EFFECTS OF PRE-GERMINATED BROWN RICE ON CELL APOPTOSIS AND CELL PROLIFERATION IN RAT LIVER TOXICITY INDUCED BY DEXTROMETHORPHAN

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A Thesis Submitted to the Graduate School of Naresuan University

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Thesis entitled "Effect of pre-germinated brown rice on cell apoptosis and cell proliferation in rat liver toxicity induced by dextromethorphan"

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has been approved by Graduate School as partial fulfillment of the requirements for the Master of Science Degree in Anatomy of Naresuan University

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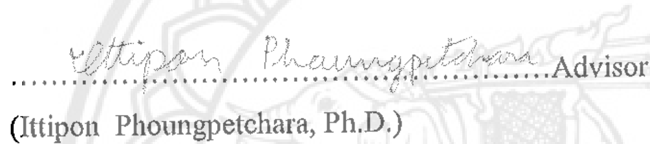

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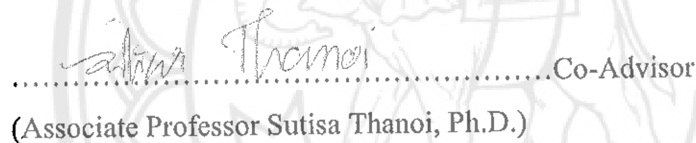

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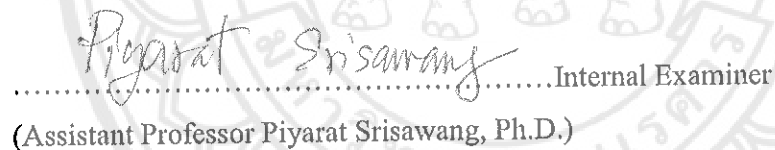

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ABSTRACT

Free radicals that occur during dextromethorphan metabolism can induce hepatotoxicity. Pre-germinated brown rice (PGBR) has many important nutrients that include antioxidants and γ-aminobutyric acid (GABA). This study aimed to investigate the effects of pre-germinated brown rice on cell apoptosis, cell proliferation and cellular activity in rat liver toxicity induced by dextromethorphan. A total of 70 male Sprague-Dawley rats were divided into five major groups: control, dextromethorphan 30 mg/kg (DXM), drug withdraw (DW) group, GABA 0.8 mg/kg treatment (DG) group and PGBR 5 g/kg treatment (DP) group. Each group was divided three periods including 15, 30 and 60 days. All rats were treated once a day orally. After sacrifice, livers were collected for histochemical investigation. The results showed a significant decreased in normol morphology of liver in DXM when compared with the control. The liver showed significantly increase in protein intensity, glycogen accumulation and cell proliferation in DW30, DW60, DG15, DG30, DG60, DP15, DP30 and DP60 compared with the DXM. The hepatic sinusoid dilation and vacuolization in hepatocyte showed significantly decrease in DW15, DW30, DW60, DG15, DG30, DG60, DP15, DP30 and DP60 when compared with the DXM. The percentage of apoptotic index showed significantly decreased in DW30, DW60, DG15, DG30, DG60, DP15, DP30 and DP60 when compared with the DXM. These results of this study indicated that DXM have negative effects to induced hepatotoxicity. On the other hand, PGBR can enhance the recovery from DXM-induced hepatotoxicity by

decreasing cell apoptosis, increasing cellular activity and cell proliferation in the rat. Furthermore, Prolonged period of PGBR administration showed no adverse effect on the liver. It is possible that PGBR can be developed for a safe alternative product for hepatotoxicity treatments.

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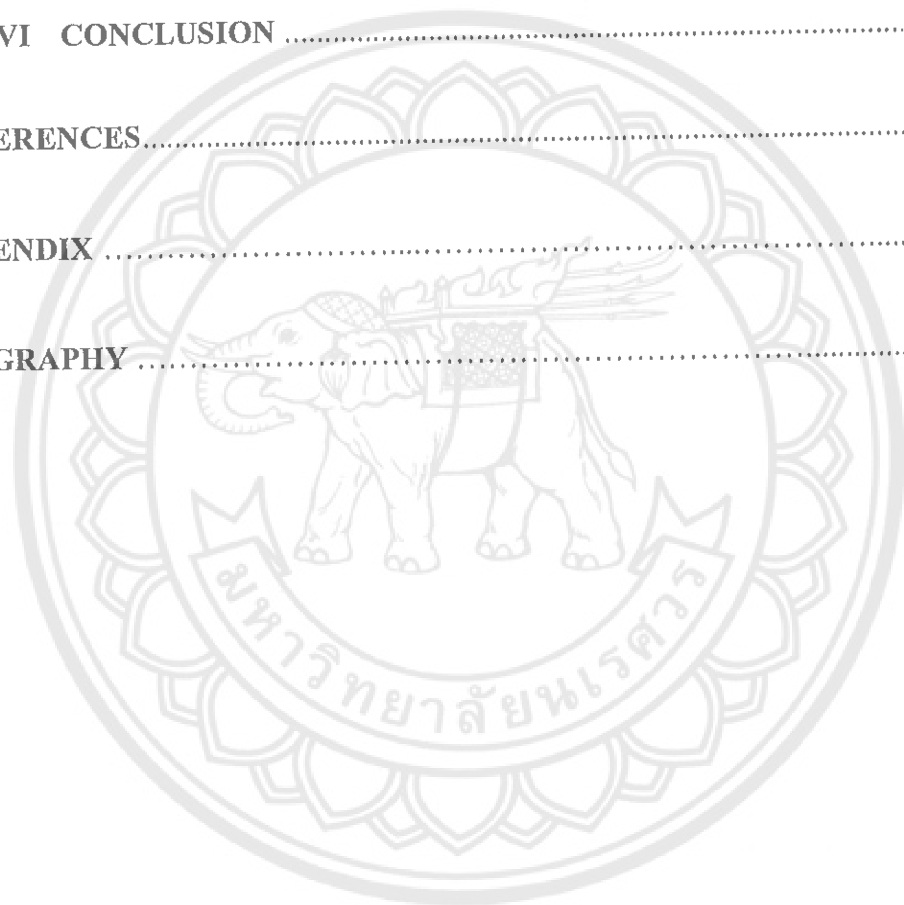

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ABBREVIATIONS

µg	=	Microgram
µl	=	Micro liter
°C	=	Degree Celsius
ANOVA	=	Analysis of variance
BW	=	Body Weight
DAB	=	3,3'-Diaminobenzidine
DXM	=	Dextromethorphan
i.p.	=	Intraperitoneal
g	=	Gram
g/kg	=	Gram per kilogram
GABA	=	Gamma (γ) aminobutyric acid
mg/kg	=	Milligram per kilogram
PBS	=	Phosphate Buffer Saline
PGBR	=	Pre-germinated brown rice
pH	=	Power of hydrogen ion concentration
WHO	=	World Health Organization

มหาวิทยาลัยนเรศวร

CHAPTER I

INTRODUCTION

Rationale and Significance of the study

Dextromethorphan (DXM) is a synthetic chemical that widely used as an antitussive (cough suppressant) and dissociative drug. Recently, cough medicine which contains DXM has been abused as a recreational drug (Bryner, et al., 2006). DXM is a synthetic analog of codeine. The effects of DXM usually include dissociation of mind and body. The use of DXM in high dosage and/or over long periods can cause several psychiatric and neurological effects such as restlessness, hallucination, delayed reaction and response times, panic, delusion, ataxia, impaired cognitive ability etc. (Romanelli, et al., 2009). Furthermore, high dosage and long term use of DXM also affect other organs in the body including the liver, a vital organ in the elimination of toxins from the body. DXM is metabolized by O-demethylation to dextrorphan, 3-methoxymorphinan through N-demethylation and 3-hydroxymorphinan through N, O-didemethylation in the liver (Schadel, et al., 1995; Beinhart, 1980; Pfaff, et al., 1983). During such detoxification processes the liver produces free radicals; excessive free radicals can cause oxidative stress. The liver is a major organ attacked by oxidative stress and causes liver injury (Sanchez, et al., 2012). However, antioxidants enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase play an important role to counter any excess of free radicals, to protect the cells against their toxic effects from oxidative stress. (Medina, et al., 2005; Mallikarjuna, et al., 2010).

Recently, there have been previous studies reported antioxidant property of capsaicin has hepatoprotective effect to diminishing the generation of MDA and inhibition of active caspase-3 induced by CCl_4 (Abdel-Salam, et al., 2006; Hassan, et al., 2011). Green tea has antioxidant properties to against diabetic cell injury induced in rat liver (Waer, et al., 2012). Moreover, curcumin had a beneficial effect on liver

regenerative capacity of the remnant liver tissue after hepatectomy, probably due to its antioxidative, antiapoptotic and proliferative properties (Toydemir, et al., 2015).

As mentioned earlier, natural products that have found highly amount of antioxidant can reduces hepatotoxicity by decreasing oxidative stress and inhibiting apoptotic enzymes. Additionally, Thailand is an agricultural country. Rice is the country's most important crop and is a major exporter in the world. Rice seeds and rice germ contain fibers and several kinds of antioxidants, such as ferulic acid, phytic acid, tocopherols, and oryzanols (Katyama, et al., 2002). Brown rice (BR) is whole grain rice, which only the hull is removed. Nowadays, The process of germination greatly enhances the content of bioactive compounds (Cornejo, et al., 2014). There are many studies found that PGBR contains a much higher nutrition than BR (Oh, et al., 2002). According to previous studies reported that PGBR contained more total ferulic acid (126%), total dietary fibers (145%), soluble dietary fibers (120%) and insoluble dietary fibers (150%) compared to the brown rice (Latifah, et al., 2010). Pre-germinated brown rice (PGBR) has found highly amount of antioxidant compounds and contains various bioactive components such as γ -oryzanol, α-tocopherol (vitamin e), dietary fibers, other important minerals and γ -aminobutyric acid (GABA) (Champagne, et al., 2004; Ohtsubo, et al., 2005; Mamiya, et al., 2007). The beneficial effects of these bioactive compounds, which includes regulation of blood pressure and heart rate, reduces liver damage, inhibits cancer cell proliferation and protects cellular stress (Oh, 2004). Therefore, PGBR has effectively reduced the levels of cholesterol (Miura, et al., 2006), reduced blood sugar levels of diabetic patients (Hagiwara, et al., 2004), prevented the β-amyloid-induced learning and memory deficits (Seki, et al., 2005) and protect cell proliferation and amyloid β-peptide-induced apoptosis of neuronal cells in rats (Ampornkul, et al., 2013). PGBR has many physiological effects, including antihyperlipidemia, antihypertension and the reduction in the risk of some chronic diseases, such as cancer, diabetes, cardiovascular disease and Alzheimer's disease (Wu, et al., 2013).

Moreover, it is documented that an important active component of PGBR is gamma aminobutyric acid (GABA) (Roohinejad, et al., 2009). GABA has been reported in high amounts in pre-germinated brown rice (Bautista, et al., 1964; Saikusa,

et al., 1994). GABA is an inhibitory neurotransmitter in the sympathetic nervous system (Wang, et al., 2006) and is widely distributed in the mammalian gastrointestinal tract (Krantis, 2000). GABA is produced by the decarboxylation of L-glutamic acid catalyzed by glutamate decarboxylase (Komatsuzaki, et al., 2007). It plays a role to promote growth and development in animals (Jonaidi, 2012). GABA provides many beneficial effects for human health such as decreasing blood pressure and controlling stress (Chung, et al., 2009). Dietary GABA has been shown to efficiently prevent the accumulation of body fat in rats (Powers, et al., 2008) and protective against the cytotoxicity of ethanol in isolated rat hepatocytes (Norikura, et al., 2006). In addition, GABA can increase the activities of antioxidant enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase in hogs (Hu, et al., 2008). However, scientific research supporting the beneficial effects of PGBR on liver toxicity is limited. Therefore, this purpose of this study was to investigate the effects of PGBR on rat liver toxicity induced by dextromethorphan.

This study will provide more scientific knowledge in finding an alternative product for treatment of hepatotoxicity. Finally, this study is also beneficial to enhance the value of Thai rice and also helps to promote rice consumption and export.

Purpose of the study

Objective of the study

1. A general experiment objective

This study aimed to investigate the effects of pre-germinated brown rice on rat liver toxicity induced by dextromethorphan.

2. Specific objectives

2.1 To determine the effect of pre-germinated brown rice on the histological pathology of rat liver toxicity induced by dextromethorphan.

2.2 To determine the effect of pre-germinated brown rice on the hepatic sinusoid and hepatic vacuoles in rat liver toxicity induced by dextromethorphan.

2.3 To determine the effect of germinated brown rice on accumulation of glycogen in the rat liver toxicity induced by dextromethorphan.

2.4 To determine the effect of germinated brown rice on protein content in rat liver toxicity induced by dextromethorphan.

2.5 To determine the effect of germinated brown rice on cell apoptosis in rat liver induced by dextromethorphan.

2.6 To determine the effect of germinated brown rice on cell proliferation in rat liver toxicity induced by dextromethorphan.

Hypothesis

Pre-germinated brown rice can recover the liver from hepatotoxic effects by decreasing cell apoptosis, increasing cell proliferation and cellular activity in rat liver.

Keywords

pre-germinated brown rice, hepatotoxicity, glycogen, protein content, cell proliferation, cell apoptosis, dextromethorphan

Anticipated outcomes

1. To support the negative effect of dextromethorphan on liver

2. To provide the understanding about the alteration of structure of the rat liver toxicity

3. To provide the novel knowledge of pre-germinated brown rice on cell apoptosis and cell proliferation in hepatotoxicity

4. To provide the novel knowledge of pre-germinated brown rice on glycogen accumulation, protein content and DNA content in hepatocytes

5. To enhance the value of Thai rice and also helps to promote rice consumption and export

6. To develop knowledge about pre-germinated brown rice for further research

Conceptual framework

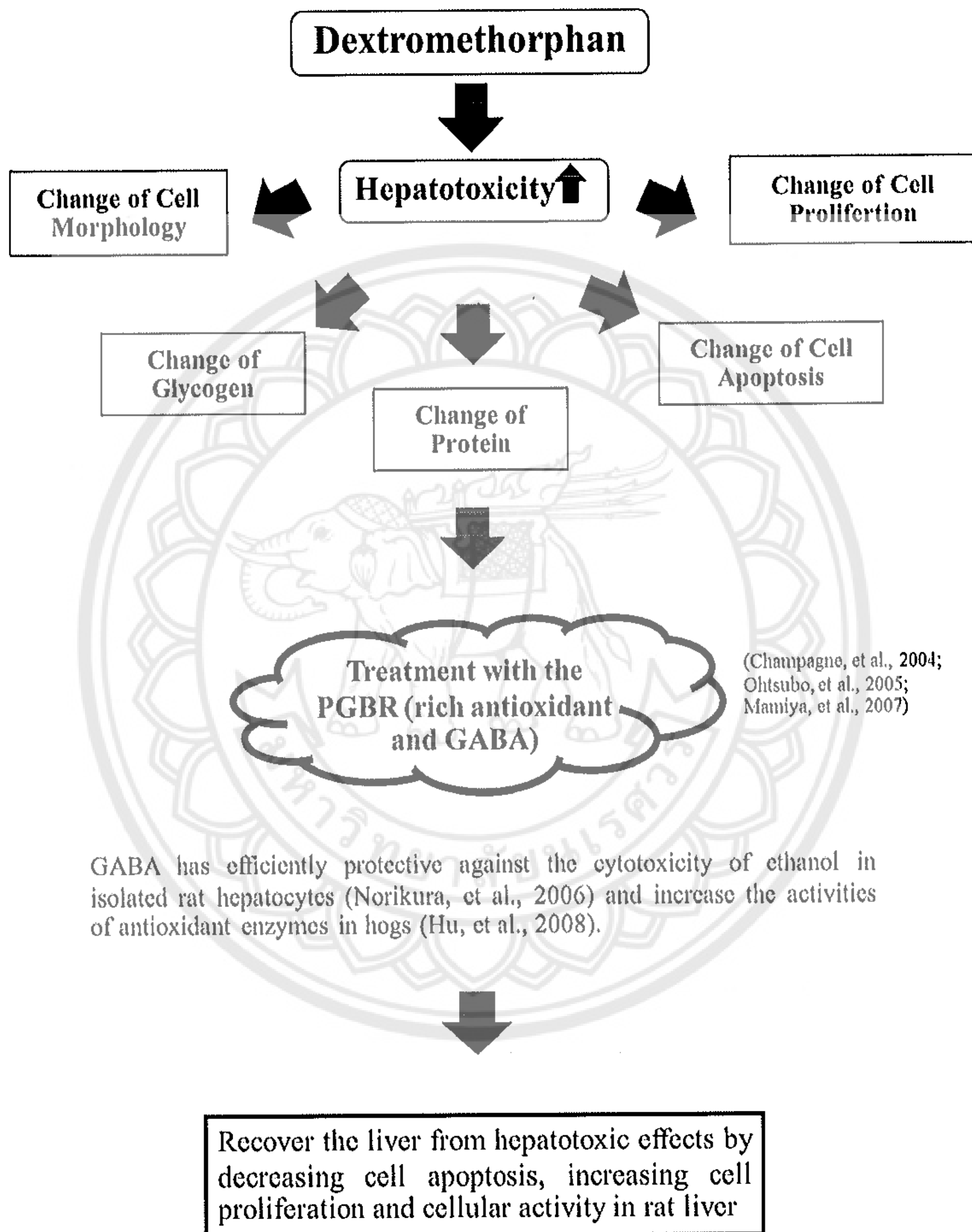


Figure 1 Conceptual framework of this study

CHAPTER II

LITERATURE REVIEW

Pre-germinated brown rice

Rice is a staple food source for many Asian countries especially Thailand. Rice is the main product to export of Thailand. There are over 40,000 different varieties of cultivated rice. This study used the rice with strain of *Oryza sativa var. glutinosa* (Purple rice, which obtain from Kheknoi, Khaoko, Phetchabun, Thailand. The characteristics of *Oryza sativa var. glutinosa* or in Thai call "kao kum" are purple pigments in the husk, and seed bran, slender grains.

Figure 2 ***Oryza sativa var. glutinosa***

Source: https://upload.wikimedia.org/wikipedia/commons/a/ac/Oryza_sativa_glutinosa_var.JPG

The rice seed consists mainly of the seed coat, embryo, and endosperm. Rice bran (seed coat) contains protein, γ-oryzanol, vitamins E (α-tocopherol and tocotrienol), K and B complex, while polished rice (without the seed coat) contains

about 25% carbohydrate, amounts of iodine, iron, magnesium, phosphorus, protein and fat (Madamba, et al., 2002; Ponciano, et al., 2005). The most popular rice in Thailand, includes brown rice and white jasmine rice. Rice has contained many nutritional components such as carbohydrate, vitamins, minerals and dietary fibers (Oh, et al., 2010). The white rice or milled rice is removed hull (husk), bran (seed coat), and germ (embryo) by polishing and milling. The major component of white rice is an endosperm, only carbohydrate. Brown rice (BR) is whole grain rice, which remove only the hull. BR remains the rice bran and rice germ that abound of nutritional components.

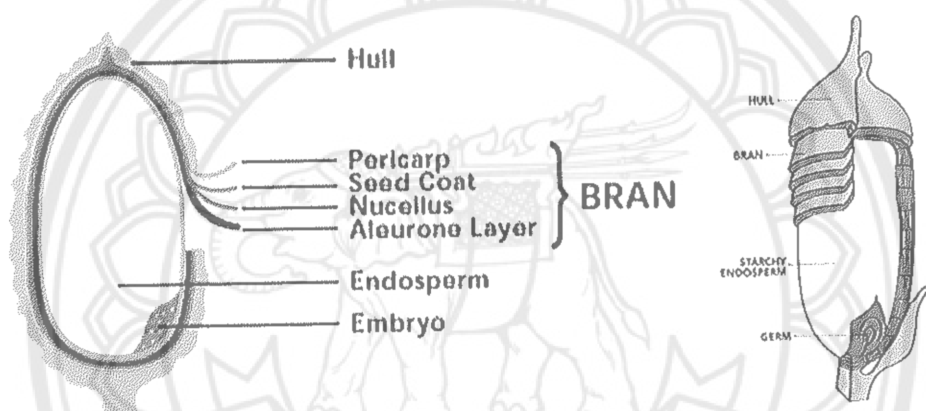


Figure 3 Structure of the rice grain

Source: http://www.ricebranoil.info/images/grafix/rice.gif

The both of milling and polishing processes remove nutritional components in rice. The complete milling and polishing that converts brown rice into white rice destroys 67% of the vitamin B3, 80% of the vitamin B1, 90% of the vitamin B6, half of the manganese, half of the phosphorus, 60% of the iron, all of the dietary fibers and essential fatty acids (Pankaj, 2008). Therefore, brown rice grains contain higher nutritional components than white rice (Champagne, et al., 2004).

Recently, germination is an effective and common process used to enhance the nutritional value of the brown rice (Yang, et al., 2001; Nakamura, et al., 2004; Wu, et al., 2013). During the germinatied grains process, polysaccharides and proteins are

decomposed by hydrolytic enzymes leads to the increase of oligosaccharides and amino acids, dietary fibers, minerals, vitamin and γ-aminobutyric acid (GABA) in pre-germinated brown rice (PGBR) (Manna, et al., 1995; Ohtsubo, et al., 2005). Following germination, the levels of biochemical and bioactive components within the grain increase, for example zinc, magnesium, potassium, ferulic acid, thiamine, riboflavin, niacin, ascorbic acid, phytate, α-tocopherol especially γ-oryzanol and GABA, which are the lipophilic antioxidant (Kayahara, et al., 2000; Frias, et al., 2005). Bioactive contents of un-germinated and PGBR are shown in Table 1. Therefore, PGBR is a higher nutritional value than brown rice.

Table 1 Bioactive contents of un-germinated and germinated brown rice

Composition (mg 100 g^{-1})	Un-germinated brown rice	PGBR
GABA	2.64 ± 0.11	44.53 ± 1.93
γ-oryzanol	64.16 ± 1.10	63.61 ± 2.40
Ferulic acid	21.75 ± 0.64	31.02 ± 1.02
Phytate	860.77 ± 7.55	609.17 ± 4.48

* The different letter in each column means significant differences at $p < 0.05$ level.

Source: Islam, et al., 2012

PGBR has been produced by soaking BR in water to induce slight germination with 0.5-1 mm height. Previous studies showed that the PGBR significantly inhibits the proliferation of cancer cells (Oh, 2004). PGBR (Oryza sativa var. glutinosa) have effectively reduced the depression behavior in rat (Thaweethee, et al., 2012) and recuded psychotoxic behavior in rat addiction that exposure with dextromethorphan (Weerasakul, et al., 2012). On the other hand, PGBR can recovery sperm quality, morphological changes of seminiferous tubules and AR expression in stress-induced rats (Roboon, et al., 2016) and can protect cell proliferation and amyloid β-peptide-induced apoptosis of neuronal cells in rats (Mamiya, et al., 2004; Ampornkul, et al.,

2013). The reduction of cholesterol levels (Miura, et al., 2006) and the reduction of blood sugar levels were observed in patients after PGBR administration (Hagiwara, et al., 2004; Seki, et al., 2005). Furthermore, PGBR can prevent chronic alcohol–related diseases (Oh, et al., 2003), reducing lipid peroxidation in hypercholesterolaemia rabbit, preventing the formation of atherosclerotic plaques and also reduce the level of hepatic enzymes (Esa, et al., 2013). These results suggested that PGBR may effect on liver toxicity.

Figure 4 Pre-germinated purple glutinous rice

Source: http://care4u.comoj.com/images-rice

According to previous studies, it was documented that an important active component of PGBR is GABA (Roohinejad, et al., 2009). GABA is a major inhibitory neurotransmitter for the regulation of presynaptic transmission in the central nervous system (CNS), neurons that secrete GABA is GABAergic (Wang, et al., 2006) It widely distributed in the mammalian gastrointestinal tract instance pancreas (Gilon, et al., 1987) and liver (Minuk, et al., 1993). GABA is produced by the decarboxylation of L-glutamic acid catalysed by glutamate decarboxylase (Komatsuzaki, et al., 2007). GABA plays a role to promote growth and development in animals (Jonaidi, 2012). It

provides many beneficial effects for human health, for example decreasing blood pressure, controlling stress (Chung, et al., 2009). Dietary GABA has been shown to efficiently prevent the accumulation of body fat in rats (Powers, et al., 2008). The previous studies showed that GABA involved maturation of germ cell and spermiogenesis (Kanbara, et al., 2005; Kanbara, et al., 2010), GABA can increase the activities of antioxidant enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase and decrease malondialdehyde concentation (MDA) in hogs (Hu, et al., 2008). GABA was found to promote synaptogenesis in the superior cervical ganglion of the adult rat (Wolff, et al., 1978), influence cell proliferation in Schwann cells (Magnaghi, et al., 2004), involved in the regulation of spermiogenesis (Kanbara, et al., 2005) and involved in epithelial cell differentiation in rat jejunum (Wang, et al., 2004). GABA has effectively to promote cell proliferation, migration, and cell survival (Barker, et al., 1998; Waagepetersen, et al., 1999; Ben-Ari, 2002; Owens, et al., 2002; Ben-Yaakov, et al., 2003). Moreover, $GABA_A$ receptor expression also has been reported in rat and human liver (Minuk, et al., 1987; Erlitzki et al., 2000), and GABA exerts an inhibitory effect via $GABA_A$ receptors on liver injury (Minuk, 1993; Zhang, et al., 1996; Kaita, et al., 1998). $GABA_B$ and $Serotonin_{2A}$ receptors coupled signalling elements induced liver cell proliferation which has therapeutic significance in liver disease (Shilpa, et al., 2013). There have been reported that baclofen is a GABA agonist, induced epidermal growth factor mediated DNA synthesis in hepatocytes in vitro (Biju, et al., 2002). Additionally, GABA has effective to protect the cytotoxicity induced by ethanol in isolated rat hepatocytes (Norikura, et al., 2006). These results suggested that GABA may effect on liver toxicity.

Dextromethorphan

In the past few years, have been reported cough medicine which contains dextromethorphan (DXM: d-3-methoxy-N-methylmorphinan) has been abused as a recreational drug (Bryner, et al., 2006). DXM is the d-isomer analog of codeine and an antitussive drug. After oral administration, dextromethorphan is quickly absorbed in the gastrointestinal tract with peak serum levels reached within 2-2.5 hours.

Dextromethorphan is absorbed into the bloodstream and crosses the blood-brain into the cerebral spinal fluid by approximately 33-80 % (Hollander, et al., 1994). The half live activity of dextromethorphan for approximately 5-6 hours (Pender, et al., 1991). High dosage and/or over long periods use of DXM can cause several psychiatric and neurological effects such as restlessness, hallucination, delayed reaction and response times, panic, delusion, ataxia, impaired cognitive ability etc. (Romanelli, et al., 2009). Its mechanism of action is as an NMDA receptor antagonist, that producing effects similar to ketamine and phencyclidine (PCP) (Hammamet, et al., 2012). Furthermore, the use of DXM in high dosage and long term also affects other organs in the body including the liver. DXM is metabolized by O-demethylation to dextrorphan, 3-methoxymorphinan through N-demethylation and 3-hydroxymorphinan through N, O-didemethylation in the liver (Beinhart, 1980; Pfaff, et al., 1983). During such detoxification processes the liver produces free radicals; excessive free radicals can cause oxidative stress.

Liver

The liver is the second largest and vital organ of human and some other animals. In the rat, it is located in the upper quadrant of the abdominal cavity, which under the diaphragm. There are four parts to the rat liver, including median lobe, left lateral, right lateral lobe and caudate lobe as shown in Figure 5.

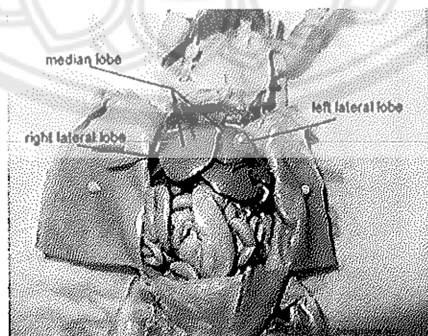


Figure 5 Located of rat liver

Source: http://www.biologycorner.com/worksheets/rat_dissection06.html

The liver is connected to the hepatic artery and the hepatic portal vein. The hepatic artery carries oxygen-rich blood from the aorta, whereas the hepatic portal vein carries blood rich in nutrients that have been extracted from food and also filters toxins that may have been ingested with the food (Chung, et al., 2008). These blood vessels subdivide into small capillaries known as liver sinusoids or hepatic sinusoids, which lead to a hepatic lobule or lobule. Lobules are the functional units of the liver. The lobules are roughly hexagonal and consist of plates of hepatocytes radiating from a central vein. Each lobule is made up of millions of hepatic cells (hepatocytes) which are the basic metabolic cells. A distinctive component of a lobule is the portal triad, which can be found running along each of the lobule's corners. The portal triad consists of three structures includes a branch of the hepatic artery, a branch of the hepatic portal vein and a bile duct.

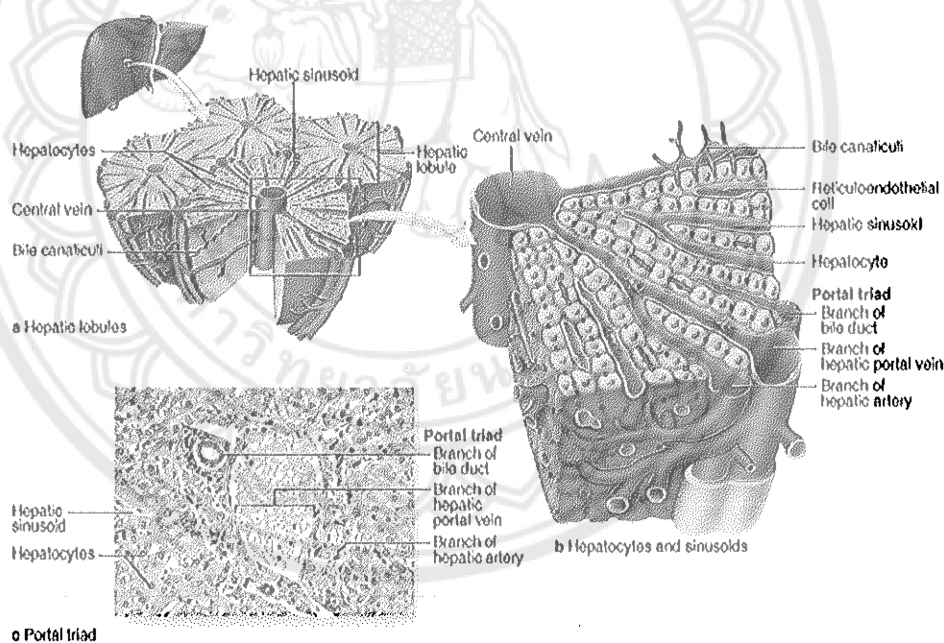


Figure 6 Portal triad in liver

Source: http://i.stack.imgur.com/9DDdF.png

The study of microscopic anatomy shows two major types of liver cell includes parenchymal cells and non-parenchymal cells. 70–85% of the liver is parenchymal hepatocytes. Non-parenchymal cells constitute 40% of the total number of liver cells (Kmieć, 2001). The liver sinusoids are lined with two types of cell, sinusoidal endothelial cells and phagocytic Kupffer cells (Pocock, et al., 2006). Hepatic stellate cells are non-parenchymal cells, which found in the perisinusoidal space (or space of Disse), between a sinusoid and a hepatocyte. Additionally, intrahepatic lymphocytes are often present in the sinusoidal lumen (Kmieć, 2001).

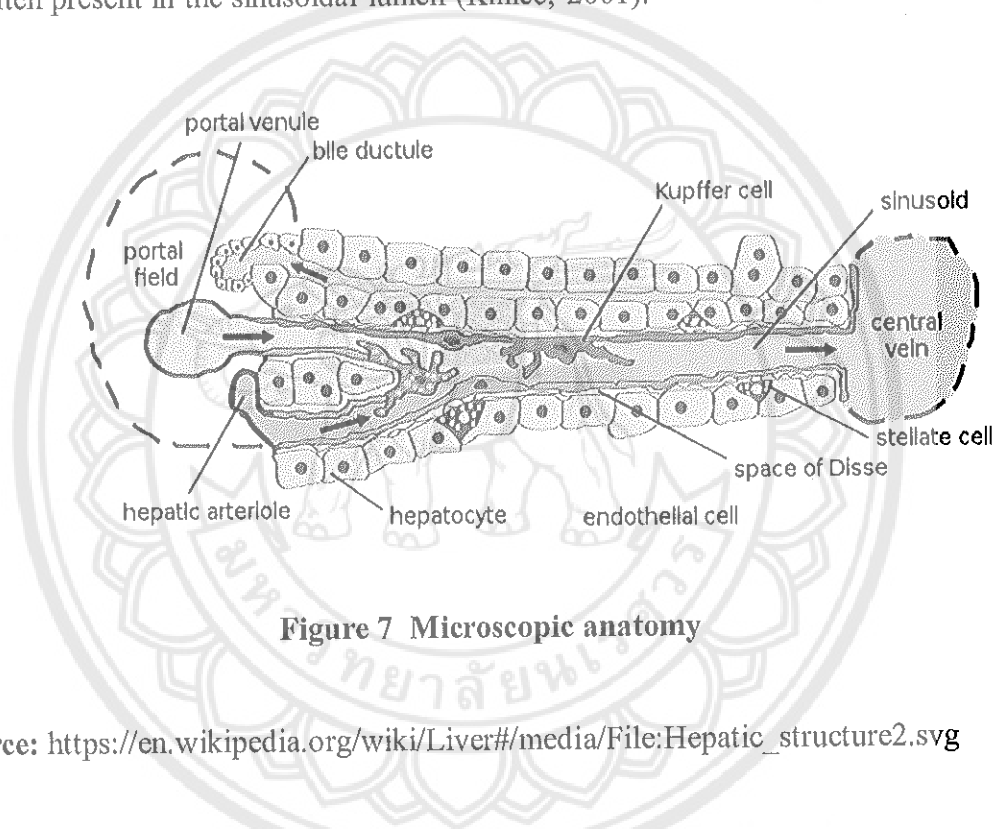


Figure 7 Microscopic anatomy

Source: https://en.wikipedia.org/wiki/Liver#/media/File:Hepatic_structure2.svg

The liver is an essential organ that has many functions in the body, including regulation of glycogen storage, storing of minerals, iron and Vitamin A, decomposition of red blood cells, plasma protein synthesis, hormone production, production of biochemical necessary for digestion and detoxification (Abdel-Misih, et al., 2010). Detoxification (detox) is the physiological process to removal of toxic substances from a living organism, which is mainly carried out by the liver. Additionally, it can refer to the period of withdrawal during, which returns to homeostasis after long-term use of an addictive substance. There are two major detoxification pathways inside the liver cells, which are called the Phase 1 and Phase 2

detoxification pathways. Phase one detoxification consists of oxidation reduction and hydrolysis. Phase one detoxification is catalysed by enzymes referred to as the cytochrome P450 enzyme group or Mixed Function Oxidase enzymes (MFO). These enzymes reside on the membrane system of the hepatocytes. This pathway converts a toxic chemical into a less harmful chemical. This is achieved by various chemical reactions (such as oxidation, reduction and hydrolysis). Phase two detoxification is called the conjugation pathway. The hepatocytes add another substance (eg. glutathione, sulphate, glycine, glucuronide conjugations) to a toxic chemical or drug for render it less harmful. This makes the toxin or drug water-soluble can be eliminated from the body via watery fluids such as bile, urine and stool (Grant, 1991).

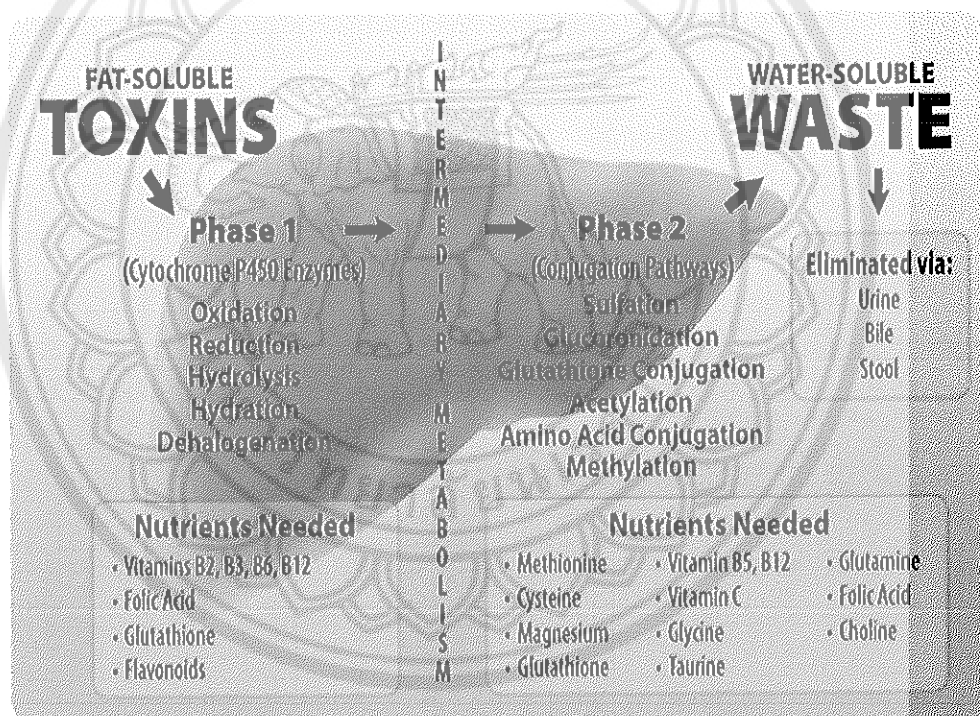


Figure 8 Liver detoxification pathways

Source: http://www.healthbywholefoods.com.au/wp-content/uploads/2016/11/Phase-1-and-11-Liver-Detoxification.jpg

However during phase one detoxification process free radicals are produced which, if excessive, can damage the hepatocytes. Antioxidants (such as vitamin C and E and natural carotenoids) reduce the damage caused by these free radicals. If antioxidants are lacking and toxin exposure is high resulting in oxidative stress, may cause damage to proteins, RNA, and DNA within the cell. Oxidative stress plays a critical role in liver diseases and other chronic and degenerative disorders (Li, et al., 2014).

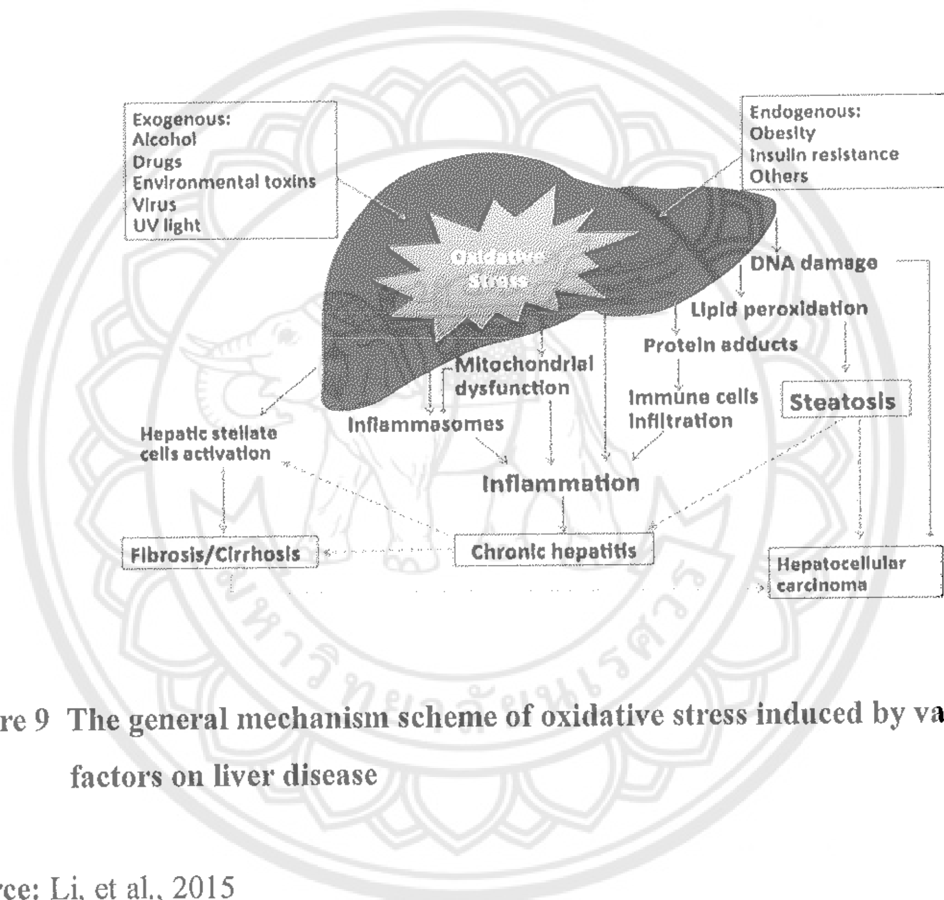


Figure 9 **The general mechanism scheme of oxidative stress induced by various factors on liver disease**

Source: Li, et al., 2015

Liver is an interesting organ with high regenerative capacity (Michalopoulos, et al., 1997; Taub, 2004; Michalopoulos, et al., 2005; Fausto, et al., 2006). Liver regeneration is highly controlled process regulated by complex network on highly redundant signals. Several signaling pathways are known to stimulate regeneration in the liver including cytokines, growth factors, hormones, and nuclear receptors (Michalopoulos, et al., 2013). After liver injury, several signals are initiated

simultaneously in the liver. Lipopolysaccharide (LPS) is a gut-derived factors, which upregulated after liver injury and reach the liver through the portal blood supply. LPS activate Kupffer cells to increase the production of tumor necrosis factor alpha (TNFα) and interleukin (IL)-6. The interleukin 6 (IL6) and activate stellate cells to produce hepatocyte growth factor (HGF). Additionally, other factors are released from the pancreas (insulin), duodenum or salivary gland (epidermal growth factor; EGF), adrenal gland (norepinepherine) and thyroid gland (triodothronine; T3). Cooperative signals from these factors allow the hepatocytes to overcome cell-cycle checkpoint controls and move from G0, through G1, to the S phase of the cell cycle. This leads to DNA synthesis and hepatocyte proliferation (Sadri, et al., 2015).

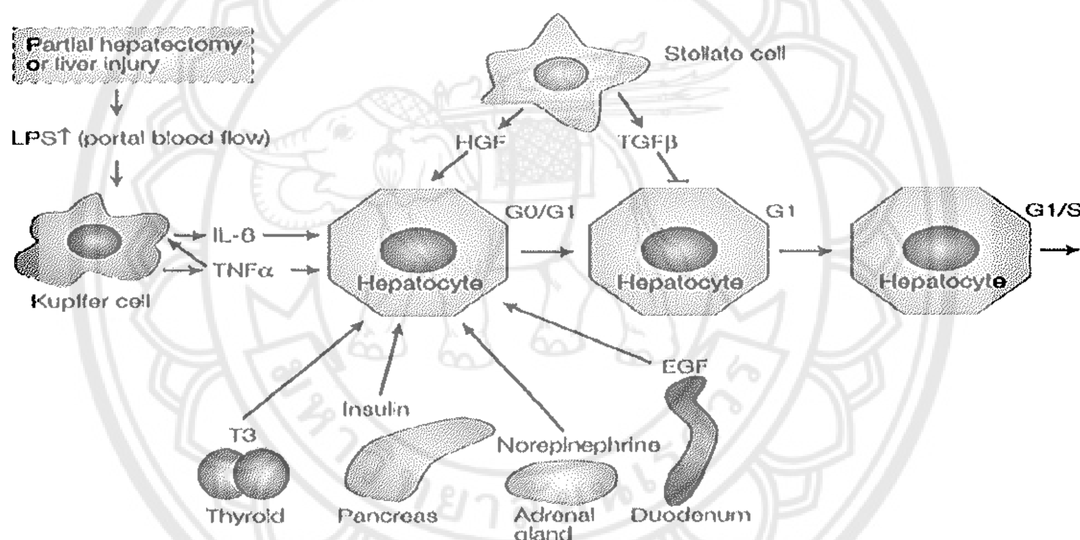


Figure 10 The liver regeneration

Source: http://www.nature.com/nrm/journal/v5/n10/images/nrm1489-f1.jpg

Proliferating cell nuclear antigen (PCNA)

Protein was observed in the nucleus of dividing cells is proliferating cell nuclear antigen, which used as a proliferation marker (Miyachi, et al., 1978). PCNA is an evolutionarily well-conserved protein found in all eukaryotic species. PCNA was first shown to act as a processivity factor of DNA polymerase, which is required for

DNA synthesis during replication (Tan, et al., 1986; Bravo, et al., 1987; Prelich, et al., 1987). However, besides DNA replication, PCNA functions are associated with other vital cellular processes such as chromatin remodelling, DNA repair, sister-chromatid cohesion and cell cycle control (Maga and Hubscher, 2003). PCNA plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery. One of the well-established functions for PCNA is its role as β subunit of DNA polymerase III (Kelman, 1997). In normal liver has found less PCNA expression (Hall et al., 1995). Therefor, PCNA used to check the cell proliferation.

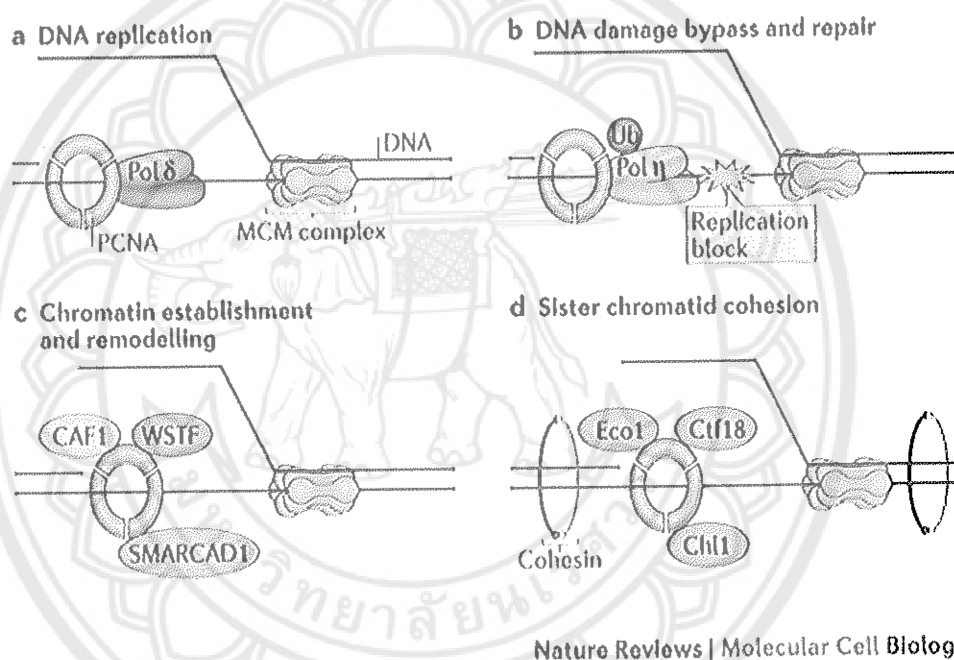


Figure 11 PCNA as a master coordinator of replication fork-associated processes

Source: http://www.nature.com/nrm/journal/v14/n5/fig_tab/nrm3562_F1.html

CHAPTER III

RESEARCH METHODOLOGY

This study used liver tissue from the project under title "Effects of pre-germinated brown rice on GABA, glutamate and dopamine neurotransmission after drug withdrawal in rats" of my co-laboratory that approved by the Ethics Committee for Animal Experimentation at the NU-AE540418. The methodology of this study, including animals, experimental design, equipment and methods as follows;

Animals

Seventy healthy male Sprague Dawley rats, weighing 200-250 g at average age of 5 weeks were utilized. All animals were obtained from National Animal Center, Salaya, Nakorn Pathom. Animals were placed under automatic temperature ($24\pm1^{0}C$) and lighting control (12 hours light/12 hours dark cycle) with humidity ranging from 50±10%. Food and water were available *ad libitum*. The study was approved by the Institutional Animal Care and Use Committee of Naresuan University, Thailand.

Reagents administration

The reagents of this study were described below;

1. Dextromethorphan, dextromethorphan hydrobromide $C_{18}H_{25}NO.HBr.H_2O$ was purchased from Sigma-Alorich® Lot#090M1298V.

2. Gamma aminobutyric acid, GABA ($C_4H_9NO_2$) was purchased from Sigma Chemical Company, St. Louis, USA. Amount of synthetic GABA was equaled with the GABA found in PGBR. GABA was dissolved in distilled water.

The components of PGBR were evaluated by the Laboratory of Faculty of Agriculture Natural Resources and Environment, Naresuan University and Central Laboratory, Bangkok, Thailand. Pre-germinated brown rice *(Oryza sativa var. glutinosa)* from KhekNoi, KhaoKho, Phetchabun (Thailand) was soaked for 24 hours until germinated. PGBR were dried and produced to powder and dissolved in distilled water (Table 2).

Table 2 The components of PGBR

Substances	Volumes (mg/100g)
GABA	16.512
Gamma Oryzanol	29.608
Alpha-tocopherol (Vitamin E)	0.91
Pyridoxine (Vitamin B_6)	0.11
Thiamine (Vitamin B_1)	0.05

Experimental design

70 rats were classified into fourteen groups as (Figure 12);

1. Control group (C): 5 rats received normal saline daily by intraperitoneal injection (i.p.) for 75 days.

2. Dextromethorphan group (DXM): 5 rats received DXM 30 mg/kg (i.p.) for 15 days.

3. DXM and withdrawal 15 days group (DW15): 5 rats received DXM 30 mg/kg for 15 days (i.p.) and withdrawal for 15 days.

4. DXM and withdrawal 30 days group (DW30): 5 rats received DXM 30 mg/kg for 15 days (i.p.) and withdrawal for 30 days.

5. DXM and withdrawal 60 days group (DW60): 5 rats received DXM 30 mg/kg for 15 days (i.p.) and withdrawal for 60 days.

6. Pre-germinated brown rice control 15 days group (CP15): 5 rats received normal saline for 15 days (i.p.) and received PGBR 5 g/kg for 15 days daily by oral administration.

7. Pre-germinated brown rice control 30 days group (CP30): 5 rats received normal saline for 15 days (i.p.) and received PGBR 5 g/kg for 30 days daily by oral administration.

8. Pre-germinated brown rice control 60 days group (CP60): 5 rats received normal saline for 15 days (i.p.) and received PGBR 5 g/kg for 60 days daily by oral administration.

9. DXM and synthetic GABA 15 days group (DG15): 5 rats received DXM 30 mg/kg for 15 days (i.p.) and received synthetic GABA 0.8 mg/kg for 15 days daily by oral administration.

10. DXM and synthetic GABA 30 days group (DG30): 5 rats received DXM 30 mg/kg for 15 days (i.p.) and received synthetic GABA 0.8 mg/kg for 30 days daily by oral administration.

11. DXM and synthetic GABA 60 days group (DG60): 5 rats received DXM 30 mg/kg for 15 days (i.p.) and received synthetic GABA 0.8 mg/kg for 60 days daily by oral administration.

12. DXM and PGBR 15 days group (DP15): 5 rats received DXM 30 mg/kg for 15 days (i.p.) and received PGBR 5g/kg for 15 days daily by oral administration.

13. DXM and PGBR 30 days group (DP30): 5 rats received DXM 30 mg/kg for 15 days (i.p.) and received PGBR 5g/kg for 30 days daily by oral administration.

14. DXM and PGBR 60 days group (DP60): 5 rats received DXM 30 mg/kg for 15 days (i.p.) and received PGBR 5g/kg for 60 days daily by oral administration.

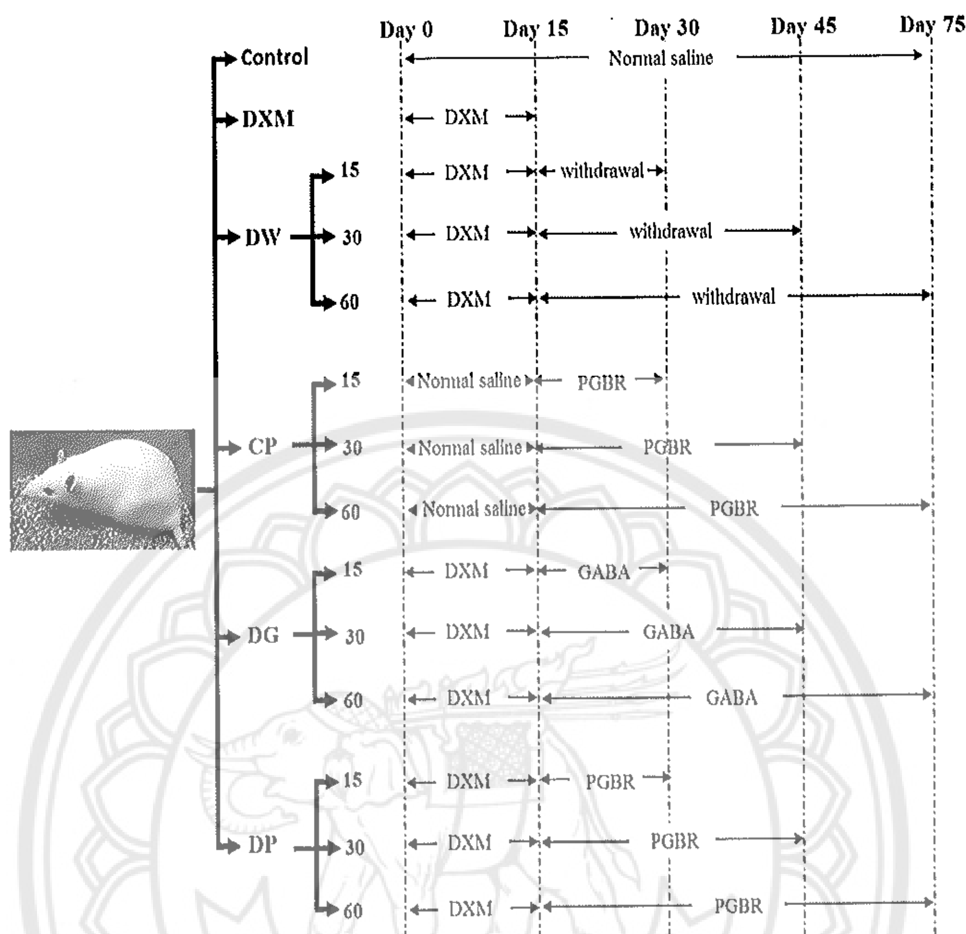


Figure 12 The schematic diagram of experiment in rat model of toxicity

Tissue preparation

After treatments, all of the rats were sacrificed by cervical dislocation. The livers were immediately removed then immersed into 10% neutral buffered formalin to preserve tissue structure. The livers were cut, placed in cassettes followed by washing in distilled water 3 times for 5 minutes each to clean the 10% neutral buffered formalin. Next, the tissues were dehydrated by 70% ethanol, 80% ethanol, 90% ethanol, 95% ethanol and 100% ethanol for removing the water from the tissue, followed by immersing in xylene and paraffin (Shandon Citadel 1000 Tissue Processor, Thermo Scientific, Bridgewater, New Jersey, USA). The liver tissues were placed into molds for infiltrating with the molten paraffin. The embedded tissues were left on the cold plate 20-30 minutes approximately, followed by removing the tissue

block from the molds. The tissue blocks were kept at 4 °C until section. The tissue sections were cut at 5 µm thickness, followed by mounting onto slides coated with 3-Aminopropyl triethoxy-silane solution (Sigma-Aldrich, St. Louis, MO, USA). The sections were allowed to dry overnight at room temperature that were evaluated the histopathology and histochemical changes in the liver.

Histopathological preparation

The morphological changes of liver were analyzed by hematoxylin-eosin (H & E) staining technique. This uses a combination of hematoxylin and eosin for demonstration of nucleus and cytoplasm. The sections were deparaffinized with xylene followed by passing through decreasing concentration of ethanol (100%, 100%, 95%, 70%) and distilled water. The sections were stained with Hematoxylin for 5 minutes, followed by washing with tap water for 5 minutes. Stain sections with Eosin for 2 minutes, followed by dehydrated with 70% ethanol, 95% ethanol and 100% ethanol and removed alcohol with xylene. Lastly, the tissues were mounted with mounting media (SP15-500 Toluene Solution UN1294, Fisher scientific, Bridgewater, New Jersey, USA). Hematoxylin-Eosin stained sections were evaluated under a light microscope (Nikon eclipse 08i; Nikon, Bangkok, Thailand, Co., Ltd.) and taken a picture with image capture system (Nikon digital camera DXM1200c, Nikon, Bangkok, Thailand, Co., Ltd.). The data were shown as morphology changes of liver in each section. The quantitative data of hepatic sinusoid and vacuolization in rat liver showed the percentages of hepatic sinusoid dilation and vacuolization. Each of different section was randomly selected four central vein per section. At four areas per one central vein were evaluated in each animal. Each area is 4,915,200 pixels squared.

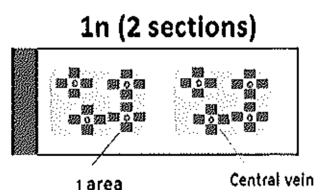


Figure 13 Evaluation of randomly selected areas

Distribution of general carbohydrates (Glycogen)

General carbohydrates were demonstrated by Periodic Acid Schiff's (PAS) technique (Mc-Manus J. 1946). This method involves the oxidation of carbohydrate with 0.5% periodic acid, which leads to the liberation of aldehydes. These aldehydes then react with the Schiff reagent to give a purple-magenta colour (Figure 13).

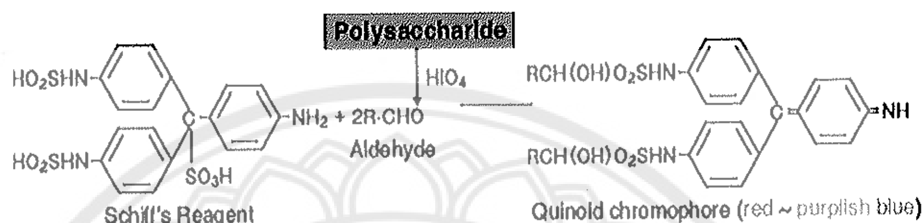


Figure 14 Reaction Formula of Schiff's reagent

Source: http://www.wako-chem.co.jp/english/labchem/journals/pathology2013/image/38099.png

The liver sections were deparaffinized with xylene three times for 5 minutes, followed by rehydration through series of ethanol (100%, 100%, 95%, 70%) and distilled water for 3 minutes. The sections were immersed in 1% periodic acid for 10 minutes, followed by washing with distilled water for 1 minute. The sections were immersed in Coleman's Feulgen solution for 30 minutes at room temperature followed by washing with water for 5 minutes. The sections were counterstained with Hematoxylin for 5 seconds, followed by washing with water for 5 minutes. The tissue sections were dehydrated with ethanol and clearing with xylene, respectively. Finally, the tissues were mounted with mounting media (SP15-500 Toluene Solution UN1294, Fisher scientific, Bridgewater, New Jersey, USA).

The distribution of glycogen were evaluated under a light microscope at magnification 20X. (Nikon eclipse 08i; Nikon, Bangkok, Thailand, Co., Ltd.) and taken a picture with image capture system (Nikon digital camera DXM1200c, Nikon, Bangkok, Thailand, Co., Ltd.) The distribution of glycogen were analysed by Image J software (http://rsb.info.nih.gov/ij/). Each of different section was randomly selected

four central vein per section. At four areas per one central vein were evaluated in each animal. Relative optical density (ROD) of glycogen accumulation was evaluated and calculated according to the following formula:

$$\text{ROD} = \log(256/\text{Intensity mean})$$

Protein staining

Total of proteins in hepatocytes were demonstrated by bromophenol blue method (Dubeyl W. and Trivedi P.C. 2016). Bromophenol blue is an acidic dye which in aqueous solution is capable of reactive groups (100 milligrams of Bromphenol blue per 100 ml.) for 10 minutes. The acidic groups of the dye react with basic groups of the protein to give blue colour.

The liver sections were deparaffinized with xylene three times for 5 minutes, followed by rehydration through ethanol (100%, 100%, 95%, 70%) and distilled water for 3 minutes. The sections were immersed in Bromophenol blue solution for 5 minutes, followed by washing with distilled water for 5 minutes. The tissue sections were dehydrated and deparaffinized with ethanol and xylene, respectively. Finally, the tissues were mounted with mounting media (SP15-500 Toluene Solution UN1294, Fisher scientific, Bridgewater, New Jersey, USA).

The total of protein were evaluated under a light microscope at magnification 20X. (Nikon eclipse 08i; Nikon, Bangkok, Thailand, Co., Ltd.) and taken a picture with image capture system (Nikon digital camera DXM1200c, Nikon, Bangkok, Thailand, Co., Ltd.) The total of protein intensity were analysed by Image J software (http://rsb.info.nih.gov/ij/). Each of different section was randomly selected four central vein per section. At four areas per one central vein were evaluated in each animal. Relative optical density (ROD) of protein content was evaluated and calculated according to the formula previously.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL assay is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids. This study used an apoptosis detection kit (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, Code: S7101, Millipore Corp., Bedford, MA). All reagents listed below were from the kit and were prepared following the manufacturer's instructions (Figure 14).

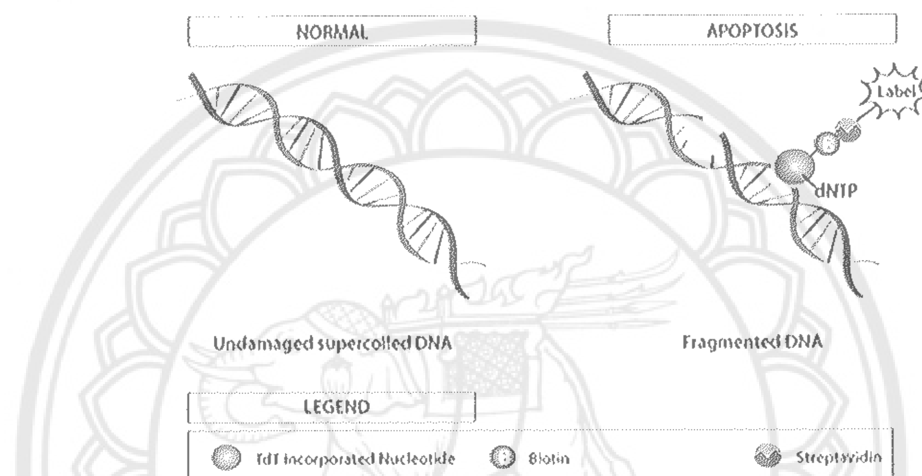


Figure 15 TUNEL assay principle

Source: https://resources.rndsystems.com/images/site/4810-11-12-assay-tacs-tdt.png

The liver sections were deparaffinized with xylene three times for 5 minutes, followed by rehydration through ethanol (100%, 100%, 95%, 70%) and distilled water for 3 minutes. The sections were incubated with 20 mg/ml proteinase K for 30 minutes at 37°C. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide for 15 minutes, followed by incubation with equilibration buffer for 20 minutes. The sections were incubated with a terminal deoxynucleotidyl transferase enzyme (TdT), in a humidified chamber at 37°C, for 2 hours, followed by a stop / wash buffer at room temperature for 10 minutes then incubated with anti-digoxigenin for 2 hours. The sections were developed with DAB for 3 minutes at room temperature followed by washing in distilled water for 5 minutes. The sections were

counterstained with Hematoxylin followed by washing with tap water for 5 minutes. The tissue sections were dehydrated with ethanol and xylene, respectively. Finally, the tissues were mounted with mounting media (SP15-500 Toluene Solution UN1294, Fisher scientific, Bridgewater, New Jersey, USA).

The apoptotic sections were evaluated under a light microscope at magnification 20X. (Nikon eclipse 08i; Nikon, Bangkok, Thailand, Co., Ltd.) and taken a picture with image capture system (Nikon digital camera DXM1200c, Nikon, Bangkok, Thailand, Co., Ltd.) Apoptotic cells were counted by Image J software (http://rsb.info.nih.gov/ij/). An apoptotic index (AI) was calculated for each sample from the ratio of the number of TUNEL-positive cells to the total number of hepatocytes. At least sixteen areas were counted in one section of each animal.

Immunohistochemistry analysis (Proliferating cell nuclear antigen: PCNA)

Immunohistochemistry technique is used for detection of specific antigens in tissue sections. The proliferating cell nuclear antigen (PCNA) expression was detected in rat liver by indirect immunohistochemistry technique. The immunohistochemical protocol was performed according to Toydemir, et al., 2015.

The sections were deparaffinized with xylene three times for 5 minutes, followed by rehydrating through decreasing concentration of ethanol (100%, 100%, 95%, 70%) and distilled water, respectively for 3 minutes. For antigen retrieval, the sections were heated in PBS three times for 5 minutes in a microwave oven at 850 W and allowed to cool. Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide in methanol (10% methanol, 0.3% H_2O_2, 1% triton-X and 1X PBS) for 30 minutes. The sections were blocked for nonspecific binding protein by incubating with 1% normal goat serum in PBS (PCN5000-Thermo Fisher Scientific, USA) for 2 hours, followed by incubation with a specific Rabbit polyclonal anti-proliferating cell nuclear antigen antibody (PCNA) (Cat. 07-2162, Millipore Corp., Bedford, MA), diluted 1:200 for 12 hours at 4°C. The sections were incubated with HRP-conjugated goat anti-rabbit immunoglobulin G (Jackson Immuno Research, 111-035-003), diluted 1:200 for 2 hours at room temperature followed by washing in PBS three times for 5 minutes. Peroxidase enzyme was detected with 3,3'-diaminobenzidine (DAB) followed

by stopping reaction in distilled water for 5 minutes. The sections were counterstained with Hematoxylin for 3 seconds, followed by dehydration with ethanol and xylene, respectively. Finally, the tissues were mounted with mounting media (SP15-500 Toluene Solution UN1294, Fisher scientific, Bridgewater, New Jersey, USA) (Figure 15).

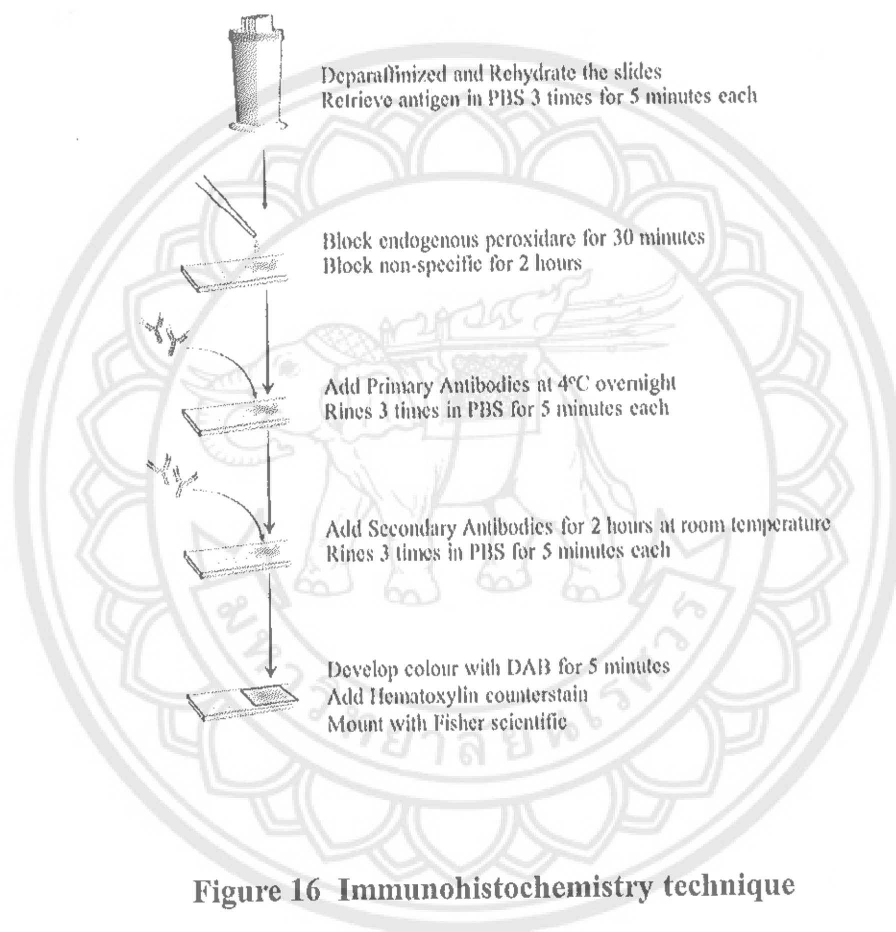


Figure 16 Immunohistochemistry technique

Source: https://resources.rndsystems.com/images/site/Protocol-6-Fluorescent-frozen6625.jpg

The immunoreactivity on tissue sections were evaluated under a light microscope at magnification 10X. (Nikon eclipse 08i; Nikon, Bangkok, Thailand, Co., Ltd.) and taken a picture with image capture system (Nikon digital camera DXM1200c, Nikon, Bangkok, Thailand, Co., Ltd.) Hepatocyte nuclei labeled brown

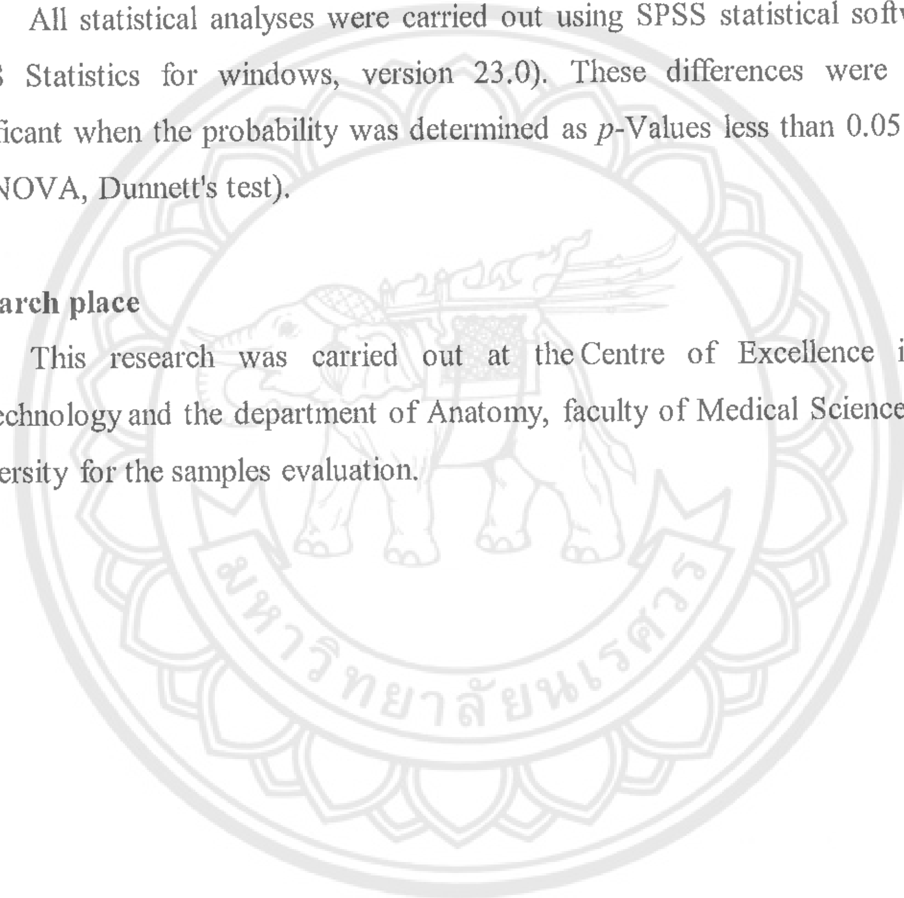
colour were PCNA-positive cells and were counted by Image J software (http://rsb.info.nih.gov/ij/). A proliferation index (PI) was calculated for each sample from the ratio of the number of PCNA-positive hepatocytes to the total number of hepatocytes were counted, at least sixteen areas were counted in one section of each animal.

Statistical analysis

All statistical analyses were carried out using SPSS statistical software (IBM SPSS Statistics for windows, version 23.0). These differences were considered significant when the probability was determined as *p*-Values less than 0.05 (Post Hoc in ANOVA, Dunnett's test).

Research place

This research was carried out at the Centre of Excellence in Medical Biotechnology and the department of Anatomy, faculty of Medical Science, Naresuan University for the samples evaluation.



CHAPTER IV

RESULTS

This study showed effects of pre-germinated brown rice on morphological changes of the liver, cell apoptosis, cell proliferation, distribution of glycogen and protein content in rat liver toxicity induced by DXM.

The effect of pre-germinated brown rice on morphological changes of the rat liver toxicity induced by DXM.

In the qualitative study, the results were showed 4 characteristics of rat liver changes, including normal morphology of rat liver, vacuolization in hepatocytes, sinusoidal congestion and nuclear fragmentation in 60 days (Figure 17).

The morphological changes of rat liver of control and PGBR groups were showed well defined cell membranes, clear cytoplasm and prominent cell boundaries. The most of the cell contains a central rounded nucleus while some hepatocytes found binucleated. (Figure 17A & C). In histological of rat livers that received DXM and withdrawal, cells with enlarged shape, vacuolization were prevalented in hepatocytes. Sinusoidal congestion was evident (Figure 17B & D). In addition, DXM treated with PGBR and GABA have found vacuolization and sinusoidal congestion to be better and close to the control group (Figure 17 E & F).

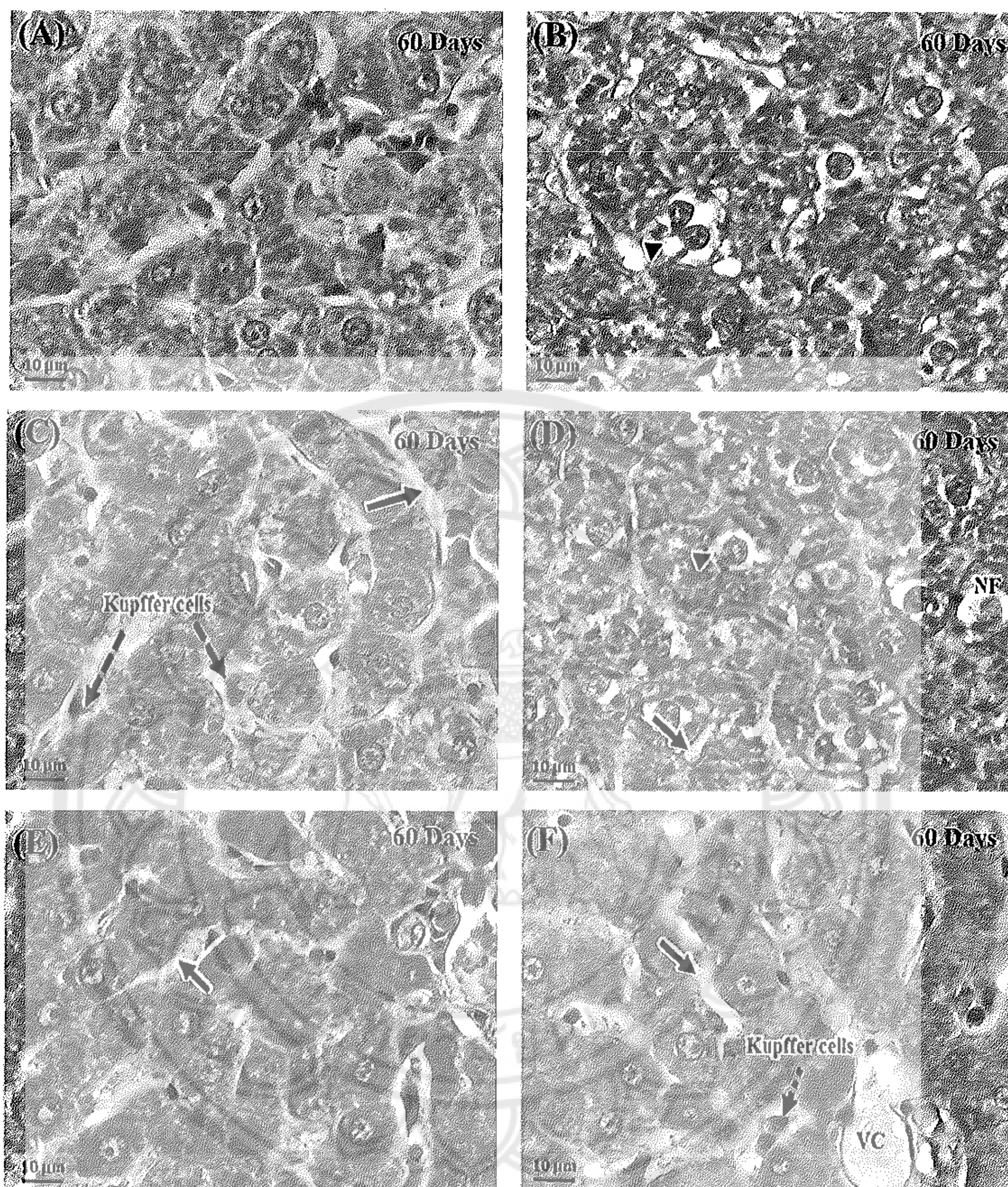


Figure 17 Hematoxylin-eosin (H&E) staining for revealing morphological changes of liver cells in rat treated for 60 days ; C(A), DXM(B), CP(C), DW(D), DG(E) and DP(F) (▲: vacuoles, ✦: sinusoidal congestion, ↑: Kupffer cell, NF: nuclear fragmentation).

The effect of pre-germinated brown rice on hepatic sinusoid and vacuolization in the rat liver toxicity induced by DXM.

The hepatic sinusoid and vacuolization in rat liver were analyzed by hematoxylin-eosin (H & E) staining technique. The results showed morphological changes of hepatic sinusoid and vacuolization in all groups in rat treated for 60 days (Figure 18).

The morphological changes of hepatic sinusoid and vacuolization were found no significant difference in PGBR for 15 days, 30 days and 60 days when compared with control group (Table 3-4). In 15 days DXM-treated group, it was significantly increased in the percentage of sinusoidal dilation and vacuolization when compared with control group (p=0.000), a significant increase in the percentage of sinusoidal dilatation and vacuolization was found in 15 days withdrawal group when compared with control group (p=0.000). However, the 15 days GABA-treated and PGBR-treated groups showed a significant decrease in the percentage of sinusoidal dilatation and vacuolization when compared with DXM group (p=0.000) (Figure 19-20).

The morphological changes of hepatic sinusoid and vacuolization were showed a significant decrease in 30 days withdrawal group when compared with DXM group (p=0.000). Moreover, the 30 days GABA-treated and PGBR-treated groups showed a significant decrease in the percentage of sinusoidal dilation and vacuolization when compared with DXM group (p=0.000) (Figure 21-22).

In addition, 60 days withdrawal group showed a significant decrease in the percentage of sinusoidal dilation and vacuolization when compared with DXM group (p=0.000). Furthermore, the 60 days GABA-treated and PGBR-treated groups showed a significant decrease in the percentage of sinusoidal dilation and vacuolization when compared with DXM group (p=0.000) (Figure 23-24).

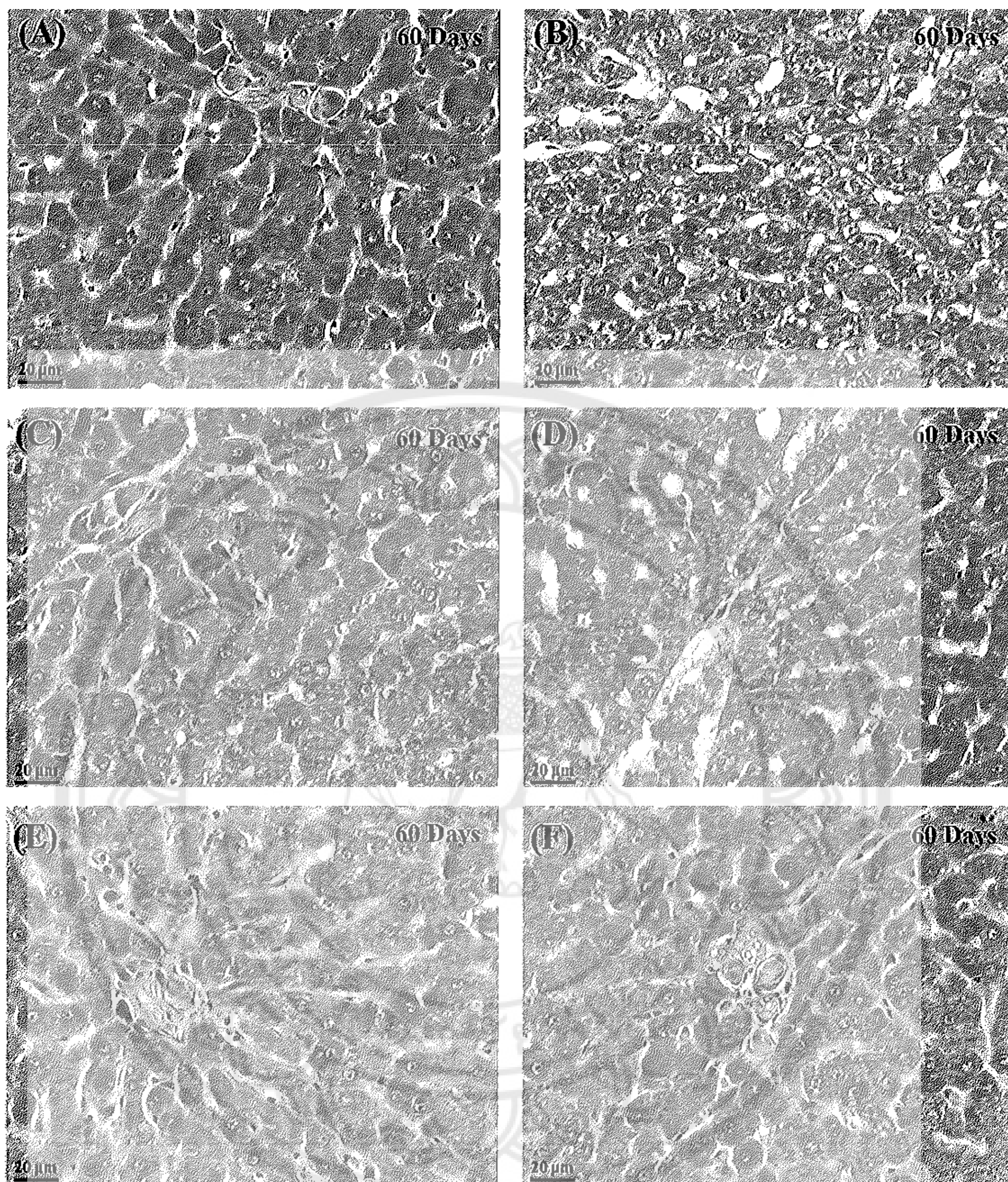


Figure 18 **Morphological changes of hepatic sinusoid and vacuolization in 60 days in all groups of rat liver were analyzed by hematoxylin-eosin (H&E) staining technique; C(A), DXM(B), CP(C), DW(D), DG(E) and DP(F).**

Table 3 Hepatic siusoid dilation in rat liver in control, PGBR (CP15), PGBR (CP30), PGBR (CP60) groups

Groups	% Hepatic sinusoid dilation
Control	9.0876±0.0112
CP 15	9.0881±0.0087
CP 30	9.0884±0.0132
CP 60	9.0869±0.0063

Note: Data are expressed as mean ± SEM.

Table 4 Hepatic vacuolization in rat liver in control, PGBR (CP15), PGBR (CP30), PGBR (CP60) groups

Groups	% Hepatic vacuolization
Control	9.0161±0.0202
CP 15	9.0143±0.0130
CP 30	9.0170±0.0044
CP 60	9.0166 ±0.0176

Note: Data are expressed as mean ± SEM.

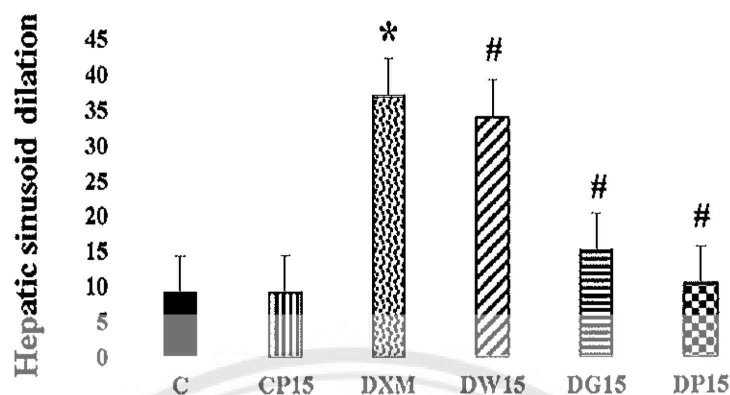


Figure 19 % Hepatic sinusoid dilation in control, PGBR (CP15), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 15 days Data are presented as mean±SEM. * $p<0.05$ versus control group and # $p<0.05$ versus DXM group

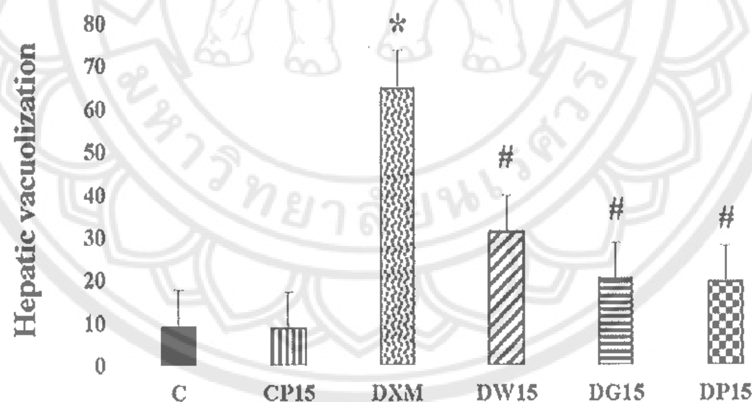


Figure 20 % Hepatic vacuolization in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 15 days Data are presented as mean±SEM. * $p<0.05$ versus control group and # $p<0.05$ versus DXM group

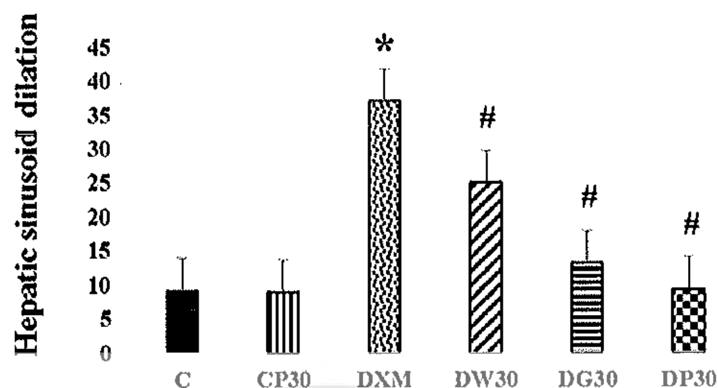


Figure 21 % Hepatic sinusoid dilation in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 30 days Data are presented as mean±SEM. * $p<0.05$ versus control group and # $p<0.05$ versus DXM group

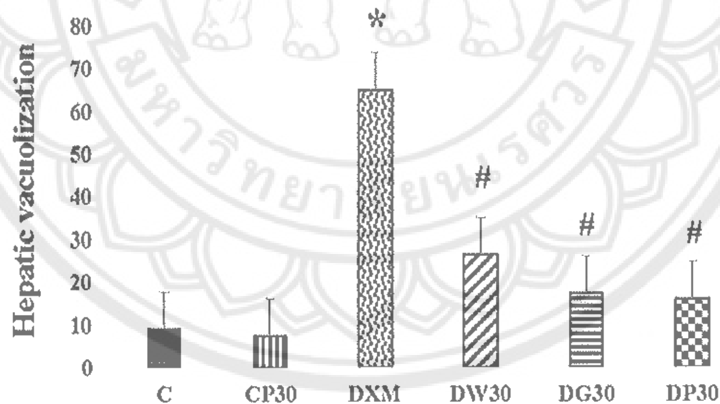


Figure 22 % Hepatic vacuolization in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 30 days Data are presented as mean±SEM. * $p<0.05$ versus control group and # $p<0.05$ versus DXM group

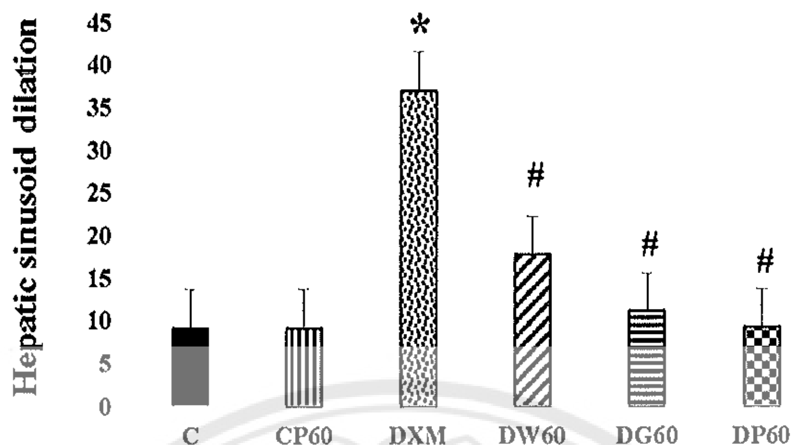


Figure 23 % Hepatic sinusoid dilation in control, PGBR (CP15), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 60 days Data are presented as mean±SEM. * p<0.05 versus control group and # p<0.05 versus DXM group

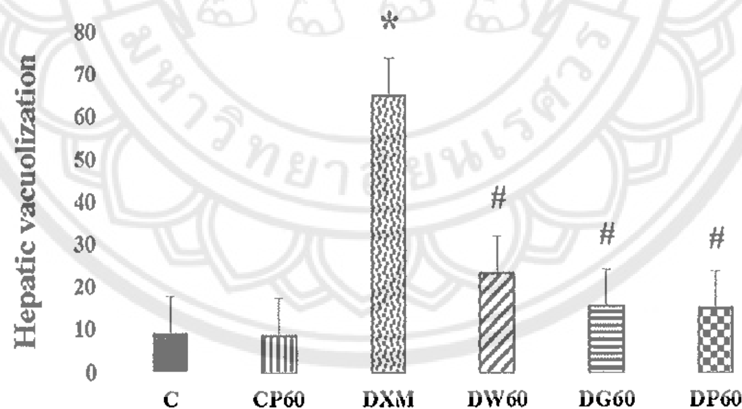


Figure 24 % Hepatic vacuolization in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 60 days Data are presented as mean±SEM. * p<0.05 versus control group and # p<0.05 versus DXM group

Table 5 Hepatic vacuolization and sinusoid dilation in rat liver in all groups of control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP)

Groups	% Hepatic vacuolization	% Hepatic sinusoid dilation
Control	9.0161±0.0202	9.0876±0.0112
CP 15	9.0143±0.0130	9.0881±0.0087
CP 30	9.0170±0.0044	9.0884±0.0132
CP 60	9.0166±0.0176	9.0869±0.0063
DXM	64.7561±0.1561*	36.8820±1.6310*
DW 15	31.5674±0.2126#	33.8342±0.5767#
DW 30	26.2513±0.2123#	24.9351±0.2646#
DW 60	23.1354±0.3377#	17.6334±0.5846#
DG 15	20.7913±0.3162#	14.8913±0.2843#
DG 30	17.2512±0.1220#	13.0378±0.2992#
DG 60	15.2503±0.2133#	11.0233±0.1245#
DP 15	19.2534±0.0888#	10.2610±0.2669#
DP 30	16.6411±0.0689#	9.2207±0.0914#
DP 60	14.7540±0.1303#	9.1291±0.1124#

Note: Data are expressed as mean ± SEM. Statistical significance is indicated as *p<0.05 versus control group, #p<0.05 versus DXM group

The effect of pre-germinated brown rice on glycogen accumulation in rat liver toxicity induced by DXM.

The glycogen accummulation in rat liver was analyzed by Periodic Acid Schiff's (PAS) technique. The results showed morphological changes of glycogen accumulation in all groups in rat treated for 60 days (Figure 25). The control and PGBR groups showed normal pattern distribution of glycogen granules (Figure 25 A & C). In DXM and all withdrawal groups showed glycogen depletion granules (Figure 25B). In addition, increased of glycogen accumulation in DXM treated with PGBR and GABA were observed (Figure 25 E & F).

The glycogen intensity was found no significant difference in PGBR for 15 days, 30 days and 60 days when compared with control group (Figure 26). In 15 days DXM-treated group, it was significantly decreased in the glycogen intensity when compared with control group (p=0.000), a significant decrease in the glycogen intensity was found in 15 days withdrawal group when compared with control group (p=0.000). However, the 15 days GABA-treated and PGBR-treated groups showed a significant increase in the glycogen intensity when compared with DXM group (p=0.000) (Figure 27).

The glycogen intensity was showed a significant increase in 30 days withdrawal group when compared with DXM group (p=0.000). Moreover, the 30 days GABA-treated and PGBR-treated groups showed a significant increase in the glycogen intensity when compared with DXM group (p=0.000) (Figure 28).

In addition, in 60 days withdrawal group showed a significant increase in the glycogen intensity when compared with DXM group (p=0.000). Furthermore, the glycogen intensity were showed significantly increased in 60 days GABA-treated and PGBR-treated groups when compared with DXM group (p=0.000) (Figure 29).

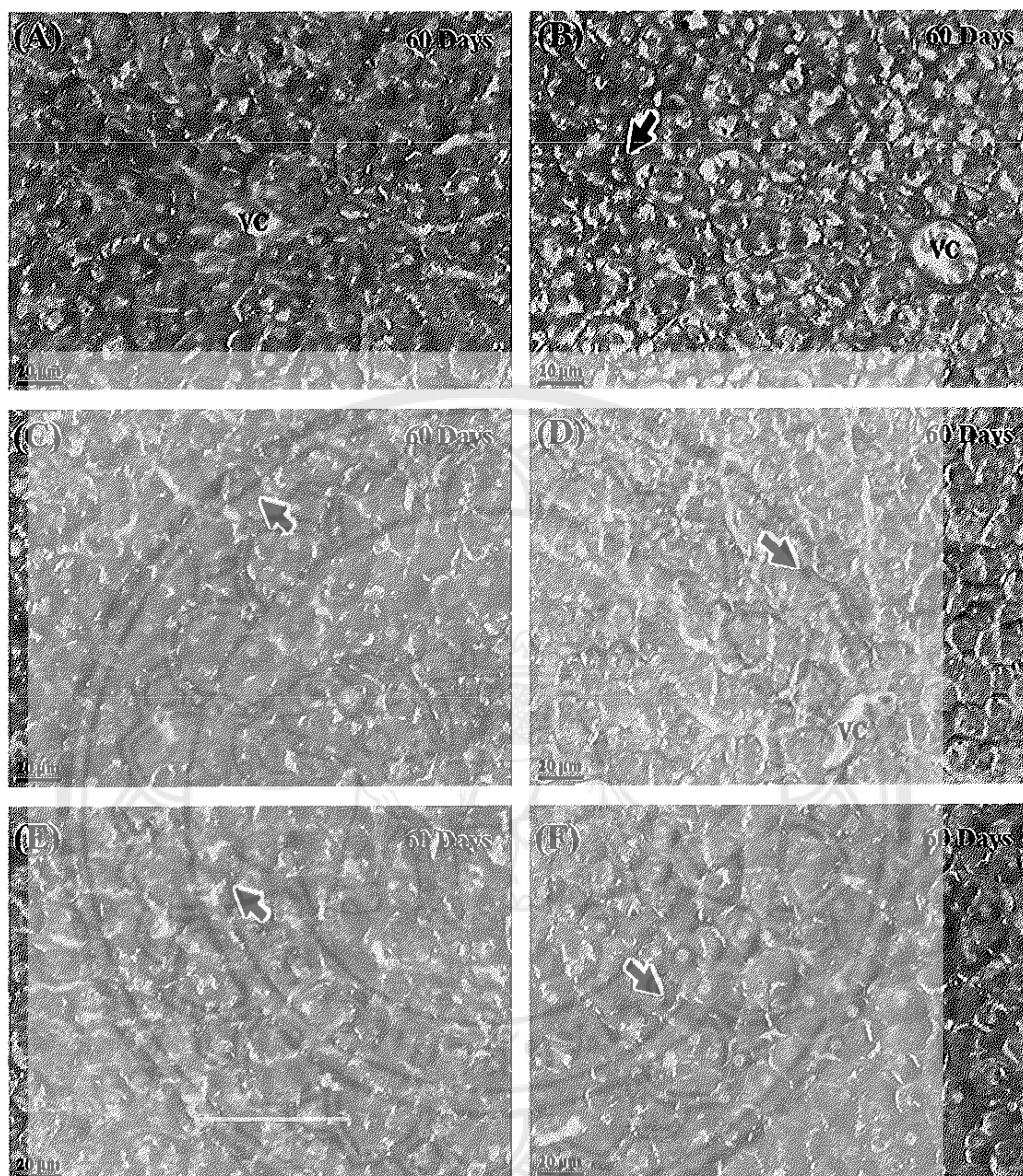


Figure 25 Morphological changes of glycongen accumulation in 60 days in all groups of rat liver were analyzed by PAS staining technique; C(A), DXM(B), CP(C), DW(D), DG(E) and DP(F) (🡅: glycogen granules, VC: vena centralis).

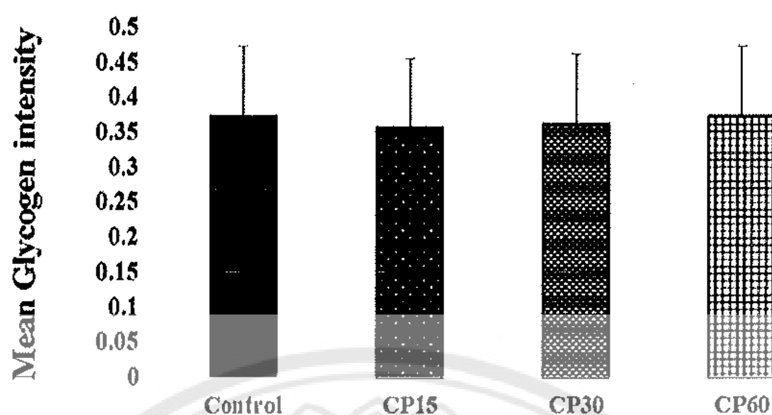


Figure 26 Glycogen intensity in control, PGBR (CP15), PGBR (CP30), PGBR (CP60) groups. Data are presented as mean±SEM.

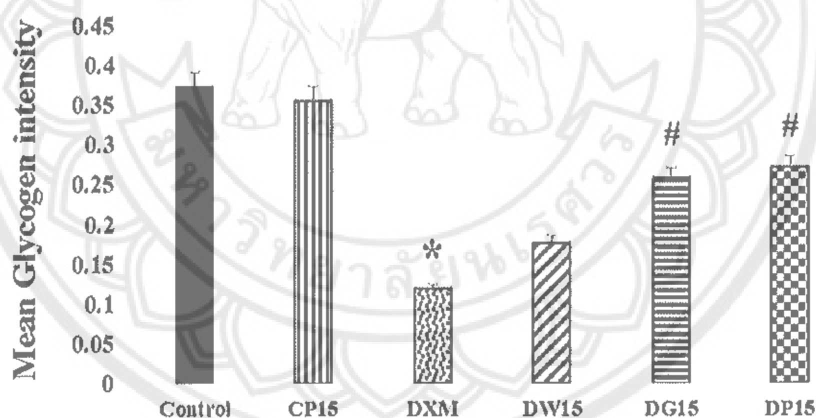


Figure 27 Glycogen intensity in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 15 days Data are presented as mean±SEM. * $p<0.05$ versus control group and # $p<0.05$ versus DXM group

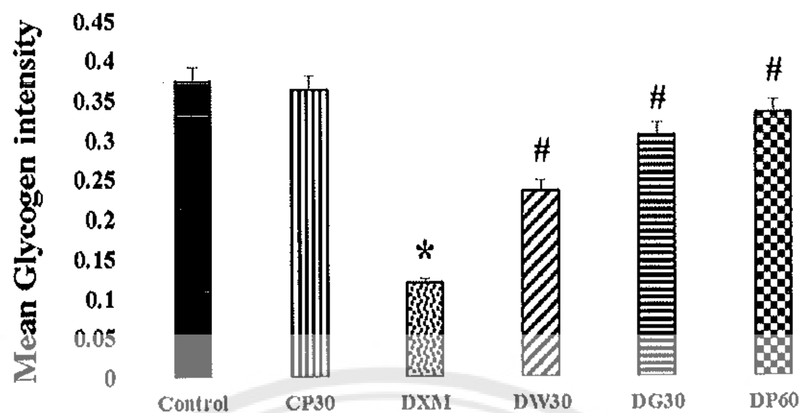


Figure 28 Glycogen intensity in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 30 days Data are presented as mean±SEM. $^{*}p<0.05$ versus control group and $^{\#}p<0.05$ versus DXM group

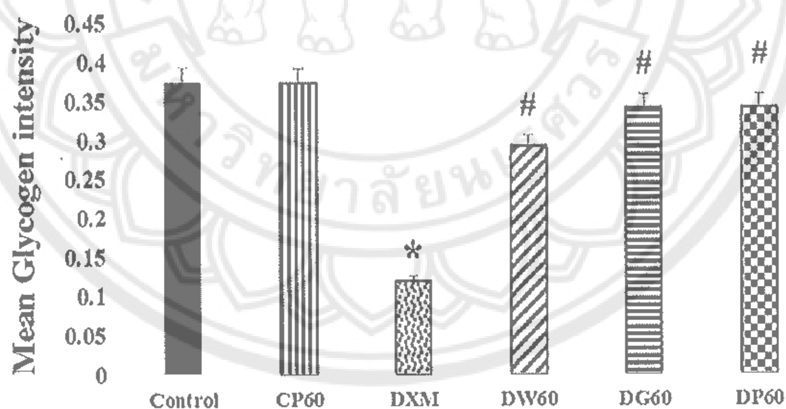


Figure 29 Glycogen intensity in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 60 days Data are presented as mean±SEM. $^{*}p<0.05$ versus control group and $^{\#}p<0.05$ versus DXM group

Table 6 Glycogen intensity in rat liver in all groups of control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP)

Groups	Mean glycogen intensity
Control	0.3725±0.0026
CP 15	0.3554±0.0041
CP 30	0.3616±0.0038
CP 60	0.3721±0.0035
DXM	0.1182±0.0012*
DW 15	0.1764±0.0022
DW 30	0.2349±0.0010#
DW 60	0.2904±0.0023#
DG 15	0.2580±0.0007#
DG 30	0.3039±0.0009#
DG 60	0.3399±0.0010#
DP 15	0.2713±0.0011#
DP 30	0.3317±0.0024#
DP 60	0.3397±0.0002#

Note: Data are expressed as mean ± SEM. Statistical significance is indicated as *p<0.05 versus control group, #p<0.05 versus DXM group

The effect of pre-germinated brown rice on protein distribution in rat liver toxicity induced by DXM.

The protein distribution in hepatocytes was analyzed by bromophenol blue method. The results showed the distribution of protein in all groups in rat treated for 60 days (Figure 30). The cytoplasm contained numerous dark blue stained protein granules in hepatocytes in control and PGBR groups (Figure 30 A & C). In DXM and all withdrawal groups, protein distribution moderately decreased and the cytoplasm appeared more vacuolated (Figure 30 B & C). In addition, increased protein distribution in DXM treated with PGBR and GABA groups were observed (Figure 30 E & F).

The protein intensity was found no significant difference in PGBR for 15 days, 30 days and 60 days when compared with control group (Figure 31). In 15 days DXM-treated group, it was significantly decreased in the protein intensity when compared with control group (*p*=0.000), a significant decrease in the protein intensity when compared with control group (*p*=0.000). However, the 15 days GABA-treated and PGBR-treated groups showed a significant increase in the protein intensity when compared with DXM group (*p*=0.000) (Figure 32).

The protein intensity was showed a significant increase in 30 days withdrawal group when compared with DXM group (*p*=0.000). Moreover, the 30 days GABA-treated and PGBR-treated groups showed a significant increase in the glycogen intensity when compared with DXM group (*p*=0.000) (Figure 33).

In addition, in 60 days withdrawal group showed a significant increase in the protein intensity when compared with DXM group (*p*=0.000). Furthermore, the protein intensity were showed significantly increased in 60 days GABA-treated and PGBR-treated groups when compared with DXM group (*p*=0.000) (Figure 34).

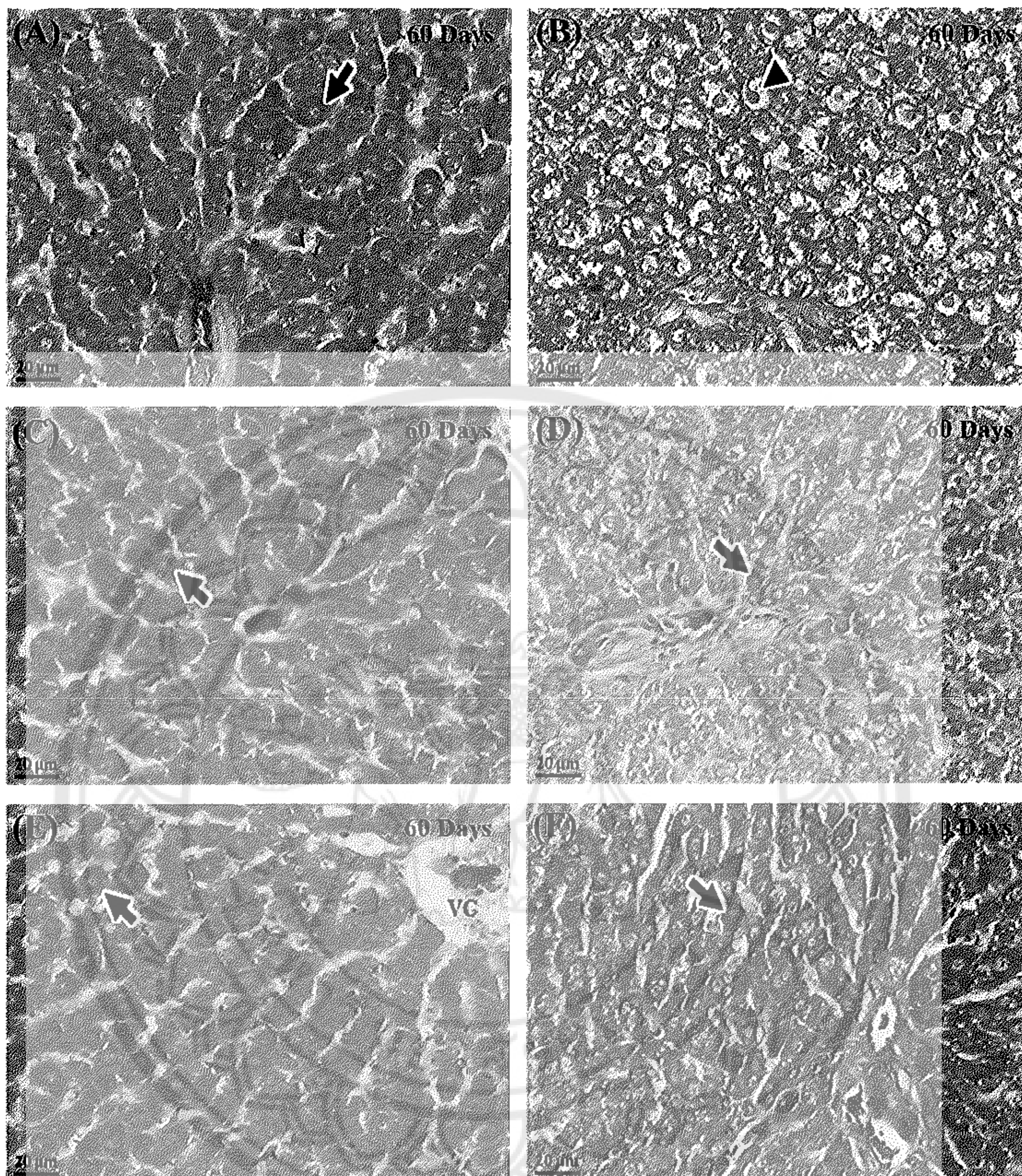


Figure 30 Morphological changes of protein distribution in 60 days in all groups of rat liver were analyzed by bromophenol blue staining technique; C(A), DXM(B), CP(C), DW(D), DG(E) and DP(F) (▲: hepatic vacuoles, 🡅: protein content, VC: vena centralis).

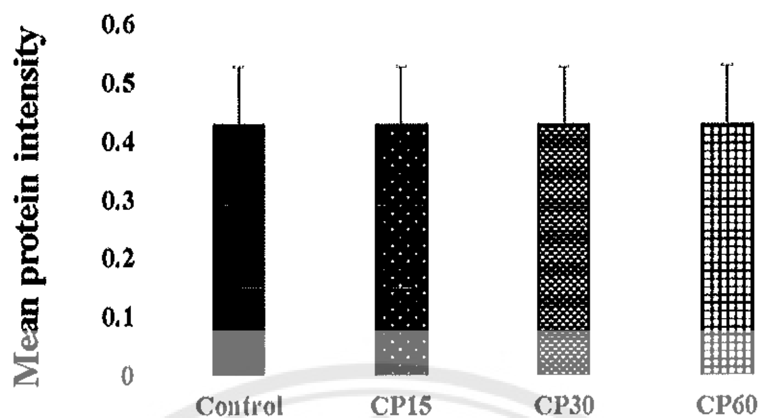


Figure 31 Protein intensity in control, PGBR (CP15), PGBR (CP30), PGBR (CP60) groups. Data are presented as mean±SEM.

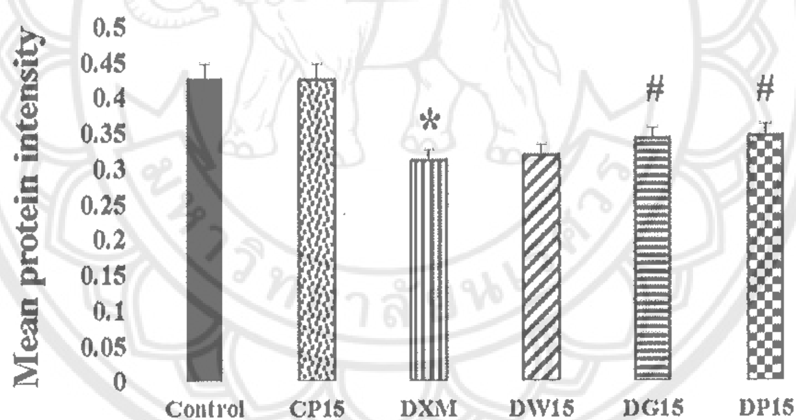


Figure 32 Protein intensity in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 15 days Data are presented as mean±SEM. * p<0.05 versus control group and $^{\#}$ p<0.05 versus DXM group

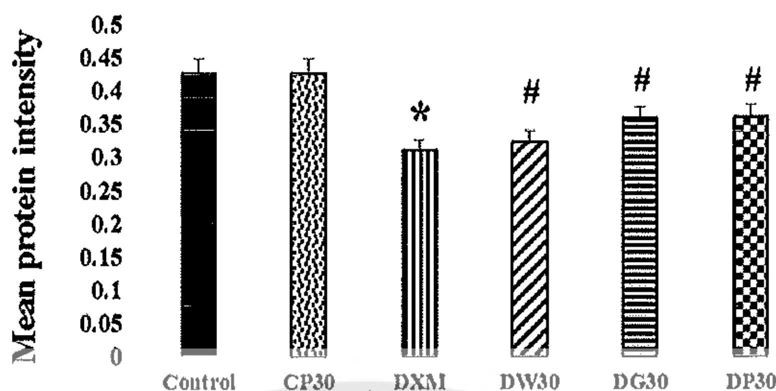


Figure 33 **Protein intensity in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 30 days Data are presented as mean±SEM. * p<0.05 versus control group and $^{\#}$ p<0.05 versus DXM group**

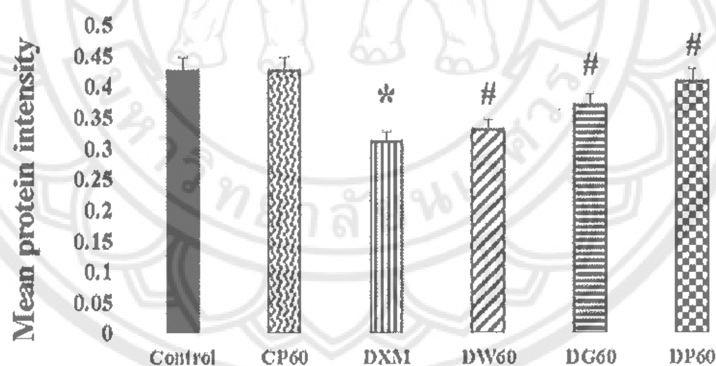


Figure 34 **Protein intensity in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 60 days Data are presented as mean±SEM. * p<0.05 versus control group and $^{\#}$ p<0.05 versus DXM group**

Table 7 Protein intensity in rat liver in all groups of control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP)

Groups	Mean protein intensity
Control	0.4259±0.0007
CP 15	0.4249±0.0005
CP 30	0.4260±0.0005
CP 60	0.4262±0.0004
DXM	0.3103±0.0004*
DW 15	0.3171±0.0005
DW 30	0.3229±0.0001#
DW 60	0.3291±0.0001#
DG 15	0.3404±0.0002#
DG 30	0.3547±0.0004#
DG 60	0.3700±0.0005#
DP 15	0.3453±0.0006#
DP 30	0.3597±0.0003#
DP 60	0.4072±0.0006#

Note: Data are expressed as mean ± SEM. Statistical significance is indicated as *$p<0.05$ versus control group, #$p<0.05$ versus DXM group

The effect of pre-germinated brown rice on cell apoptosis in the rat liver toxicity induced by DXM.

The cell apoptosis in rat liver was analyzed by TUNEL assay. The results showed Morphological changes of cell apoptosis, was localized in the DNA of apoptotic cell in all groups in rat treated for 60 days (Figure 35). The cell apoptosis was negligible in control and PGBR groups (Figure 35A & C). In DXM and all withdrawal groups, plenty of apoptotic cells were presented in the liver (Figure 35B & D). In addition, the apoptotic cells were markedly reduced in DXM treated with PGBR and GABA groups (Figure 35 E & F).

The positive apoptotic cell was showed an apoptotic index (AI; percentage of TUNE-positive hepatocytes). The AI was found no significant difference in PGBR for 15 days, 30 days and 60 days when compared with control group (Figure 36). In 15 days DXM-treated group, it was significantly increased in the AI when compared with control group (p=0.000), a significant increase in the AI was found in 15 days withdrawal group when compared with control group (p=0.000). However, the 15 days GABA-treated and PGBR-treated groups showed a significant decrease in the AI when compared with DXM group (p=0.000) (Figure 37).

The AI was found a significant decrease in 30 days withdrawal group when compared to the DXM group (p=0.000). Moreover, the 30 days GABA-treated and PGBR-treated groups showed a significant decrease in the AI when compared with DXM group (p=0.000) (Figure 38).

In addition, the AI was showed significantly decreased in 60 days withdrawal group when compared with DXM group (p=0.000). Furthermore, the 60 days GABA-treated and PGBR-treated groups showed a significant decrease in the AI when compared with DXM group (p=0.000) (Figure 39).

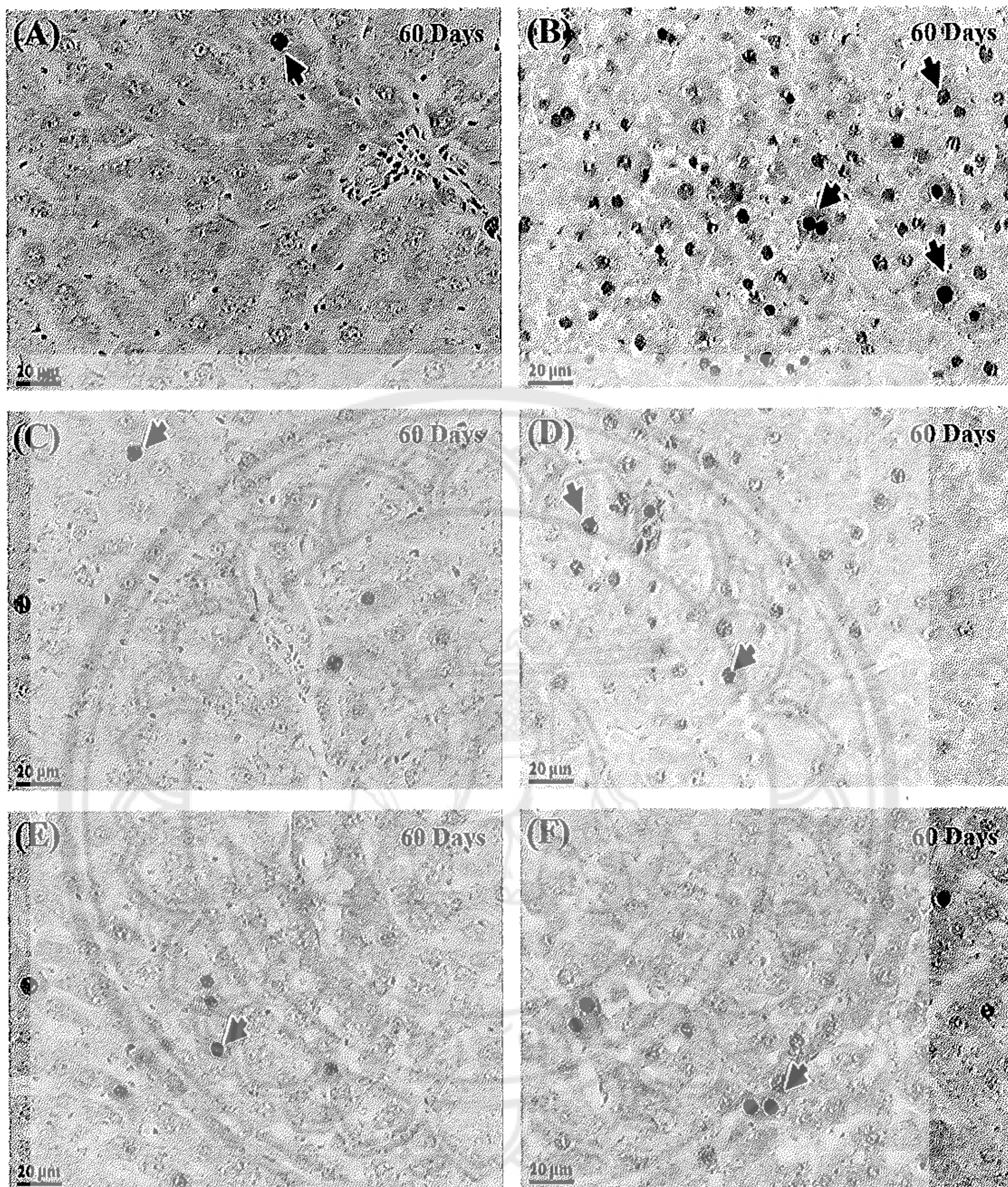


Figure 35 **Morphological changes of cell apoptosis in 60 days in all groups of rat liver were analyzed by TUNEL Assay technique; C(A), DXM(B), CP(C), DW(D), DG(E) and DP(F) (🡅: TUNEL-positive apoptotic hepatocytes).**

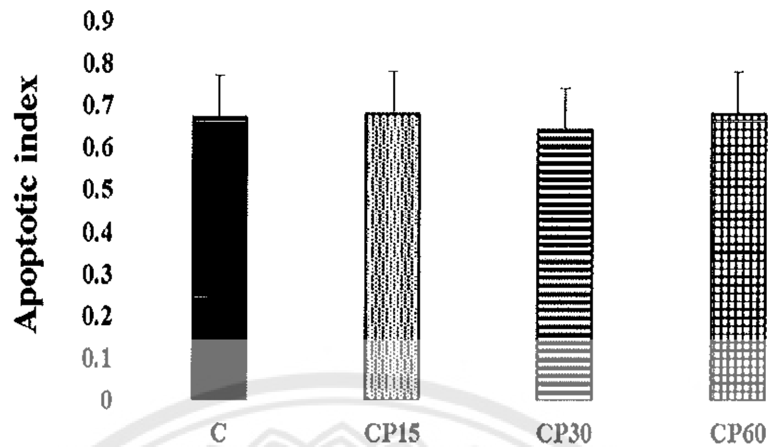


Figure 36 **Apoptotic index in control, PGBR (CP15), PGBR (CP30), PGBR (CP60) groups. Data are presented as mean±SEM.**

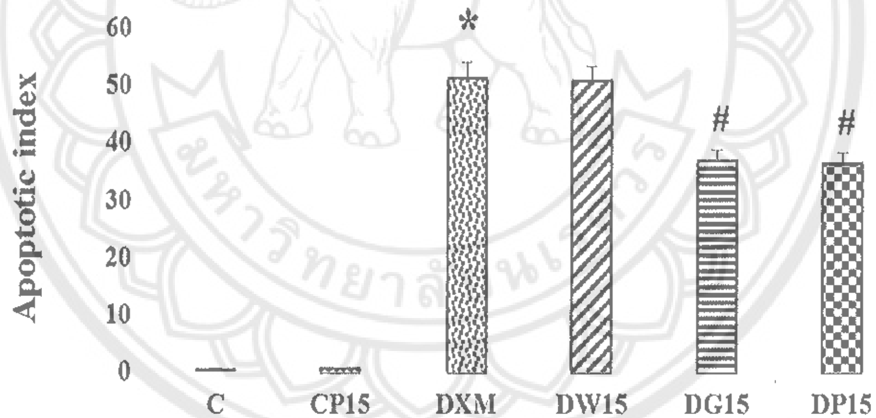


Figure 37 **Apoptotic index in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 15 days Data are presented as mean±SEM. * p<0.05 versus control group and # p<0.05 versus DXM group**

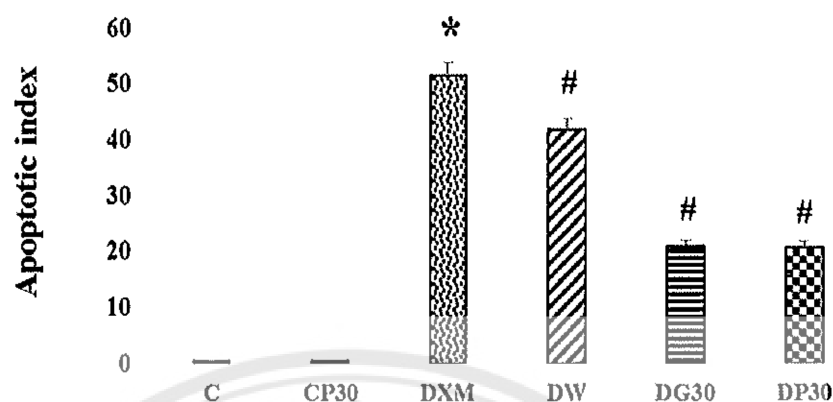


Figure 38 **Apoptotic index in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 30 days Data are presented as mean±SEM. * *p*<0.05 versus control group and $^{\#}$ *p*<0.05 versus DXM group**

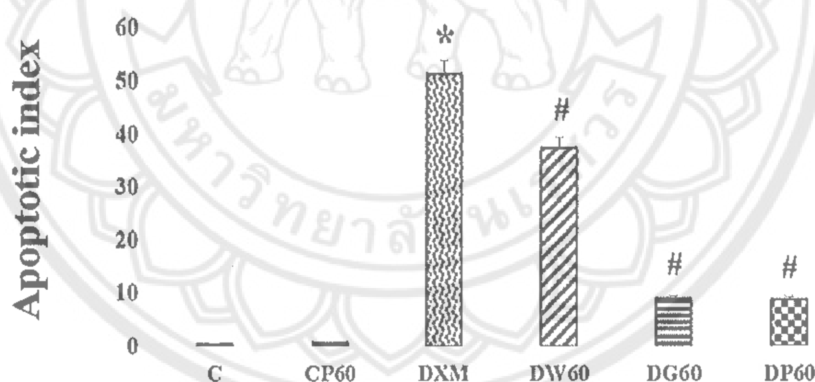


Figure 39 **Apoptotic index in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 60 days Data are presented as mean±SEM. * *p*<0.05 versus control group and $^{\#}$ *p*<0.05 versus DXM group**

Table 8 Apoptotic index in rat liver in all groups of control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP)

Groups	%Apoptotic index
Control	0.6212±0.0521
CP 15	0.6854±0.0134
CP 30	0.6489±0.0124
CP 60	0.7142±0.1145
DXM	51.4345±0.1494*
DW 15	44.2256±0.2179
DW 30	41.8017±0.2678#
DW 60	37.4476±0.2114#
DG 15	36.9221±0.1244#
DG 30	20.9207±0.0789#
DG 60	9.2170±0.0157#
DP 15	36.5344±0.1547#
DP 30	20.7046±0.0675#
DP 60	8.7740±0.0214#

Note: Data are expressed as mean ± SEM. Statistical significance is indicated as *p<0.05 versus control group, #p<0.05 versus DXM group

The effect of pre-germinated brown rice on cell proliferation in the rat liver toxicity induced by DXM.

The cell proliferation was evaluated by immunohistochemistry staining of PCNA (a marker of cell proliferation). The results showed Morphological changes of positive cell proliferation in all groups in rat treated for 60 days (Figure 40). The cell proliferation was presented in control and PGBR groups (Figure 40 A & C). In DXM and all withdrawal groups showed reduce cell proliferation (Figure 40 B & D). The cell proliferation was showed markedly increased in DXM treated with PGBR and GABA groups (Figure 40 E & F).

The cell proliferation showed an proliferation index (PI; percentage of PCNA-positive hepatocytes). The PI were found no significant difference in PGBR for 15 days, 30 days and 60 days when compared with control group (Figure 41). In 15 days DXM-treated group, it was significantly decreased in the PI when compared with control group (p=0.000), a significant decrease in the PI was found in 15 days withdrawal group when compared with control group (p=0.000). However, the 15 days GABA-treated and PGBR-treated groups showed a significant increase in the PI when compared with DXM group (p=0.000) (Figure 42).

The PI was found a significant increase in 30 days withdrawal group when compared with DXM group (p=0.000). Moreover, the 30 days GABA-treated and PGBR-treated groups showed a significant increase in the PI when compared with DXM group (p=0.000) (Figure 43).

In addition, the PI was showed significantly increased in 60 withdrawal group when compared with DXM group (p=0.000). Furthermore, the 60 days GABA-treated and PGBR-treated groups showed a significant increase in the PI when compared with DXM group (p=0.000) (Figure 44).

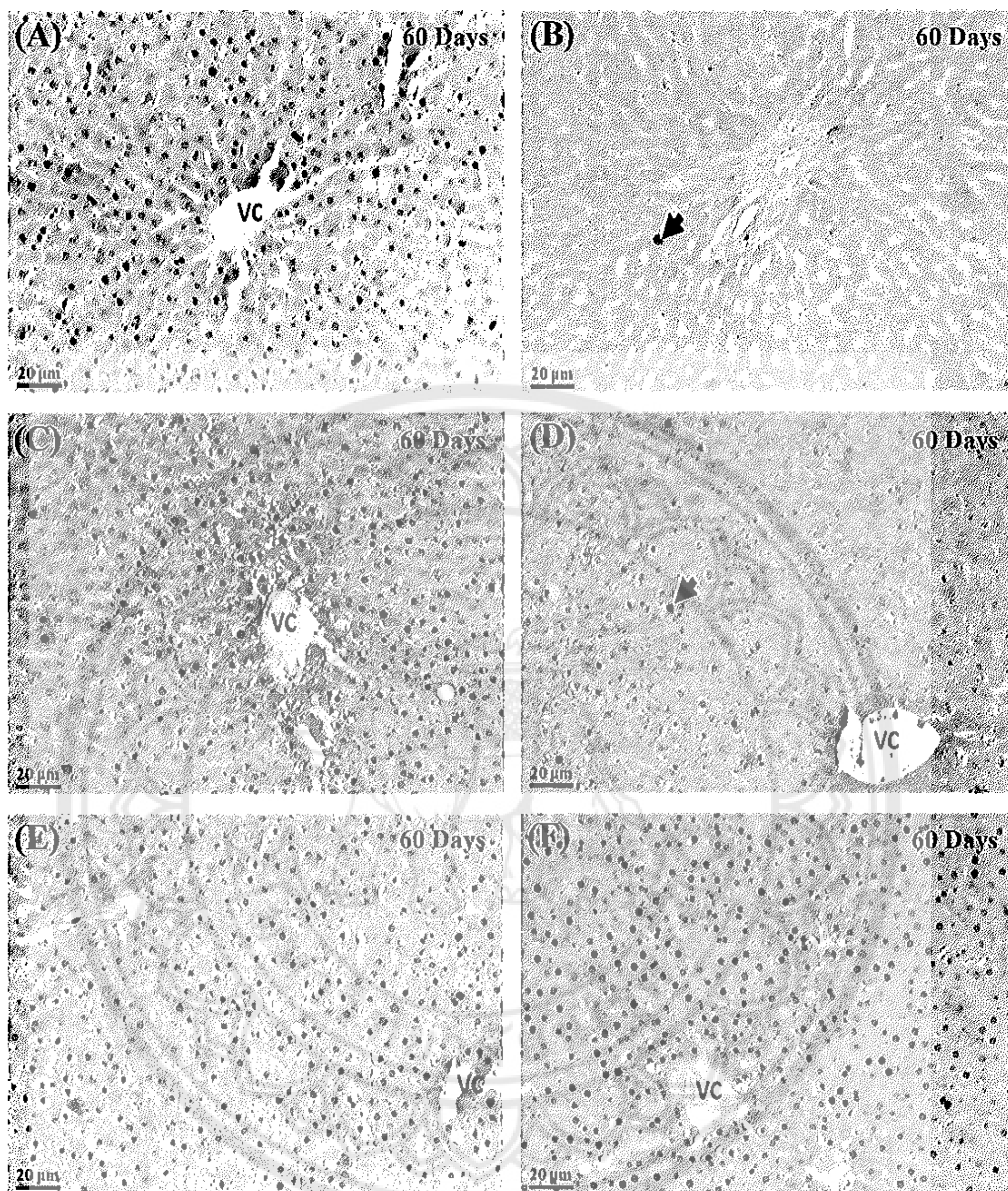


Figure 40 Morphological changes of cell proliferation in 60 days in all groups of rat liver were analyzed by Immunohistochemistry staining technique; C(A), DXM(B), CP(C), DW(D), DG(E) and DP(F) (↑: PCNA-positive cell).

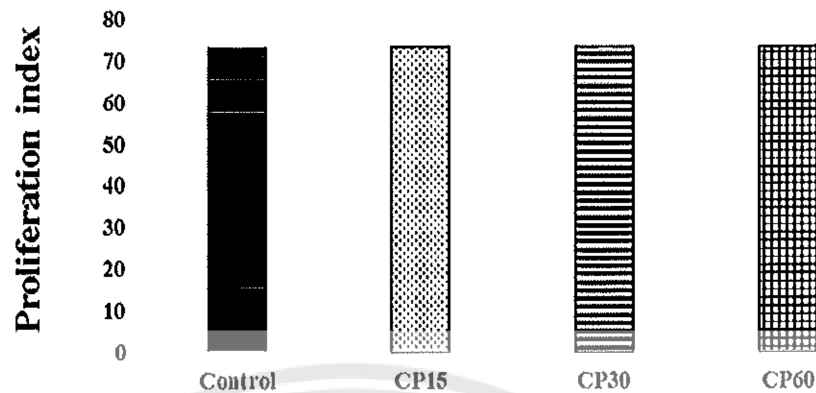


Figure 41 Proliferation index in control, PGBR (CP15), PGBR (CP30), PGBR (CP60) groups. Data are presented as mean±SEM.

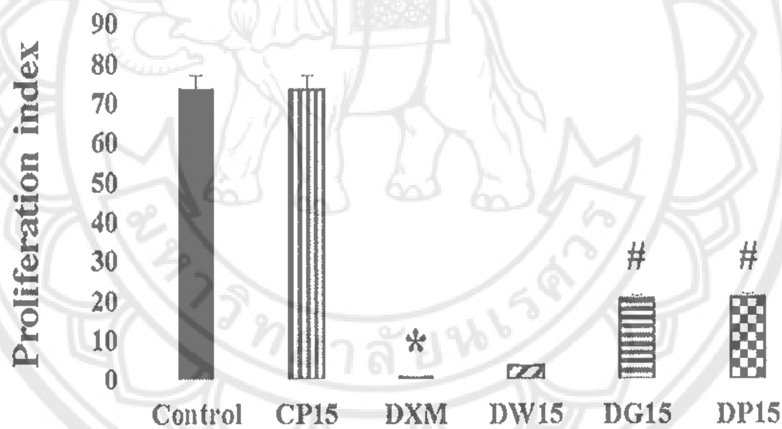


Figure 42 Proliferation index in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 15 days Data are presented as mean±SEM. * $p<0.05$ versus control group and # $p<0.05$ versus DXM group

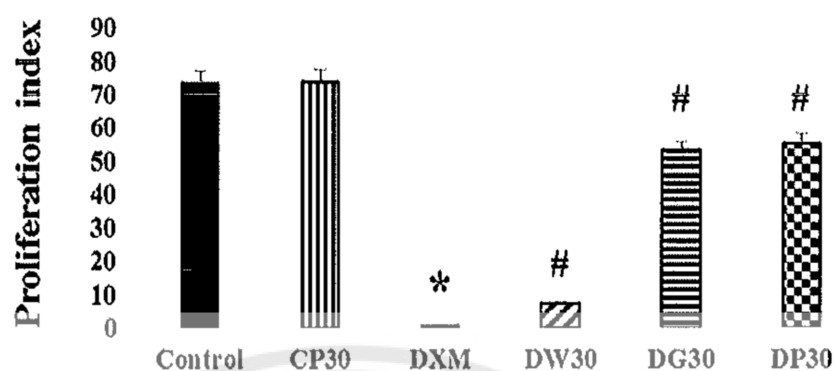


Figure 43 Proliferation index in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 30 days Data are presented as mean±SEM. $^{*}p<0.05$ versus control group and $^{\#}p<0.05$ versus DXM group

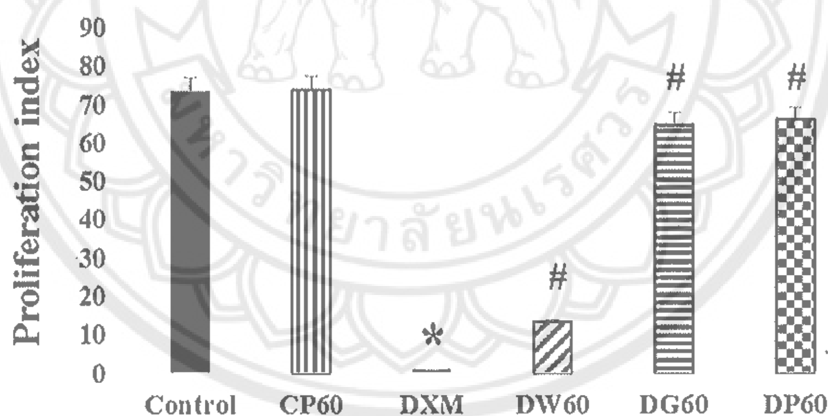


Figure 44 Proliferation index in control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP) for 60 days Data are presented as mean±SEM. $^{*}p<0.05$ versus control group and $^{\#}p<0.05$ versus DXM group

Table 9 Proliferation index in rat liver in all groups of control, PGBR (CP), DXM, DXM withdrawal (DW), DXM treated with GABA (DG) and DXM treated with PGBR (DP)

Groups	% Proliferation index
Control	72.2431±0.0124
CP 15	72.2124±0.0971
CP 30	72.8541±0.0642
CP 60	73.4673±0.0374
DXM	0.8647±0.1243*
DW 15	3.2510±0.1975
DW 30	7.2201±0.1664#
DW 60	13.2374±0.1287#
DG 15	20.2371±0.0417#
DG 30	53.2014±0.1491#
DG 60	64.4367±0.1746#
DP 15	20.6204±0.0042#
DP 30	55.2674±0.0073#
DP 60	65.8100±0.0541#

Note: Data are expressed as mean ± SEM. Statistical significance is indicated as *$p<0.05$ versus control group, #$p<0.05$ versus DXM group

CHAPTER V

DISCUSSION

Effect of DXM on cell morphology, cell apoptosis, cell proliferation, distribution of glycogen and protein intensity in hepatocytes

The use of DXM in high dosage and/or over long periods can cause oxidative stress. DXM is rapidly metabolized and detoxified by the liver that can release free radicals leading to cellular stress and cellular injury (Pfaff, et al., 1983; Larrey, et al., 1989; Liu, et al., 1999; Lobo, et al., 2010; Sanchez-Valle, et al., 2012). Consequently, cellular stress can destroy the balance of inflammatory cytokines (e.g., IL-12) and enhancing biochemical lesions that promote cell apoptosis (Lovell, et al., 1995; Tanaka, et al., 1996; Rowell, et al., 1997; Nanji, et al., 1999).

The present study illustrated that DXM had negative effects on rat liver by increasing cell apoptosis when compared with the control. The resulting of cell apoptosis was characterized by cellular shrinkage leading to sinusoidal dilatation, suggesting that DXM could induce cell apoptosis. The finding is consistent with the results from a previous study that found cell apoptosis makes morphology changes includes shrinkage and blebbing of cells (Gerlag, et al., 1985). A possible reason is that an increase of free radicals can cause protein cross-linking or protein fragment leading to the production of protein deficit, dysfunction of enzyme and/or dysfunction of the receptor (Freeman, et al., 1982; Awasthi, et al., 1984). This is consistent with this study which found reduced protein intensity in rats treated with DXM.Oxidatively damage protein products may contain very reactive groups that contribute to membrane damage and many cellular dysfunctions (Lobo, et al., 2010). The decreasing protein intensity in the cell cytoplasm could be attributed to the disruption of lysosomal membranes under the effect of various toxicants leading to the liberation of their hydrolytic enzymes in the cytoplasm resulting in marked lysis and the dissolution of the target material (Awasthi, et al., 1984).

Glycogen granules were observed by PAS staining that depending on the intensity of the colored compound formed which is directly proportional to the glycogen in the cell (Waer, et al., 2012). In the results of the present study, distribution of glycogen had a significant decrease in rats treated with DXM, that is consistent with the amounts of glycogen in the liver have decreased in toxicity rat. (Cattley, et al., 2002). Moreover, previous studies have found that free radicals can cause cellular injury (Sanchez-Valle, et al., 2012). Losing of cytoplasmic content occurred in cellular injury may reflect to vacuolization in the hepatic cells that found in rats treated with DXM. This is consistent with CCl_4-induced injury to hepatocytes resulting present cytoplasmic vacuolation of hepatocytes (Nayak, et al., 1996) and loss of cytoplasmic glycogen (Bannasch, et al., 1980; Recknagel, et al., 1973).

Furthermore, there has been reported that reactive oxygen species (ROS) or free radicals may influence liver regeneration (Albright, et al., 2003; Beyer, et al., 2008). ROS mediate cell growth arrest and activate proteins inhibiting cell cycle (Barnouin, et al., 2002; Horimoto, et al., 2004). Therefore, ROS production in the liver may play a key role in the negative control of regeneration. Therefore, a decrease of cell proliferation following DXM treatment may result from DNA fragment from cell apoptosis which can inhibit cell proliferation (Cao, et al., 2014).

Effect of DXM withdrawal on cell morphology, cell apoptosis, cell proliferation, distribution of glycogen and protein intensity in hepatocytes

After oral administration, DXM is quickly absorbed in the gastrointestinal tract within 2-2.5 hours (Hammamet, et al., 2012). The antitussive activity of DXM lasts for approximately 5-6 hours with a plasma half-live of 2-4 hours (Pender, et al., 1991; Shaul, et al., 1997). The cytotoxic effects of DXM depend on dose and tolerance. In this study used DXM at dose 30 mg/kg/day for 15 days that suggested dose in adult human (Bem, et al., 1992). DXM is metabolized to dextrophan by the liver (Pfaff, et al., 1983; Larrey, et al., 1989). DXM is eliminated via the urine (Pender, et al., 1991; Shaul, et al., 1997). Consequently,The DXM withdrawal group reduced DXM metabolize leading to reduced free radicals in the cells. The present study illustrated that DXM withdrawal can slightly recover the liver from hepatotoxicity. This is

consistent with a previous study found morphological changes of seminiferous tubules has showed a significant increase in rats treated with DXM. Moreover, DXM induce DNA fragmentation in human sperm (Safarinejad, 2008; Tanrikut, et al., 2010). Therefore, DXM may cause cellular toxicity in the liver as well. However, It is well known that hepatocytes can recovery after injury. The regenerative capacity of the liver is typically triggered by hepatic injury, including partial hepatectomy (Bucher, 1963; Bucher, et al., 1984; Nagasue, et al., 1987).

These results suggested that pathological changes and cellular dysfunctions can recover followed duration of withdrawal.

Effect of GABA on cell morphology, cell apoptosis, cell proliferation, distribution of glycogen and protein intensity in hepatocytes

Antioxidation function in animal body requires the participation of the antioxidation enzyme system, which includes glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) (Hu, et al., 2008). GSH-Px, an important antioxidat enzyme in the body (Wei, et al., 2011), specifically catalyzes reduced glutathione (GSH) to clear H_2O_2 and reduce the generation of lipid peroxides, thus protecting the structure and functions of cell membrane (Pompella, et al., 2003; Ahmad, et al., 2012). In addiction, GSH can prevent damage to important cellular components caused by reactive oxygen species such as free radicals (Pompella, et al., 2003). There has been previous study found that the increasing of GABA can promote the glutamate level in the body that as the raw material for the synthesis of glutathione (GSH) in the antioxidation system(Chen, et al., 2013). Moreover, the result from Castelli's report has found GABAB receptor expression in the hepatocyte (Castelli, et al., 1999). GABAB receptor plays a role to phosphorylation in the epidermal growth factor receptor (EGFR) (Biju, et al., 2002; Watanabe, et al., 2006). EGFR downstream signaling proteins initiate several signal transduction cascades, principally the MAPK, Akt and JNK pathways, leading to DNA synthesis and cell proliferation (Oda, et al., 2005; Garay, et al., 2015). The present study illustrated that oral administration of GABA was greatly increased cell proliferation, distribution of glycogen and protein intensity in the hepatocyte. This is consistent with a previous study showed that

GABA has protective against the cytotoxicity of ethanol in isolated rat hepatocytes (Norikura, et al., 2006). GABA induced liver cell proliferation which has therapeutic significance in liver disease (Shilpa, et al., 2013) and also improved antioxidation enzyme activity in intestinal tract (Huang, 2011).

As mentioned earlier, EGFR can induced DNA synthesis and cell proliferation. On the other hand, EGFR can inhibited pro-apoptotic protein leading to reduced cell apoptosis (Leary, et al., 2013; Bhatt, et al., 2013). These findings are in line with the present study have found that oral administration of GABA was greatly reduced pathological changes in the livers. Furthermore, GABA was markedly decreased the number of apoptotic cells, percentage of sinusoidal dilatation and vacuolization in the rats liver.

Therefore, GABA has recoverable effects on pathological changes, cell apoptosis and promote cell proliferation, distribution of glycogen and protein content in hepatocytes. The alterations of cellular dysfunction including, cell pathology, cell apoptosis, cell proliferation, distribution glycogen and protein intensity were ameliorated by GABA.

Effect of PGBR on cell morphology, cell apoptosis, cell proliferation, distribution of glycogen and protein intensity in hepatocytes

A number of studies have demonstrated that antioxidant supplements may be an excellent prevention strategy for many diseases, including liver injury, liver fibrosis, aging, cancer and diabetes (Anandakumar, et al., 2008; Ekambaram, et al., 2008; Ismail, et al., 2009; Rodrigo, et al., 2007; Wu, et al., 2008). The antioxidants can eliminate free radicals and protect the liver from oxidative stress (Augustyniak, et al., 2005; Fu, et al., 2011; Guo, et al., 2012; Bourogaa, et al., 2013; Deng, et al., 2013; Li, et al., 2014). In recent years, many foods and plants have abundant natural antioxidants and possess the ability of eliminating free radicals and protecting the liver from oxidative stress (Augustyniak, et al., 2005). These natural products, many bioactive compounds have been investigated for their role in eliminating oxidative stress, such as L-theanine, vitamin E (α-tocopherol), N-acetyl cysteine, raxofelast and betaine (Li, et al., 2012). Vitamin E could restore the redox status that prevented

oxidative stress and reduced cell apoptosis caused by ethanol-induced hepatic oxidative injury (Kaur, et al., 2010). Vitamin E had the ability to inhibited lipid peroxidation in mice exposed to ethanol (Altavilla, et al., 2005). Moreover, PGBR has antioxidant properties and many bioactive compounds such as γ-oryzanol and vitamin E that can reduced free radicals leading to reduced pathology of the liver (Ampornkul, et al., 2013). Both γ-oryzanol and vitamin E can prevented lipid peroxidation of cell membrane (Arlas, et al., 2008 and Akiyama, 1999). The present study provided the evidence that oral administration of PGBR reduced pathological changes caused by DXM. This is consistent with a previous study found PGBR may prevent CCl_4-induced liver oxidative stress and injury through enhancement of the antioxidant capacities, which may be due to complex actions of various bioactive compounds, including phenolic acids, γ-oryzanol, vitamin E, and GABA (Wunjuntuk, et al., 2015). Moreover, PGBR has high amount of GABA that can increase the activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase. The present study illustrated that GABA was greatly increased hepatocyte proliferation, distribution of glycogen, protein content and markedly decreased the number of apoptotic cells, percentage of sinusoidal dilatation and vacuolization. This is consistent with the results from a previous study that found GABA can induced cell proliferation in liver disease (Shilpa, et al., 2013). Consequently, The liver recovery after PGBR administration may results from antioxidants and GABA. The antioxidants may stimulated hepatocytes to shift from G0 to G1 phase in the cell cycle (Huang, et al., 1998). Furthermore, prolonged treatment of PGBR showed no effects on the rat liver.

These results demonstrated that PGBR had recoverable effects on pathological changes, cell apoptosis and promote cell proliferation, distribution of glycogen and protein intensity in hepatocytes. Therefore, PGBR could be developed as a safe alternative product for treatment hepatotoxicity caused by DXM.

CHAPTER VI

CONCLUSION

In summary, the present study showed that dextromethorphan (DXM) can induce morphological changes of the liver, cell apoptosis, increase in the percentage of sinusoidal dilatation and vacuolization. Whereas, reduce cell proliferation, distribution of glycogen and protein content. Moreover, DXM withdrawal can slightly reverse the liver toxicity in 30 and 60 days withdrawal. These results suggested that free radicals occur during the detoxification process induces oxidative stress, cause liver injury.

The present study showed that DXM treated with PGBR and DXM treated with GABA can reverse the toxic effects of DXM on the liver resulting in reducing hepatic vascular degeneration, sinusoid expansion and decreased cell apoptosis. Moreover, PGBR and GABA can promote liver tissue recovery by increased living cell, cellular activities (including; accumulation of glycogen and protein content) and cell proliferation. These results suggested that PGBR which containing high amount of GABA and antioxidants may contribute to its ability to reverse the hepatotoxic effects of a drug of abuse. In addition, prolonged period of PGBR administration showed no adverse effect on the liver.

Therefore, PGBR can enhance a recovery of the morphological changes of the liver, cell apoptosis, cell proliferation, distribution of glycogen and protein content to normality in rats, received DXM.

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APPENDIX

APPENDIX A DOCUMENTS OF THE PROJECT

เอกสารรับรองโครงการ

คณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์ มหาวิทยาลัยนเรศวร

ชื่อโครงการ	ผลของข้าวกล้องงอกต่อเซลล์อะพอพโทซิสและการแบ่งเซลล์ในตับหนูที่ถูกเหนี่ยวนำให้เกิดพยาธิสภาพด้วยเด็กโตรเมโธเฟน Effect of Pre-germinated Brown Rice on Cell Apoptosis and Cell Proliferation in the Toxicity Rat Liver which Induced by Dextromethorphan
เลขที่โครงการ	NU-AEE590304
เลขที่เอกสารรับรอง	59 02 004
ประเภทการรับรอง	ยกเว้น(1)
ชื่อหัวหน้าโครงการ/ผู้ยื่นขอฯ	ดร.อิทธิพล พวงเพชร
สังกัดหน่วยงาน /คณะ	วิทยาศาสตร์การแพทย์
วันที่รับรอง	23 พฤษภาคม 2559
วันสิ้นสุดการรับรอง	23 พฤษภาคม 2562

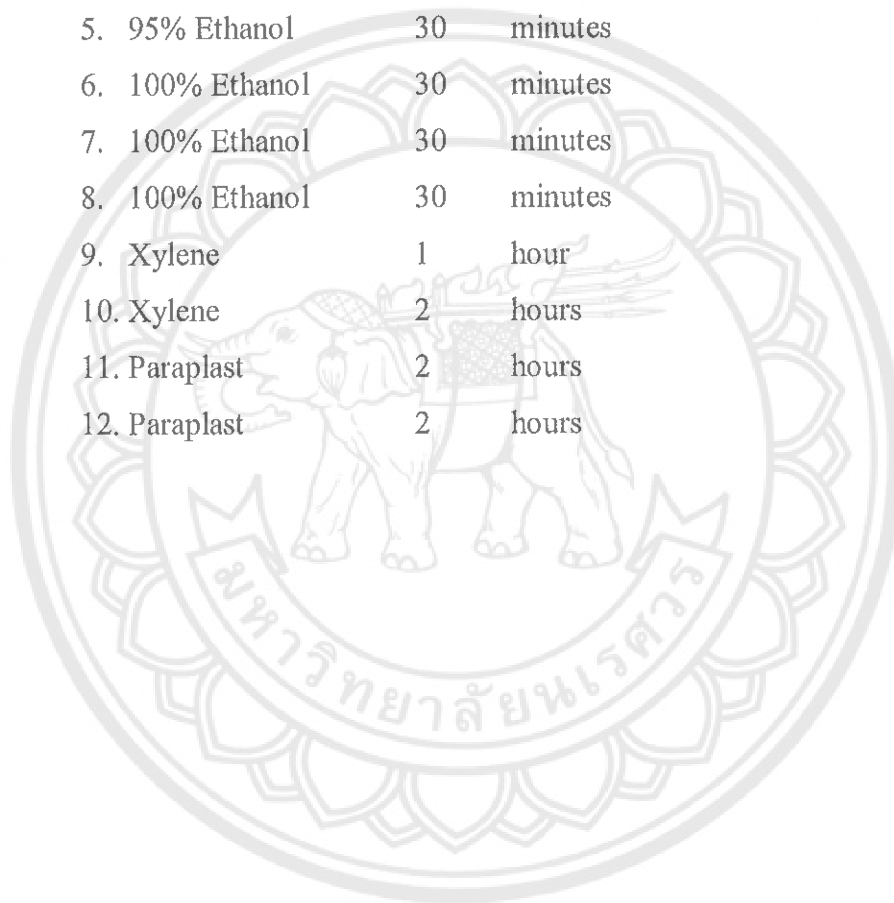
ขอรับรองว่าโครงการวิจัยนี้ ได้รับการรับรองด้านจรรยาบรรณการใช้สัตว์
จากคณะกรรมการกำกับการเลี้ยงและการใช้สัตว์ มหาวิทยาลัยนเรศวร

....
(รองศาสตราจารย์ ดร.รัตติมา จีนาพงษา)
ประธานคณะกรรมการกำกับการเลี้ยงและการใช้สัตว์
มหาวิทยาลัยนเรศวร

....
(ผู้ช่วยศาสตราจารย์ ดร.ภูพงษ์ พงษ์เจริญ)
รองอธิการบดีฝ่ายวิจัย
มหาวิทยาลัยนเรศวร

APPENDIX B PROTOCAL AND LIST OF SOLUTION IN TISSUE PROCESSING

No.	Solution	Time	Unit
1.	70% Ethanol	30	minutes
2.	80% Ethanol	30	minutes
3.	90% Ethanol	30	minutes
4.	95% Ethanol	30	minutes
5.	95% Ethanol	30	minutes
6.	100% Ethanol	30	minutes
7.	100% Ethanol	30	minutes
8.	100% Ethanol	30	minutes
9.	Xylene	1	hour
10.	Xylene	2	hours
11.	Paraplast	2	hours
12.	Paraplast	2	hours



APPENDIX C PROTOCAL AND LIST OF SOLUTION AND BUFFER IN HEMATOXYLIN AND EOSIN STAINING TECHNIQUE

1. Rehydration and De-paraffin
    1) Xylene 5 minutes
    2) Xylene 5 minutes
    3) Xylene 5 minutes
    4) 100% Ethanol 3 minutes
    5) 100% Ethanol 3 minutes
    6) 95% Ethanol 3 minutes
    7) 70% Ethanol 3 minutes
    8) Distilled water 3 minutes

2. Hematoxylin 5 minutes

3. Tap water 5 minutes

4. Eosin 2 minutes

5. Dehydration
    1) 70% Ethanol 3 minutes
    2) 95% Ethanol 3 minutes
    3) 95% Ethanol 3 minutes
    4) 100% Ethanol 3 minutes
    5) 100% Ethanol 3 minutes
    6) Xylene 5 minutes
    7) Xylene 5 minutes
    8) Xylene 5 minutes

6. Mounted with mounting media and closed with cover glass

APPENDIX D PROTOCAL AND LIST OF SOLUTION AND BUFFER IN GLYCOGEN STAINING

1. Rehydration and De-paraffin
    1) Xylene 5 minutes
    2) Xylene 5 minutes
    3) Xylene 5 minutes
    4) 100% Ethanol 3 minutes
    5) 100% Ethanol 3 minutes
    6) 95% Ethanol 3 minutes
    7) 70% Ethanol 3 minutes
    8) Distilled water 3 minutes

2. 1% periodic acid 10 minutes

3. Distilled water 1 minutes

4. Coleman's Feulgen solution 30 minutes

5. Dehydration
    1) 70% Ethanol 3 minutes
    2) 95% Ethanol 3 minutes
    3) 95% Ethanol 3 minutes
    4) 100% Ethanol 3 minutes
    5) 100% Ethanol 3 minutes
    6) Xylene 5 minutes
    7) Xylene 5 minutes
    8) Xylene 5 minutes

6. Mounted with mounting media and closed with cover glass

APPENDIX E PROTOCAL AND LIST OF SOLUTION AND BUFFER IN PROTEIN STAINING

1. Rehydration and De-paraffin

1)	Xylene	5	minutes
2)	Xylene	5	minutes
3)	Xylene	5	minutes
4)	100% Ethanol	3	minutes
5)	100% Ethanol	3	minutes
6)	95% Ethanol	3	minutes
7)	70% Ethanol	3	minutes
8)	Distilled water	3	minutes

2. Bromophenol blue 5 minutes

3. Distilled water 5 minutes

4. Dehydration

1)	70% Ethanol	3	minutes
2)	95% Ethanol	3	minutes
3)	95% Ethanol	3	minutes
4)	100% Ethanol	3	minutes
5)	100% Ethanol	3	minutes
6)	Xylene	5	minutes
7)	Xylene	5	minutes
8)	Xylene	5	minutes

5. Mounted with mounting media and closed with cover glass

APPENDIX F PROTOCAL AND LIST OF SOLUTION AND BUFFER IN TUNEL assay TECHNIQUE

1. Rehydration and De-paraffin
   1) Xylene 5 minutes
   2) Xylene 5 minutes
   3) Xylene 5 minutes
   4) 100% Ethanol 3 minutes
   5) 100% Ethanol 3 minutes
   6) 95% Ethanol 3 minutes
   7) 70% Ethanol 3 minutes
   8) Distilled water 3 minutes

2. 20 mg/ml proteinase K for 30 minutes at 37oC
   Washed with 1X PBS 5 minutes for 3 times

3. Block endogenous peroxidase
   1) 1X PBS
   2) 10% Methanol
   3) 30% H_2O_2
   4) 0.1% Triton-X

   Incubated 15 minutes at room temperature
   Washed with 1X PBS 5 minutes for 3 times

4. Equilibration buffer 20 minutes

5. Terminal deoxynucleotidyl transferase enzyme (TdT) in a humidified chamber at 37°C for 2 hours
   Washed with 1X PBS 5 minutes for 3 times

6. Stop / wash buffer at room temperature for 10 minutes

7. Anti-digoxigenin incubated 2 hours at room temperature
   Washed with 1X PBS 5 minutes for 3 times

8. Chromogen by DAB (3,3'-Diaminobenzidine)
   Incubated in dark chamber 3 minutes at room temperature
   Stop reaction with distilled water for 5 minutes

9. Counterstained with hematoxylin for 3 seconds

10. Dehydration

1)	70% Ethanol	3	minutes
2)	95% Ethanol	3	minutes
3)	95% Ethanol	3	minutes
4)	100% Ethanol	3	minutes
5)	100% Ethanol	3	minutes
6)	Xylene	5	minutes
7)	Xylene	5	minutes
8)	Xylene	5	minutes

11. Mounted with mounting media and closed with cover glass

APPENDIX G PROTOCAL AND LIST OF SOLUTION AND BUFFER IN IMMUNO-HISTOCHEMISTRY TECHNIQUE

1. Rehydration and De-paraffin

1) Xylene	5	minutes
2) Xylene	5	minutes
3) Xylene	5	minutes
4) 100% Ethanol	3	minutes
5) 100% Ethanol	3	minutes
6) 95% Ethanol	3	minutes
7) 70% Ethanol	3	minutes
8) Distilled water	3	minutes

2. Retrieve antigen with 1X PBS by 70P microwave 5 minutes for 3 times

   Washed with 1X PBS 5 minutes for 3 times

3. Block endogenous peroxidase

   5) 1X PBS
   6) 10% Methanol
   7) 0.3% H_2O_2
   8) 1% Triton-X

   Incubated 30 minutes at room temperature

   Washed with 1X PBS 5 minutes for 3 times

4. Block non-specific protein (1% Normal Goat Serum)

   1) 1X PBS
   2) Normal Goat Serum

   Incubated 2 hours at room temperature

5. Primary antibody; Rabbit polyclonal anti-proliferating cell nuclear antigen antibody (PCNA) 1:200, Incubated 12 hours at 4°C followed overnight
    1) 1X PBS
    2) Normal Goat Serum
    3) Anti-PCNA

   Washed with 1X PBS 5 minutes for 3 times

6. Secondary antibody; HRP-conjugated goat anti-rabbit immunoglobulin G 1:200, Incubated 2 hours at room temperature
    1) 1X PBS
    2) Normal Goat Serum
    3) HRP-conjugated goat anti-rabbit immunoglobulin G

   Washed with 1X PBS 5 minutes for 3 times

7. Chromogen by DAB (3,3'-Diaminobenzidine)

   Incubated in dark chamber 5 minutes at room temperature

   Stop reaction with distilled water for 5 minutes

8. Counterstained with hematoxylin for 3 seconds

9. Dehydration

1) 70% Ethanol	3	minutes
2) 95% Ethanol	3	minutes
3) 95% Ethanol	3	minutes
4) 100% Ethanol	3	minutes
5) 100% Ethanol	3	minutes
6) Xylene	5	minutes
7) Xylene	5	minutes
8) Xylene	5	minutes

10. Mounted with mounting media and closed with cover glass