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NEW DEVELOPMENT OF PHARMACEUTICAL CARE IN A PHARMACOGENETIC AND PHARMACOGENOMIC APPROACH

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THE INFLUENCE OF HIGH FRUCTOSE WITH LOW MAGNESIUM DIET ON THE LIVER AND RENAL TUBULAR CELLS HISTOPATHOLOGY OF MALE WISTAR RATS

Wahyu Dewi Tamayanti¹, Ervianti Dela Rosa¹, Regina Carlosono¹,

Ratna Megawati Widharna¹, Bernadette Dian Novita Krisdianto²

¹Departement of Clinical and Community Pharmacy, Faculty of Pharmacy, Widya Mandala Chatolic University Surabaya, Indonesia.

²Medical Faculty, Departement of Pharmacology, Widya Mandala Chatolic University Surabaya, Indonesia

Correspondence:
dewffua@gmail.com

ABSTRACT

Background : Diet containing high fructose with low magnesium may induce hyperglycemia which is one of the metabolic syndrome disorder related to chronic kidney disease. Additionally, high fructose diet induces the novo lipogenesis to form triglycerides and VLDL that may lead to the increased of fat storage and thus triggering insulin resistance.

Objective : This study was aimed to evaluate renal and liver histopathology by measuring the number of cells underwent necrosis and apoptosis as well as the expression of p53 gene after high fructose and low magnesium treatment.

Methods : As many as thirty two male Wistar rats were divided into 4 groups: control group (C); P1 group (high fructose diet); P2 group (low magnesium diet); and P3 group (high fructose with low magnesium diet). All groups were treated by their respective diet for 2 months. Observation was conducted on the renal tubular and liver cells in the organs that were paraffin blocked, HE stained, and bounded by p53 antibody through IHC.

Outcome measured : The percentage of necrotic, apoptotic, and p53 expression in liver and renal tubular cells of male Wistar rats after high fructose and low magnesium diet.

Results : The results that were analyzed by Oneway Anova and Duncan test showed necrosis in renal tubular cells and hepatocytes in all treatment groups. The necrosis percentages of renal tubular cells were $18.80 \pm 3.31\%$, 43.19%, 34.50%, and 50.44% respective for C, P1, P2, and P3. Whereas, the necrosis percentages in the liver cells were $12.77 \pm 4.54\%$, 27.36%, 13.73%, and 37.48% respective for C, P1, P2, and P3. However, no apoptotic and p53 gene expression both in renal tubular and liver cells was observed.

Conclusion : This study revealed that continuous high fructose with low magnesium diet may induce severe insulin resistance, which was seen through P3 group that showed the highest necrosis percentage both in liver and renal tubular cells.

Keywords : high fructose and low magnesium diet, liver and renal tubular cells, necrosis, apoptosis, p53

INTRODUCTION

Fructose is a simple sugar that has been widely used as artificial sweetener. It is used as a component of food and beverages in the form of high fructose corn syrup (HFCS). The high consumption of HFCS of 1970 to 1999 was significantly related to the increased incidence of obesity and diabetes mellitus, as had been observed by *US Food and Drug Administration* (Prahastuti, 2011; Bantle, 2009; US DAERS, 2007). Fructose is efficiently induced *de novo lipogenesis* (DNL) by serving carbon atom to form triglyceride and very low density lipoprotein (VLDL), thus improving fat storage in the liver that eventually stimulating insulin resistance which may lead to liver disorder (Prahastuti, 2011). Fructose is involved in the glycolysis process of the body. During the process, fructose is absorbed and subsequently converted into glucose. It will then metabolized in the liver and use the magnesium as cofactor in taking the phosphate group of Adenosine Tri Phospat (ATP) (Sun&Empie, 2012).

Magnesium is a cofactor in ATP transfer process. It is important in facilitating phosphorylation of insulin receptor. It is somehow believed that depletion of intracellular magnesium may lead to the defective function of tyrosine kinase of the receptor. The defect will induce the release and activation of insulin, therefore promoting the insulin resistance (Takaya *et al.*, 2004; Song *et al.*, 2003; Wiyatmoko, 2007). On the other hand, magnesium is also playing a role in trans-membrane transfer of calcium, sodium, and potassium ions in de- and repolarization phase by activating the Ca-ATPase and Na-ATPase. In that situation, intracellular low magnesium level may lead to the release of potassium out of the cell which then disrupt the conduction and metabolism of the cells. The disruption may worsen the inflammation and metabolic syndrome by stimulating the insulin resistance. The insulin resistance is believed to be the cause of increased lipid oxidation in the liver which may promote liver disfunction, such as necrosis and apoptosis (Rayssiguier *et al.*, 2006; Hastuti, 2006; Wiyatmoko, 2007; Macfarlane *et al.*, 2000). Furthermore, the poorer metabolic syndrome due to insulin resistance will also contribute to the increased disruption of renal cells which subsequently promoting the chronic kidney disease (CKD) (Chen *et al.*, 2004).

In necrosis, a group of hepatic and renal tubular cells is inflamed and/ or lethal, which can be observed by the asymmetric forming of the cells and the missing of the cells nuclear part (Kasno, 2005; Macfarlane *et al.*, 2000; Nugraha *et al.*, 2008). The apoptosis is a programmed cell death which may be occurred either as a physiologic or pathologic condition (Macfarlane *et al.*, 2000). When a cell is lethal, the DNA damage is occurred, which will subsequently induce the phosphorylation of p53 by activating ATM/ATR kinase and stimulating Chk2/Chk1 kinase (Amudson *et al.*, 1998; Syaifudin, 2007; Sukamdi *et al.*, 2010; Bartek and Lukas, 2001). The p53 gene is one of tumor suppressor genes which will be accumulated during the DNA damage. It is regulated the apoptotic process by inhibiting the cell replication to stop at the G1 phase (Lumongga, 2008). The insight regarding the fructose, magnesium, and their influenced in the alteration of renal tubular and liver cells have supported this study to be conducted.

METHODS

Animal Handling

This experimental study was conducted in Animal Laboratorium, Clinical Chemistry Laboratorium of Faculty of Pharmacy - Widya Mandala Catholic University Surabaya, and Anatomy Pathology Department of Dr. Soetomo Hospital, Surabaya, Indonesia. As many as 32 male Wistar rats (*Rattus norvegicus*) of 150-200 g body weight and 2-3 months of age, were used. The Wistar rats were obtained from the Biochemistry Animal Laboratorium of Medical Faculty, Airlangga University, Surabaya. Prior to the study, the rats were adapted to new environment for two weeks. The rats were cage in the room with 12 hours of light and 12 hours of dark, under good air circulation of 30°C temperature. The food and water was given twice a day. The rats was divided into 4 groups of 5, namely: **control group (C)** which fed normal food and 3,3% premix contained magnesium of 60 ml/day; **high fructose diet group (P1)** which fed normal food that added 60% fructose solution of 2 ml/day; **low magnesium diet group (P2)** which fed only normal food; and **high fructose and low magnesium diet group (P3)** which fed normal food added 60% fructose solution of 2 ml/day and 3.3% premix contained magnesium of 60ml/day. The treatment was given for 2 months.

Materials

Materials used were: dissection equipments, weight balance (Sartorium, Germany), microtome (Leica RM 2245), light microscope (Olympus CX521), fructose (Merck KGaA, Germany), buffer formaline, hematoxilin eosin dye, antibody p53 (Biocare, USA).

Research methods

The 4 groups of rats were treated with their respective diet for 2 months. Afterwards, the rats were sacrificed in order to obtain the liver and the kidney. Both of the organs were immersed in buffer formaline before blocked with paraffin. After blocking, the organs were sliced and stained by Hematoxillin-Eosin dye. The stained organs were then be used to analyze the necrotic and apoptotic condition. The paraffin blocked organs were also sliced and then subjected to the immunohistochemistry (IHC) process, in which the p53 antibody was conjugated into the prepared sliced organs. By using this IHC slide, the p53 expression level was counted under light microscope of 400 times magnification. The obtained data were analyzed by *Kolmogorov-Smirnov* and confirmed by *Homogeneity of Variances Test* in order to examine their normal distribution. Since the data were normally distributed, the One Way Anova was performed, and followed by Duncan test to attain the significant different between group.

RESULTS AND DISCUSSION

The research showed that high fructose and low magnesium diet was able to increase the body weight of the rats compared to other groups (Fig. 1). This phenomenon occurred due to the ability of fructose to

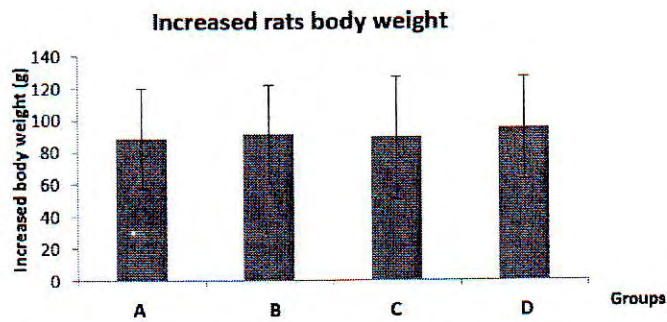


Figure 1. Increased Rats Body Weight After Treatment. A: control group B: high fructose diet group C: low magnesium diet group D: high fructose low magnesium diet group

stimulate the leptin resistance which may lead to the eating addicted effect of the rats. As the leptin effect was resisted, the rats have lost the satiated feel. Thus, they always feel hungry and eat as much as they can. This, will triggered *de novo lipogenesis* due to the formation of triglycerides (TG) as the product of fructose metabolism in the liver. If this formation remains, the TG will be accumulated in the liver. The TG accumulation will induce the formation of fat degeneration in the cytoplasm of the hepatocyte which can be observed by the increased volume of it. If this condition occurred continuously, may induce the cell disfunction by undergoing the necrotic mechanism (Hastuti, 2006; Mayes, 2013; Prahastuti, 2011).

The necrotic inside the cells was proved by counting the number of hepatocytes and renal tubular cells that showed nuclear volume alteration (Fig. 2). The figure showed that in hepatocytes, the percentages of necrotic were $12.77 \pm 4.54\%$, $27.36 \pm 4.00\%$, $13.73 \pm 3.25\%$, and $37.48 \pm 4.16\%$ respective for C, P1, P2, and P3 groups (Table 1).

Table 1. Necrotic percentages in hepatocytes and renal tubular cells

Groups	Hepatocytes (%)	Renal tubular cells (%)
Control (C)	12.77 ± 4.54	18.80 ± 3.31
High fructose diet (P1)	27.36 ± 4.00	43.19 ± 9.03
Low magnesium diet (P2)	13.73 ± 3.25	34.50 ± 6.98
High fructose low magnesium diet (P3)	37.48 ± 4.16	50.44 ± 6.67

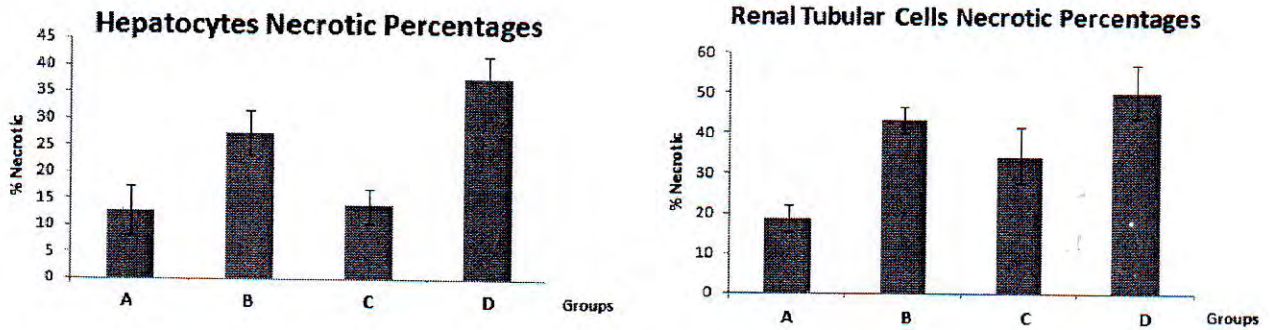


Figure 2. Necrotic Percentages of Hepatocytes and Renal Tubular Cells A: control group (C) B: high fructose diet group (P1) C: low magnesium diet group (P2) D: high fructose low magnesium diet group (P3)

The highest necrotic percentage showed by P3 might be due to the severe condition of insulin resistance. The deficiency of magnesium may thus induce phosphorylation of insulin receptor, therefore induced a defective function of tyrosine kinase and decrease the ability of insulin to transport glucose into the cells. this situation is tightly related to the occurrence of metabolic syndrome (Takaya *et al.*, 2004, Yenny dan Suastika, 2011). The decreased glucose intake into the cells may cause advanced glycation end products (AGEs) formation. The higher number of AGEs may then inhibit activity of nitric oxide (NO), thus trigger the formation of reactive oxygen species (ROS). ROS may lead to the occurrence of oxidative stress, inflammation, and endothel dysfunction, which thus disrupt the hepatocytes (Sanchez *et al.*, 2007, Uribarri *et al.*, 2010, Goldin *et al.*, 2006). On the other hands, magnesium deficiency may decrease the potassium and increase natrium concentration inside hepatocytes, thus influence the osmotic balance and may facilitate the flow of extracellular fluid into the hepatocytes. when continuously accured, this condition may cause the swallowing of the hepatocytes vacuola. This condition known as the hidropic degeneration (HD) (Fig. 3). Long term HD may trigger the energy release of hepatocytes activity, thus will eventually induce necrosis of hepatocytes (Wiyatmoko, 2007, Hastuti, 2006).

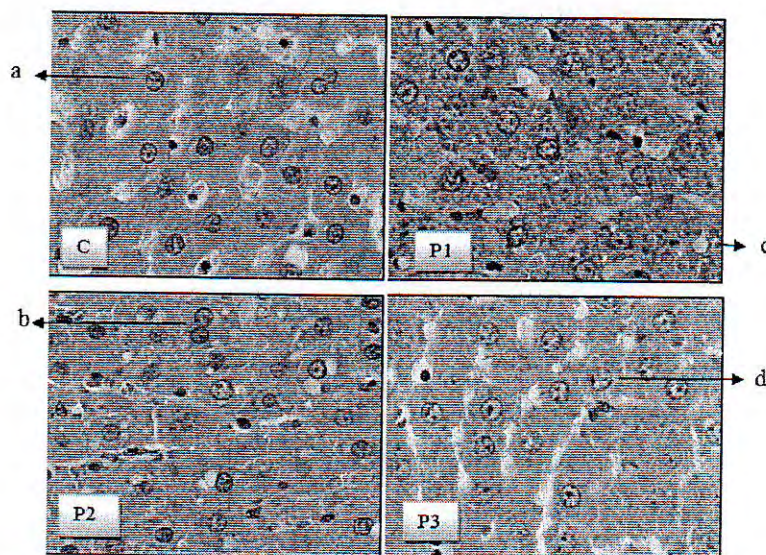


Figure 3. Hepatocytes HE-Stained (400x magnification) a. Normal hepatocytes b. Binuclei c. fat degeneration d. Hidrophic degeneration C. control group P1. high fructose diet group P2. low magnesium diet group P3. high fructose low magnesium diet group

It was shown in Fig 3. that high fructose and low magnesium diet induced the alteration of hepatocytes, such as fat degeneration, hidrophic degeneration, and turbid cells. The turbid cells were swollen due to the input of extracellular water. Moreover, cytoplasm of the turbid cells contained granules and observed as heterogenous cells in terms of shape and form. As the continuous damage occurs, the hepatocyte underwent vacuolization, in which the cytoplasm was observed to be clearer due to the flow of fluid into hepatocytes inside the vacuoles. Further, the hepatocytes underwent fat degeneration which may be a trigger of necrotic (Hastuti, 2006). In necrotic, the hepatocyte membrane was lysed thus the border between hepatocytes was not clearly observed (Hastuti, 2006, Saleh, 1979, Susilowati, 2009).

In the renal tubular cells, the necrotic percentages were $18.80 \pm 3.31\%$; $43.19\% \pm 9.03$; $34,50 \pm 6.98\%$; and $50.45\% \pm 6.67$ respective for C, P1, P2, and P3. Figure 2 shown that the necrotic of renal tubular cells in P1 was trice higher, P2 was twice higher, and P3 was 4 times higher than the C (Table 1). Necrotic cells of the renal tubular cells, that were HE-stained, underwent an alteration on their shapes and form. The necrotic cells observed as bigger or smaller in size with oval shapes compared to the normal cells (Fig. 4). Moreover, the necrotic cells shown as pale color compared to the normal cells, which color were purple-blue.

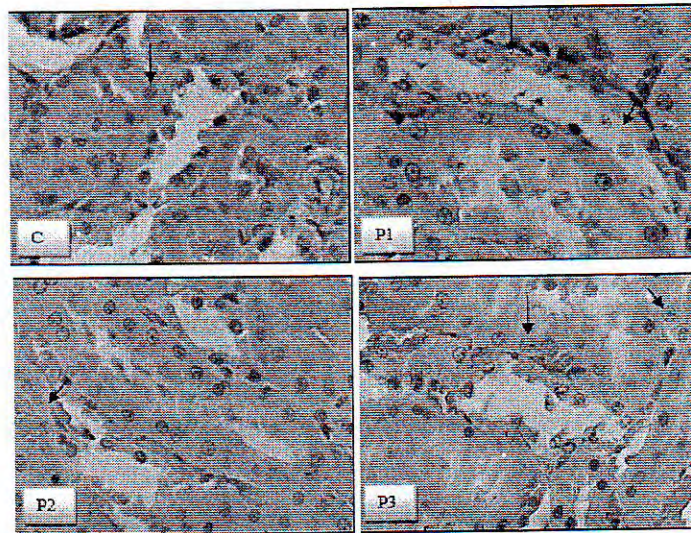


Figure 4. Renal Tubular cells HE-Stained (400x magnification) C. control group P1. high fructose diet group P2. low magnesium diet group P3. high fructose low magnesium diet group → Necrotic cells

The results indicate that low magnesium diet that occur continuously will stimulate the kidney stone formation which eventually triggering necrotic of the cells. Formation of kidney stone occurred may be due to the inhibition of cAMP activation that modified paratiroid hormone synthesis ada secretion (Shills, 1999). The modified function of paratiroid hormone will effect the hydroxylation of calsitrol and calcidiol (Fettemi *et al.*, 1991) which control the calcium amount. When the condition occurred, the calcium level will not be controlled, thus calcium will bind to citrate and oxalate in the renal tubular cells. This may decrease the excretion of citrate. The fructose diet is believed to undergo necrosis through the resistance of insulin. The combination of magnesium with fructose will worsen the condition since magnesium is the cofactor of ATP transfer reaction. Therefore, magnesium will cause defect in insulin receptor by inhibiting phosphorylation of tyrosine kinase in

the receptor. This condition will eventually lead to the diabetes mellitus which chronically may cause renal dysfunction (Takaya *et al*, 2004).

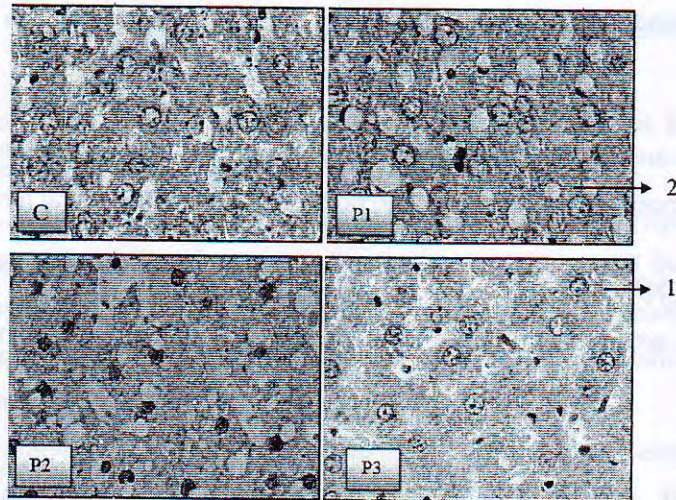


Figure 5. Hepatocytes Immunohistochemistry – p53 antibody (400x magnification) 1. Normal hepatocytes 2. Fat degeneration C. control group P1. high fructose diet group P2. low magnesium diet group P3. high fructose low magnesium diet group

The p53 wild type antibody was coupled to the hepatocytes. The necrotic hepatocytes which expressed p53 shown brown nucleus color compared to the normal cells. The results showed no p53 expression in the hepatocytes (Fig. 5). It might be caused by no mutagenic effect stimulated by the treatments. Therefore, no p53 expression was observed. The p53 gene is involved in the cells regulation by governing the cells to undergo apoptosis (Kodama *et al.*, 2011, Sukamdi *et al.*, 2010). It is one of pro-apoptotic gene that expressed when there DNA damage is occurred. It works by inhibiting cell replication and stopping the G1 phase of cell cycle in order to trigger the DNA repair (Amudson *et al.*, 1998, Bartek and Lukas, 2001, Syaifudin, 2007).

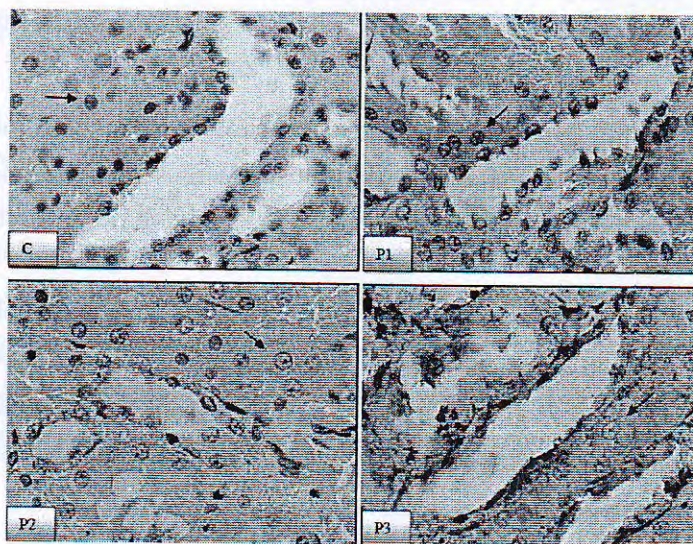


Figure 6. Renal tubular cells Immunohistochemistry – p53 antibody (400x magnification) 1. Normal hepatocytes 2. Fat degeneration C. control group P1. high fructose diet group P2. low magnesium diet group P3. high fructose low magnesium diet group → cells with no expression of p53

In the the renal tubular cells, no p53 expression was observed as well. As seen in Fig. 6 which there is no cells whose nucleus shown brown color. Therefore we can see that high fructose and low magnesium diet was not induce DNA damage thus no p53 expression was observed.

CONCLUSION

The high fructose and low magnesium diet may cause hepatic and renal tubular cells necrotic. However, no apoptotic and p53 expression was observed in both hepatocytes and renal tubular cells.

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