72 h, the tissue samples were immunohistochemically processed and then observed under a light microscope with 400x magnification. AIF expression and apoptosis were measured in the Allred/H score. **Result:** AIF expression results were: P0 = 4,35(2,2-5,4); P1 = 5,9(4,8-6,3); P2 = 5,25(2,4-5,8). The AIF expression of P2 was found to be significantly lower (p = 0.008) than P1. Apoptosis results were: P0 = 5,2(4,7-5,5); P1 = 6,4(6,1-7,1); P2 = 5,7(4,8-5,9). The apoptosis level of P2 was found to be significantly lower (p = <0.0001) than P1. Strong correlations between the decrease in AIF expression and the apoptosis level were found (p = <0.0001). **Conclusion:** Glutamine contributes to the decreased AIF expression and apoptosis in intraglomerular mesangial cells of cisplatin-exposed rats.

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Highlights

1. Glutamine, an amino acid, can help mitigate the side effects of cisplatin by boosting glutathione (GSH) levels, an antioxidant that counteracts oxidative stress.
2. By reducing AIF protein expression and apoptosis, glutamine may protect kidney cells from cisplatin toxicity, potentially improving the safety of chemotherapy.

BACKGROUND

Cancer remains one of the deadliest diseases. According to Basic Health Research (*Riset Kesehatan Dasar*), the number of cancer patients in Indonesia increased from 1.4 per 1000 people in 2013 to 1.79 per 1000 people in 2018 (Ministry of Health of Republic Indonesia, 2019). Cancer occurs when altered cells proliferate out of control while being subject to natural selection-driven evolution (Brown, et al., 2023). An anticancer drug that is widely used in cancer treatment regimens is cisplatin, a platinum-based anticancer drug. Cisplatin is effective against solid organ cancers such as breast cancer, head and neck cancer, testicular cancer, and lung cancer (Dasari &Tchounwou, 2014) The efficacy of cisplatin increases, as does its side effects with each increase in dose. Therefore, adjustments are needed for different conditions in patients. Although cisplatin's effect on glomerular damage is significantly lower than tubular damage, making it less likely to be observed in human models, except in cases of high-dose usage, it can still directly damage glomerular cells and cause proteinuria (Oh, et al., 2014). Cell death in the kidneys is a normal process that occurs to maintain function, especially at a developmental age, to maintain the number of cells in the kidneys. Cells that are no longer needed at each stage of growth should also be removed. One of the building blocks of the kidney is the mesangial cells, as they act as support for the glomerular structure (Priante, et al., 2019). However, when the kidneys are exposed to cisplatin, which is nephrotoxic, more cells will die. When these cells are damaged, the mesangial cells that hold the glomerular structure together break apart, which can cause terrible problems like proteinuria (Rabelink, et al., 2015).

As modern cancer treatments are hardly accessible to the masses, conventional chemotherapy, such as cisplatin, is still widely used. One substance that is thought to be effective in reducing the side effects of cisplatin is glutamine, an amino acid found naturally in the human body. The biggest role of glutamine is its protective response and defense in the situation of injury or stress (Dasari & Tchounwou, 2014). Previous research has shown that the existence of glutamine can maintain homeostasis as well as cell safety and survival. Glutamine deficiency may cause a decrease in lymphocyte proliferation that may induce apoptosis (Cruzat, et al., 2018). Glutamine is a precursor of a potent antioxidant called glutathione (GSH). Cisplatin exposure may increase the quantity of free radicals found in the system. Glutamine supplementation has shown an increase in GSH levels in rats with inflammatory shock (Gould & Pazdro, 2019). It is hypothesized that the administration of glutamine can reduce AIF protein expression and apoptosis, which can lead to side effects. Glutathione, which is made from glutamine, can lower AIF expression and apoptosis by blocking ROS. This means that the cisplatin-induced apoptosis pathway can be partially blocked (Fakhrinnisa, et al., 2021). This research aimed to analyze the renal protective effects of glutamine as a means to reduce the side effects of cisplatin, with the ultimate goal of developing an affordable, accessible, and side-effect-free cancer treatment in the future.

OBJECTIVE

The general objective of this research was to explain the nephroprotective role of glutamine on various protein expressions and apoptosis in renal glomerular cells. This study aimed to investigate the effects of glutamine administration as a renal protector on AIF protein levels in glomerular cells exposed to cisplatin in white rats, the impact on apoptosis rates in these cells, and the relationship between glutamine treatment with AIF protein levels and apoptosis in intraglomerular mesangial cells of renal glomeruli exposed to cisplatin in those rats.

MATERIAL AND METHOD

This study used a randomized post-test-only control group design. The data were obtained from animal testing. The following materials were used in this study: a rat cage, a water bottle, an enclosure substrate, a food ration kit, a syringe, IHC sampling tools, a light microscope, experimental animals, animal feed, cisplatin, glutamine, an Apoptotic Detection Kit POD (11684817910 ROCHE), and an anti-AIF antibody (AIF Monoclonal Antibody, Thermo Fisher Scientific, Cat. #MA5-15880).

For the methods, 30 white rats (*Rattus norvegicus*) of the Wistar strain were given 1 week to adapt, and all rats were provided with appropriate food and water. Sick or dead rats were removed from the cage. The rats were then randomized into three experimental groups, with ten rats in each group. The descriptions of each group are as follows: P0: Negative control group (receiving standard feed and water, allowed to rest); P1: Positive control group (receiving standard feed and water, injected with cisplatin); P2: Treatment group (receiving standard feed and water, injected with glutamine and cisplatin). In group P0, the rats were euthanized on the 10th day by cervical dislocation under ether anesthesia, and their kidneys were collected for immunohistochemical examination.

In group P1, the rats received a cisplatin injection on the 7th day and were then observed for 72 hours. On the 10th day, the rats were euthanized by cervical dislocation under ether anesthesia, and their kidneys were collected for immunohistochemical examination. In group P2, the rats were given glutamine injections from the 1st to the 7th day. A cisplatin injection was administered on the 7th day, followed by 72 hours of observation. The rats were euthanized on the 10th day by cervical dislocation under ether anesthesia, and their kidneys were collected for immunohistochemical examination.

The 72-hour observation period after cisplatin administration was based on research by (Tsuruya, et al., 2003), which states that signs of nephrotoxicity can be observed 3 days after cisplatin administration. A paraffin block of kidney tissue was then prepared and deparaffinized. The TUNEL assay was performed using anti-AIF antibodies and an apoptotic detection kit.

The samples were observed under a light microscope at 400x magnification. Scoring was performed using the Allred/H Score. The data were processed using Microsoft Excel 2019 and analyzed with IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA). Prior to differential analysis, normality and homogeneity tests were conducted using the Shapiro-Wilk test (α=0.05) and Levene's test (α=0.05), respectively. If the normality test indicated a normal distribution, a difference test was performed using ANOVA (α=0.05). If significant differences were found, further analysis was carried out using the LSD (Least Significant Difference) test (α=0.05). If the normality test showed that the distribution was not normal, or if the data were not homogeneous, a comparative test was conducted using the Kruskal-Wallis test (α=0.05). If differences were found, the Mann-Whitney test (α=0.05) was used for further analysis.

RESULT

The data shown below are in Allred/H score and are presented as median (min-max). The P1 group has highest median score compared to the P0 and P2 group in both variables. The P0 group which received no intervention presented the lowest score with the P2 group slotting in the middle. The calculation of apoptosis was performed using the Allred scoring guideline.

Table 1. Median score of AIF expression and apoptosis.

Variables	Median (min-max)		
	P0	P1	P2
AIF expression	4.35 (2.2-5.4)	5.9 (4.8-6.3)	5.25 (2.4-5.8)
Apoptosis	5.2 (4.7-5.5)	6.4 (6.1-7.1)	5.7 (4.8-5.9)

The Allred/H score is based on the intensity level of expression in each cell and the proportion of cells at each intensity level. The intensity levels of expression are scored as follows: "0" (no expression), "1" (low expression), "2" (moderate expression), and "3" (high expression). The proportion of cells expressing AIF protein and undergoing apoptosis is scored as "0" (0%), "1" (0–1%), "2" (1–10%), "3" (10–33%), "4" (33–67%), and "5" (67–100%) (Mazières, et al., 2013).

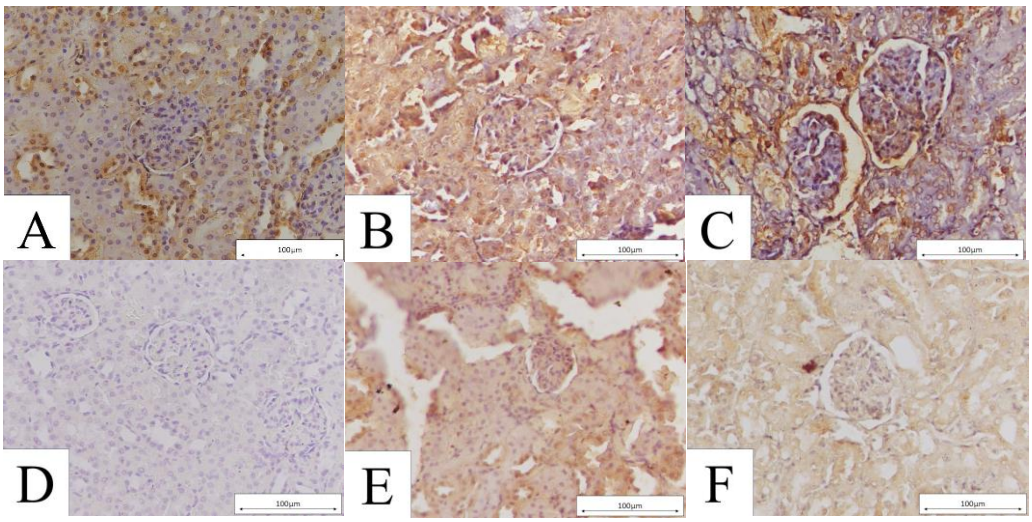

Figure 1. Cell histology under microscope after treatment with Apoptotic Detection Kit.
Legends: A: AIF P0; B: AIF P1; C: AIF P2; D: Apoptosis P0; E: Apoptosis P1; F: Apoptosis P2.

Observation of samples was conducted using a light microscope at 400x magnification on the following fields of view: 3 in the upper pole, 4 in the middle pole, and 3 in the lower pole. The mesangial cells of the intraglomerular glomerulus undergoing apoptosis and AIF protein expression were scored (Figure 1).

Table 2. Kruskal-Wallis score of AIF and apoptosis.

	Groups	n	Median (min-max)	p-value
AIF	P0	10	4.35 (2.2-5.4)	<0.0001
	P1	10	5.9(4.8-6.3)	
	P2	10	5.25(2.4-5.8)	
Apoptosis	P0	10	5.2 (4.7-5.5)	<0.0001
	P1	10	6.4 (6.1-7.1)	
	P2	10	5.7 (4.8-5.9)	

The Kruskal-Wallis one-way analysis of variance was done to determine whether there are statistically significant differences between the three groups of this research on each variable. The p-value of the Kruskal-Wallis test of difference in both variables was significant (<0.0001), which indicated that there were significant differences among all the groups in each variable (Table 2).

Table 3. Mann-Whitney score of AIF and apoptosis.

	Groups	p-values
AIF	P0-P1	<0.0001
	P0-P2	0.041
	P1-P2	0.008
Apoptosis	P0-P1	<0.0001
	P0-P2	0.007
	P1-P2	<0.0001

The p-values of the Mann-Whitney test of the difference between groups P1 and P2 in AIF expression and apoptosis was significant ($p<0.008$ and $p<0.0001$, respectively), which showed that there was a significant decrease between the group that received cisplatin only (P1) and the group that received both cisplatin and glutamine (P2). This may indicate the effect of glutamine as a renal protector (Table 3).

DISCUSSION

The effect of glutamine on AIF expression

The positive control group (P1), which was given with cisplatin injection, showed a significantly higher level of AIF expression when compared to the negative control group (P0), which did not receive special treatment. This showed that cisplatin administration affected the level of AIF expression in renal glomerular intraglomerular mesangial cells, confirming a study by (Lumintang, et al., 2020). Although there was still a significant difference between the treatment group (P2), which was given with glutamine, and the P0 group, this showed that glomeruli that had been exposed to cisplatin still experienced greater damage compared to glomeruli that had not been exposed to cisplatin. However, the P2 group, where the cisplatin injection was preceded by glutamine administration, still showed a significantly lower AIF expression level than the P1 group, which received cisplatin only. Similar findings were also found by (Fakhrinnisa, et al., 2021). This backs up the idea that giving glutamine through an IV can lower the amount of AIF that is expressed in the mesangial cells inside the renal glomeruli of cisplatin-exposed white rats. The findings in this study are important in supporting the hypothesis that intravenous glutamine can reduce AIF expression in glomerular components, especially in glomerular intraglomerular mesangial cells of cisplatin-exposed white rats (Lumintang, et al., 2020).

The positive control group (P1) had higher levels of AIF because they were exposed to cisplatin, which caused more free radicals and DNA damage. This activation of the p53 gene leads to the transcription of the AIF protein and the induction of Bcl-2, which in turn creates pores in the mitochondrial outer membrane (Zong & Liang, 2023). These pores are called mitochondrial outer membrane permeabilization (MOMP). The administration of cisplatin prompts the translocation of AIF protein from mitochondria to the nucleus of the cells, which ultimately leads to apoptosis. In the case of an infection, injury, or stress, such as cancer, apoptosis can occur without the effects of caspase proteins. This caspase-independent pathway of apoptosis is made possible by the fragmentation of DNA caused by mitochondrial damage. One of the more prominent proteins involved in such a pathway is AIF (apoptosis inducing factor); therefore, it can also be used as a unit of measurement for caspase-independent apoptosis levels (Jeong, et al., 2014; Suhaili, et al., 2017; Negara, et al., 2020).

The significant decrease in AIF expression in the treatment group (P2) was caused by glutamine as a GSH precursor in cells that binds ROS. ROS can cause mitochondrial dysfunction by creating pores in the outer walls of mitochondria. Increased ROS can also be induced by cisplatin (Ho & Shirakawa, 2022). The addition of glutamine also led to an increase in the anti-apoptotic agent Hsp70. Hsp70 averts cell death by inhibiting the maturase of p53. This prevents Bcl-2 from causing mitochondrial dysfunction, which in turn prevents the transfer of AIF from the mitochondria. Hsp70 is also able to inhibit AIF from translocating to the nucleus (Stankiewicz, et al., 2005).

These results were in line with previous studies showing that Hsp70 protects cells from apoptosis in various pathways. The Hsp70 protein separates the AIF protein, stopping it from moving to the nucleus and starting apoptosis without caspase. Other studies confirm this, showing that in primary cortical neurons and SH-SY5Y cells, the Hsp70 protein prevents caspase-independent pathway apoptosis by preventing AIF translocation to the nucleus (Sabirzhanov, et al., 2012).

The effect of glutamine on apoptosis levels

The positive control group (P1), which was given with cisplatin injection, showed a significantly higher level of cell apoptosis when compared to the negative control group (P0), which did not receive special treatment. The results showed that giving cisplatin changes the amount of cell death in the mesangial cells inside the renal glomerulus. The positive control group (P1) had more apoptosis because they had more free radicals, which can start several different cell death pathways when exposed to cisplatin. Apart from that, cisplatin also regulates the p53 gene and induces apoptosis (Basu & Krishnamurthy, 2010). Although there was still a significant difference between the treatment group (P2) that was given glutamine and the P0 group, this showed that glomeruli that had been exposed to cisplatin still experienced greater damage compared to glomeruli that had not been exposed to cisplatin. However, the P2 group, where the cisplatin injection was preceded by glutamine, still showed a significantly lower level of cell apoptosis than the P1 group, which received cisplatin only. Intravenous glutamine administration can decrease cell apoptosis in the mesangial cells of kidney glomeruli in white rats exposed to cisplatin (Fakhrinnisa, et al., 2021). The results of this study are significant in reinforcing the hypothesis that intravenous glutamine can reduce apoptosis in the glomerular component,

particularly in the intraglomerular mesangial cells of cisplatin-exposed white rat kidneys.

The results obtained from previous research stated that the administration of cisplatin prompts the translocation of AIF protein from mitochondria to the nucleus of the cells, which ultimately leads to apoptosis (Jeong, et al., 2014). Other research states that excessive levels of ROS in cells can cause damage to proteins, nucleic acids, lipids, membranes, and organelles, which can lead to apoptosis (Redza-Dutordoir & Averill-Bates, 2016).

In the treatment group (P2), the number of cells that died was lower. This was due to the presence of glutamine, which protects cells by increasing the expression of Hsp70. Glutamine itself is a powerful antioxidant and anti-inflammatory agent. Increasing Hsp70 is important because it stops the AIF protein from moving to the nucleus by blocking the release of cytochrome c. This stops apoptosis from happening. Other studies show that Hsp70 overexpression can reverse FasL-induced apoptosis and help cells survive (Vasaikar, et al., 2015; Kim, et al., 2018; Zhang & Bishop, 2020).

A decrease in apoptosis also occurs because glutamine is a precursor of glutathione (GSH), a prominent antioxidant capable of fending the effects of reactive oxygen species (ROS) and has benefits in the immune system. Glutaminolysis initiates the transition from glutamine to GSH by producing glutamate, which then combines with cysteine to trigger the production of GSH. GSH has been shown to prevent mitochondria-dependent apoptosis and effectively prevent cells from cytotoxicity caused by ROS (H2O2) (Kwon, et al., 2019; Asantewaa & Harris, 2021; Luque-Ceballos, et al., 2023). The positive control group's (P1) increase in the AIF expression test and cell apoptosis test was significantly related. Cisplatin exposure increases AIF expression by increasing the synthesis of AIF and triggering AIF translocation to the nucleus. AIF is a protein that can degrade DNA and stimulate chromatin condensation, both of which result in cell apoptosis (Bano & Prehn, 2018).

Strength and limitations

This study offers numerous strengths, including its novelty, as it addresses a topic that is both current and timeless in the field of cancer treatment, exploring innovative methods to prevent potential side effects. This study is comprehensive in its test of each variable, as every variable was tested multiple times for normality and homogeneity before differential analysis. It also has limitations, such as using healthy mice instead of ones with a cancer model and the limited apoptotic pathways examined.

CONCLUSION

Glutamine helps to lower AIF expression and apoptosis levels in the intraglomerular mesangial cells of rats that were exposed to cisplatin. Further research may require involvement of rats with cancer models and/or the examination of different apoptotic pathways is recommended.

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Conflict of Interest

All authors have no conflict of interest.

Ethic Consideration

The study obtained an ethical clearance letter from Komite Etik Penelitian Kesehatan Fakultas Kedokteran Universitas Airlangga, No. 190/EC/KEPK/FKUA/2023 on 10-07-2023.

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None.

Author Contribution

RAM contributes to conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, statistical expertise, and administrative, technical, or logistic support. IS contributes to conception and design, analysis and interpretation of the

data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, provision of study materials or patients, and administrative, technical, or logistic support. EBK contributes to analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article and administrative, technical, or logistic support. BP contributes to critical revision of the article for important intellectual content, final approval of the article, and administrative, technical, or logistic support.

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