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Original Article

EFFECT OF DIAZEPAM ON KIDNEY FUNCTION AND HISTOLOGICAL STRUCTURE OF WHITE RAT'S KIDNEY

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ABSTRACT

The use of NAPZA (Narcotics, Alcohol, Psychotropic, and other addictive substances) in Indonesia keeps increasing. One type of frequently used NAPZAs is diazepam. Diazepam is a kind of sedative-hypnotics drug which belongs to benzodiazepine. The objective of this study was to determine kidney function by examining the level of urine ureum and creatinine also histological structure in rat after treated with diazepam. Rats were divided into one control group and three diazepam treatment groups which were solvent control (PEG 1%), treatment I (62.25 mg/kg BW), treatment II (83 mg/kg BW), and treatment III (124.5 mg/kg BW) of diazepam. These doses were based on LD_{50} in human. Rat urine was taken on the D0, D7, D14, D21, and D28. The ureum level was examined by ureumse-GLDH method and the creatinine level was analyzed by Jaffe method. The histological section was made by paraffin method with Hematoxylin and Eosin (HE) staining. Results showed that diazepam given to rat for 28 days led the increase of urine ureum and creatinine levels compare to control group. Furthermore, histological damages of the kidney also detected including cellular injuries, both reversible and irreversible injuries, congestion, hemorrhage, and glomerular damage. In conclusion, the use of diazepam for 28 days affected kidney function in rat.

Key words: diazepam, ureum, creatinine, kidney.

INTRODUCTION

Indonesian National Narcotics Agency stated that the number of NAPZA (Narcotics, Alcohol, Psychotropic and Other addictive substances) users in Indonesia kept increasing in recent years. It is predicted that the number of drug users reaches 5,1 million people, and the number of people addicted to the drug in the world reaches 27 million (Anonymous, 2014).

One type of NAPZAs is diazepam. Diazepam or valium is a psychotropic belong to Benzodiazepine group. Diazepam has 82% higher level of user compared to other types of benzodiazepine (Ganiswara, 1995). Diazepam is used as an anticonvulsant anxiolysis, sedation, and myorelaxation (Griffin III *et al.*, 2013; Crowe *et al.*, 2000).

Diazepam is bound to a specific subunit of the GABA receptor at a site that is distinct from the binding site of the endogenous GABA (*Gamma Amino Butyric Acid*) molecule. GABA is a neurotransmitter inhibitor which will block impulse transmission in nerve fibers. GABA will open chloride ion gate which is negatively charged thus nerve fibers will be highly negatively charged. Therefore, it's difficult to transmit impulses through nerve fibers when the neuron inhibition of GABA stronger. Continuous use of diazepam can cause side effects, especially in livers and kidneys (Griffin III *et al.*, 2013).

Normal kidneys have three main functions, which are glomerular ultrafiltration, water, and cell reabsorption by tubules, and secretion of organic ions by tubules. In glomerulus, filtrated blood will leave glomerulus through eferen arterioles and will enter proximal convoluted tubules. When filtrate flows through tubules, substances which are beneficial to the body will be selectively reabsorbed into peritubular capillary plasma (Kumar *et al.* 2007).

Kidney functions are commonly determined by two parameters, i.e. ureum and creatinine levels in urine. Ureum is the final product of protein catabolism which comes from amino acid, in wich the amino group removed in the liver and will be filtrated in kidney glomerulus. Creatinine is the waste product of creatinine phosphate overhauling in muscles and will be filtrated in the glomerulus. Thus, ureum and creatinine levels examination is the reference to detect kidney function disorders. Furthermore, to reveal kidney damage it can be analyzed by determining histological structure (Kumar *et al.* 2007).

The objective of this study was to determine the effects of diazepam on kidney functions by analyzing urine ureum and creatinine levels and histopathological of kidneys in rat.

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METHODS

Determination of Experimental Dosages

Study was conducted in Universitas Gadjah Mada Integrated Research and Testing Laboratory (LPPT). Twelve male white rats (wistar) weighed between 150-200 grams were acclimated for one week. Diazepam was given orally to rat. Rats were divided into 4 groups which were polyethylene glycol (PEG) 1 % as solvent control group, 62.25 diazepam mg/kg per day for treatment I which is ½ LD50 in human, 83 mg/kg diazepam per day for treatment II which is 1/3 LD50 in human, and 124.5

mg/kg diazepam per day for treatment III which is ¼ LD50 in human.

Urine Sample Collection

Urine samples were collected on day 0, 7, 14, 21 and 28 by collecting urine for 24 hours by using metabolite cages.

Determination of Creatinine Level

Measurament of creatine level in urine using "Jaffe Reaction" fixed time kinetic method. Creatinine will react with picric acid in alkaline atmosphere, forming amber complex compounds (creatinine-pricate). Mixing reagents contain sodium hydroxide:picric acid (4:1). Blank solution was distilled water while standard solution was 2 mg/dl creatinine. Urine samples were analyzed by Mikrolab 300 Photometer with 490 nm wavelength.

Determination of Ureum Level

Ureum level was analyzed by ureumse- GLDH method. Ureum in urine samples will produce ammonia and carbon dioxide catalyzed by ureum enzyme. Mixing reagents contain (ureum and GLDH): (NADH) (4:1)., Blank solution was distilled water while standard solution was 2 mg/dl ureum. Urine samples were analyzed by Mikrolab 300 Photometer with 340 nm wavelength.

Histological Section of Kidney

Kidney tissues were fixed in neutral buffer formalin (NBF) 10 % and were processed using paraffin method. The 5-6 µm tissue sections were obtained and stained with Hematoxylin and Eosin (HE). The ordinal method was used for the histopathological scoring approach (Gibson-Corley et al., 2013) with modifications. The scoring system consists of histopathological condition in 4 individual types which are cellular injuries, hemorrhage, congestion, and glomerular damage (Table 1). The scoring was performed on renal cortex by 10 fields of views each slide with 40x objective lens magnification.

Table 1. Histopathology scoring system

Type of Histopathological condition	Score	Damage
Cellular Injuries	0	No damage
Reversible and Irreversible Injuries	1	<10%
	2	11%-40%
	3	41%-80%
	4	>80%
Hemorrhage	0	No damage
	1	<25% area
	2	25-50 %Area
	3	51-75% Area
	4	76-100 Area
Congestion	0	No damage
	1	<25% area
	2	25-50 %Area
	3	51-75% Area
	4	76-100% Area
Glomerular Damage	0	No damage
	1	mild capillary enlargement
	2	moderate capillary enlargement
	3	(Plus) Hypersegmented Glomerulus
	4	(plus) Glomerulus shrinkage

Table 2. Urine Ureum Level After Diazepam Treatment. ANOVA done by using (P<0.05) showed some differences of the average of ureum level on white rats urine between groups from D0 to D28 day (P=0.007).

Group	Day				
	0	**7**	**14**	**21**	**28**
Solvent Control	$7.1^{a} \pm 3.57$	$9.67^{a} \pm 3.90$	$6.17^{a} \pm 0.57$	$5.60^{a} \pm 0.78$	$6.47^{a} \pm 2.27$
62.25 mg/kg BW	$60.40^{b} \pm 0.41$	$41.9^{b} \pm 2.83$	$67.55^{b} \pm 11.16$	$108.35^{c} \pm 3.02$	$202.55^{c} \pm 4.95$
83 mg/kg BW	$5.55^{a} \pm 1.10$	$23.15^{ab} \pm 6.66$	$55.5^{b} \pm 3.76$	$27.55^{ab} \pm 3.72$	$11.3^{ab} \pm 1.23$
124.5 mg/kg BW	**$3.7^{a} \pm 23.55$**	**$4.1^{a} \pm 16.35$**	**$23.45^{ab} \pm 19.99$**	**$60.2^{b} \pm 18.56$**	**$51.45^{b} \pm 16.55$**

Table 3. Urine Creatinine Level after diazepam treatment**.** ANOVA done by using (P<0.05) showed some differences of the average of creatinine level on white rats urine between groups from D0 to D28 (P=0.04).

Group	Day				
	0	**7**	**14**	**21**	**28**
Solvent Control	$10^{a} \pm 0.0$	$11.67^{a} \pm 1.67$	$9.43^{a} \pm 0.43$	$8.33^{a} \pm 1.67$	$11.43^{a} \pm 0.51$
62.25 mg/kg BW	$17.5^{a} \pm 0.0$	$22.5^{ab} \pm 10.22$	$3.15^{a} \pm 4.50$	$25^{ab} \pm 4.09$	$13.7^{a} \pm 6.25$
83 mg/kg BW	$50^{b} \pm 8.17$	$17.5^{ab} \pm 6.13$	$45.9^{b} \pm 25.10$	$30^{ab} \pm 0.00$	$82^{c} \pm 3.27$
124.5 mg/kg BW	$10^{a} \pm 6.13$	$22.5^{ab} \pm 10.22$	$15.5^{ab} \pm 0.12$	$15^{ab} \pm 4.09$	$28^{ab} \pm 0.98$

RESULTS

The ureum level is a variable that can be used as an indicator to evaluate the kidney function. The ureum level of white rats' urine on D0, D7, D14, D21, D28 is presented in Figure 1.

Although fluctuative, all treatment groups showed an increase of ureum level on DO to D28 whereas control group showed steady ureum level.Ureum level of 62.25 mg/kg BW was higher than dose 83 mg/kg BW and 124.5 mg/kg BW from day 7 to 28. A significant difference of ureum level between control, 62.25 mg/kg BW, 83 mg/kg BW and 124.5 mg/kg BW can be seen in Table 2.

Creatinine is a variable that can be used as an indicator to evaluate kidney function. The level of urine creatinine on white rats on D0, D7, D14, D21, and D28 is presented in Figure 2.

Although fluctuative, all treatment groups showed the increased of creatinine level from D0 to D28 whereas control group showed steady creatinine level. However, the level of creatinine in treatment of diazepam 83 mg/kg BW was higher than treatment of diazepam 62.25 mg/kg BW and 124.5 mg/kg BW. A significant difference of

creatinine level between control and 62.25 mg/kg BW, 83 mg/kg BW, 124.5 mg/kg BW can be seen in Table 3.

Histological structure of the rats renal cortex which were control, solvent control (PEG 1%), and three doses of diazepam treatment (62.25 mg/kg BW, 83 mg/kg BW; and 124.5 mg/kg BW were presented in Figure 3.

The histopathological scoring of kidney on white rats in a various dose of diazepam treatment can be seen in Table 4.

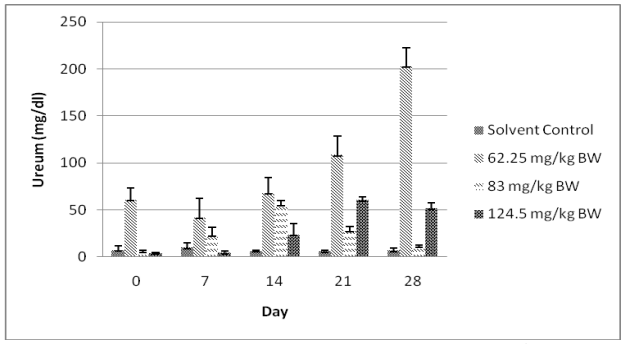

Figure 1. Urine ureum level in wistar white rats which is fed by PEG 1% (control) and diazepam with dose 62.25 mg/kg BW, 83 mg/kg BW, and 124.5 mg/kg BW .

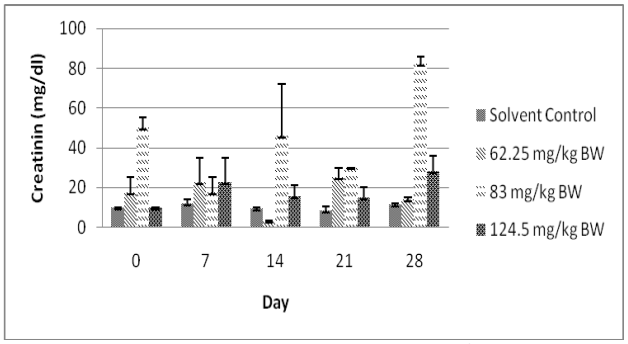

Figure 2. Urine creatinine level on wistar white rats fed using PEG 1% (control) and diazepam with dose 62.25 mg/kg BW, 83 mg/kg BW, and 124.5 mg/kg BW.

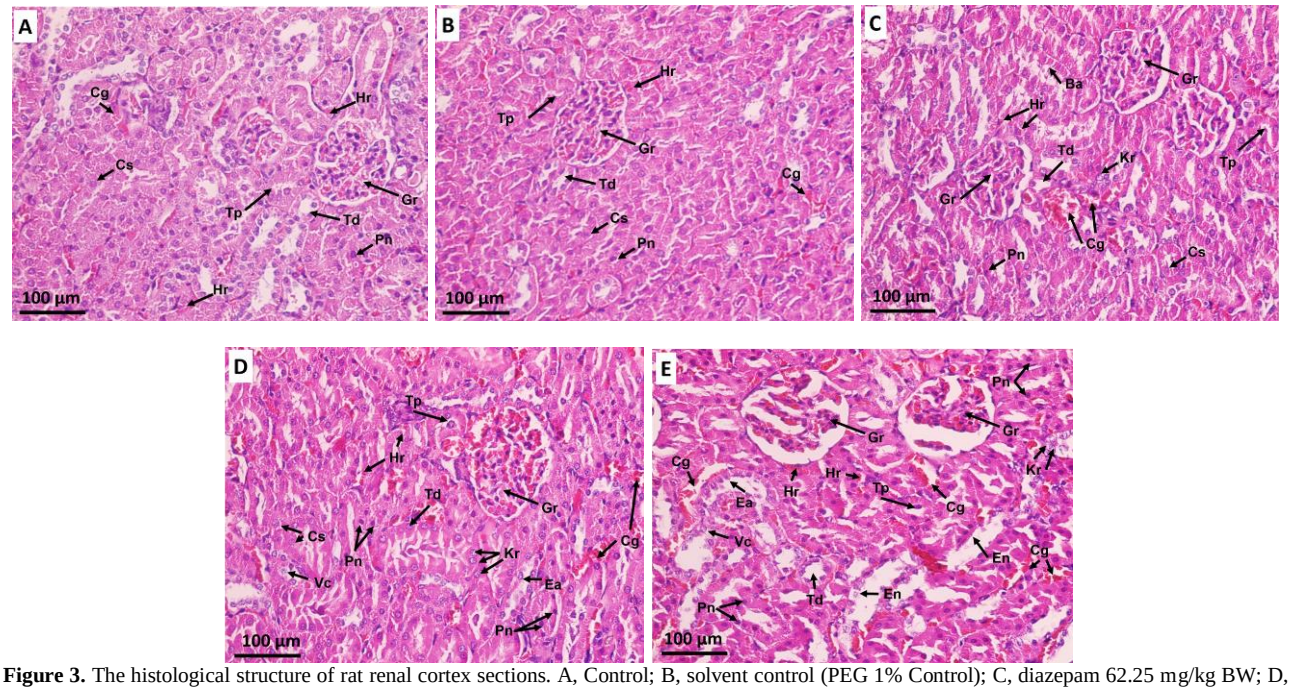

Figure 3. The histological structure of rat renal cortex sections. A, Control; B, solvent control (PEG 1% Control); C, diazepam 62.25 mg/kg BW; D, diazepam 83 mg/kg BW; and E, diazepam 124.5 mg/kg BW. Gr, glomerulus; Tp, tubulus contortus uriniferus pars proximal; Td, tubulus contortus uriniferus pars distal; Cs, cloudy swelling; Pn, pyknosis; Hr, hemorrhage; Cg, congestion; Kr, karyorrhexis; Vc, vacuolization; Ea, epithelial cells abrasion;En, epithelial cells necrosis.

Both control and PEG 1% treatment showed several mild injuries in renal cortex cells. The reversible injury appeared in mild amount on untreated control (Score 1) and a moderate amount of PEG 1% control (Score 2) dominated by cloudy swelling. The irreversible injury appears in mild amount dominated by pycnosis (irreversible injury score 1). Several hemorrhage were detected however congestion was not found in interstitial tissue (hemorrhage score 1 and congestion score 0). Furthermore, no glomerular damage was detected in both control and solvent control.

The histological 62.25 mg/kg BW showed a moderate amount of both reversible injuries (score 2) and irreversible injury (score 2). Brush borders abrasion can be observed in numerous kidney tubules. Furthermore, medium amount of hemorrhage (score 2) and some congestion (score 1) were seen in renal cortex of treatment 1. Moderate capillary enlargement appears in glomerulus (glomerular damage score 2).

Histological section of 83 mg/kg BW showed similar condition with 62.25 mg/kg BW but more hemorrhages were detected in 83 mg/kg BW. Furthermore, brush borders abrasions were common appearance and this phenomenon was continued to epithelial cells abrasion. Mild amount of capillary enlargement was also detected in glomerulus.

Rat in 124.5 mg/kg BW showed severe condition of renal cortex. Most cells were likely to experience both

reversible (score 2) and irreversible (score 3) cell injury. Hemorrhage and congestion were also detected very common in renal cortex (score 3 for both). On the other hand 124.5 mg/kg BW showed distinct phenomenon that did not appear in another group which was hypersegmented and wrinkled glomerulus (score 4).

On the other hand, rat behavior changed during diazepam treatment. This data was presented in Table 5.

Table 4. Scoring of kidney damage in white rats (*Rattus norvegicus*) treated by diazepam.

No.	Type of Histopathological Condition	Control	Solvent Control (PEG 1%)	62.25 mg/kg BW	83 mg/kg BW	124.5 mg/kg BW
1	Cellular injuries					
	Reversibel	1	2	2	2	2
	Irreversibel	1	1	2	2	3
2	Congestion	0	0	1	1	3
3	Hemorrhage	1	1	2	2	3
4	Glomerular Damage	0	0	2	1	4

Table 5. Rat Behaviour

Group	Solvent Control	62.25 mg/kg BW	83 mg/kg BW	124.5 mg/kg BW
Behaviour	Rats were active	Rats decreased their movement	Rats in limped condition	Rats were often fallen asleep

Rats in all groups showed normal behavior before treatment, active and did not show any indication of pain. During treatment rats in diazepam treatment group got more drowsiness and a decrease in activity compared to control group. Moreover according to subjective observation, rats in treatment groups have more concentrated urine which was indicated by dark yellow color. Whereas rats in control group, the color of urine was normally yellow. Furthermore body weight of rat during treatment was presented in Figure 4.

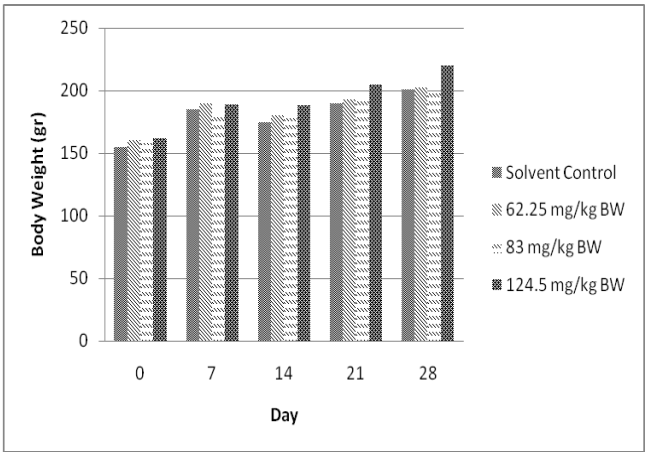

Figure 4. Rats body weight

Body weight of rat in both control and treatment groups were increased start from 2nd weeks and keep increasing until the end of treatment. There was no significant difference in body weight of control and treatment group. Hence, diazepam treatment did not affected to body weight but affected to behavior and physiological condition.

DISCUSSION

Key property of psychotropic drugs was their analgesic effect via the central nervous system. Consequently, this action has an impact on other functions, such as heart rate, breathing rate, and blood pressure. The majority of these substances or their metabolites are excreted by the kidneys, and renal complications of drug abuse are common. They include a wide range of glomerular, interstitial and vascular diseases. The damage may be acute and reversible or chronic and can lead to end-stage renal failure. The involvement of the kidney in the use of drugs is either attributed to their elimination through the kidney, or a direct nephrotoxic effect, or through other mechanisms (Pantelias, K., and Grapsa, E, 2011).

In common, chemical medicine shows dose dependent phenomenon. Hence the higher dose the effects will getting stronger. However in this study, result showed that 124.5 mg/kg BW (1/2 LD50) had ureum level lower than the 62.25 mg/kg BW (1/4 LD50). Meanwhile, the creatinine level in the 124.5 mg/kg BW (½ LD50) was lower than 83 mg/kg BW (1/3 LD50). This possibly has a relation with the kidney condition. Although urea and creatinine levels of rats in group of 124.5 mg/kg BW lower than 83 mg/kg BW and 62.25 mg/kg BW respectively, but histological section revealed more destructive damage in kidney structure detected in 124.5 mg/kg BW.

The histopathological severity of renal cortex was dose dependent manner which increased due to the increase of diazepam's dose of treatment. Therefore, the filtration process does not normally run due to clogs in the glomeruli. Meanwhile, the 62.25 mg/kg BW and 83 mg/kg BW of the glomerular histological structure was not severely damaged, allowing the filtration process to normally run, thus, the level of ureum and creatinine in urine is higher than in 124.5 mg/kg BW.

The filtration process does not run normally may also be resulted from diazepam modulating postsynaptic effects of GABA transmission, thereby increasing presympatic barriers and affect capillary pressure and blood plasma colloid pressure. These two factors play an important role in glomerular filtration rate, as the lower the capillary pressure and plasma colloid osmotic pressure, it will lead to decreasing glomerular filtration rate, thus the level of ureum and creatinine detected in urine is low (Pantelias and Grapsa, 2011).

Cellular injury was detected on control groups of rats (untreated and solvent) renal cortex which dominated by reversible injuries. It can be caused by dietary aspect. The condition of control rat's renal cortex was relatively not severe compared to diazepam treatment groups. Rat in

62.25 mg/kg BW and 83 mg/kg BW groups have a similar histopathological condition of renal cortex. The cellular injury seems to be equal between reversible and irreversible injuries in tubular and interstitial region. Epithelial abrasion becomes a distinct histopathological appearance in renal cortex of 62.25 mg/kg BW and 83 mg/kg BW groups.

On the other hand, the most severe histopathological condition of renal cortex was detected in 124.5 mg/kg BW group. Irreversible cells injuries are quite commons detected in this group. Furthermore, there are many hypersegmented and wrinkled glomerulus in renal cortex. This condition can be caused by mesengial cells disorders that may lead to membran proliferative glomerulonephritis and also glomerulosclerosis (Kumar et al., 2007).

Diazepam is a benzondiazepine derivative that is used as an anticonvulsant anxiolysis, sedation, and myorelaxation (Griffin III et al., 2013; Crowe et al., 2000). There are two general ways of diazepam being responsible for acute renal injury. They are affecting the hemodynamic aspect and destructing cells by its chemical compounds and their activities. Diazepam can decrease the blood pressure and lower blood flow to the kidney. This condition may lead to an ischemic condition of kidney which induces hypoxia condition in renal cortex cells (Bellomo et al., 2012; Khajuria et al., 2014). The ischemic condition can be detected by the existence of congestion (Kumar et al., 2007). Several congestions appear in rat treated with diazepam treatments. However, congestion being much severe in 124.5 mg/kg BW. Hypoxia may cause renal cortex cells experienced several stress conditions thus the cell injury can't be avoided (Bellomo et al., 2012; Finlay and Jones, 2013).

The Early stage of epithelial abrasion is characterized by epithelial cell's membrane disruption than continued to cells abrasion and may end to cells necrosis. Epithelial cells abrasion may be caused by the chemical compound of diazepam that possibly nephrotoxic. Diazepam, a long-acting BZD, produces the active metabolites such as oxazepam, desmethyldiazepam, and temazepam. These metabolites could increase the duration of drug action (Griffin III et al., 2013). Furthermore, they will affect the elimination process/excretion by the kidneys and interfere process of glomerular filtration, reabsorption, and tubular secretion since there are many Peripheral Benzodiazepine Receptors (PBR) in several parts of the nephron (Bribes et al., 2001). Diazepam, which is known can interact with equal affinity on Benzodiazepine Receptor, will be accumulated into several region of nephron with the help of PBR binding. Epithelial cells abrasion also may be caused by the increase of urinary retention.

Previous study revealed that drug abuse may lead to the increase infection risk by various bacteria, viruses, and fungi and may cause glomerulonephritis (GN) due to deposition of immune nephrotic syndrome (Crowe et. al, 2000; Howse and Bell, 2011). Therefore, rat with glomerulonephritis possibly decreases their immune ability thus easily infect by various bacteria, viruses and fungi.

In the body, the diazepam is being catalyzed by the enzyme CYP2C19 that will transform the diazepam into dimethyl diazepam while the enzyme CYP3A4 will transform the desmethyldiazepam into oxazepam. Desmethyldiazepam is an active metabolite of diazepam having pharmacological effects on the nervous system, i.e. the central nervous system. If liver does not function well, then the drug which is usually be transformed in the liver will not be changed or only be transformed partially. It makes the drug effects last longer and become more toxic.

Diazepam should not be used in the long term, for it may do harm to the body. This drug also causes sleeping and lose of consciousness accompanied by nystagmus and slow speech (Griffin III et al. 2013, Niendya, W et al., 2011).

Diazepam is a fat-soluble drug, thus takes longer to be excreted than those of drugs with polar property. So it will affect the elimination process by the kidneys (excretion) and interfere with the process of glomerular filtration, reabsorption, and tubular secretion, so as to the diazepam will be accumulated into the nephron. It is due to the ability of the kidneys to accumulate xenobiotic compounds in the cells. Diazepam will be secreted from the blood into the urine and will be accumulated in the glomeruli and proximal tubules, and if being reabsorbed from the urine, it will go through the tubular epithelial cells with a high concentration. As a result, these toxic substances are accumulated in the kidneys and may result in kidney damage (Bribes et al., 2001).

In conclusion, diazepam treatment on rats for 28 days increased the levels of ureum and creatinine in the urine compare to control group. Furthermore diazepam treatment caused histological damage in the kidneys of rats. Since test only done in 28 days it couldn't analyze chronical effect of diazepam; thus, longer treatment is needed to get more comprehensive data.

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