行政院國家科學委員會補助專題研究計畫成果報告

脂肪肝對部份肝臟切除後肝細胞凋亡及再生的影響

THE EFFECTS OF FATTY LIVER ON HEPATOCYTE APOPTOSIS AND LIVER REGENERATION AFTER PARTIAL HEPATECTOMY

主持人：賴鴻緒

計畫類別：個別型計畫

計畫編號：NSC 90-2314-B-002-321

執行期限：90 年 8 月 1 日至 91 年 7 月 31 日

本成果報告包括以下應繳交之附件：

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執行單位：台大醫學院　外科

中　華　民　國　91 年　8 月　　日

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中文摘要

脂肪肝由於肝細胞內堆積過多脂肪，細胞質內許多酵素與化學反應無法正常運作，肝功能因此變差。脂肪肝常因營養不均衡，脂肪攝取太多而引起。食物中增加纖維素含量，可促進膽汁分泌增加，進而幫助脂肪之代謝。然而，脂肪肝病患能否借低脂肪飲食，或高纖維飲食的攝取而恢復，目前尚未有定論。國內台灣地區脂肪肝患者已超過 35% 。而台灣地區十大死因之首為惡性腫瘤，其中肝癌在男性占第一位，女性占第二位；慢性肝病及肝硬化，也是十大死因之第六名，這些病患在開刀等治療過程中，若加上脂肪肝因素，預後將更不良，對國人健康威脅相當大。

細胞凋亡是一種階段式細胞死亡，亦為細胞死亡前型態學之改變，包括了細胞萎縮、細胞核凝結、細胞膜起泡及細胞吞噬現象等。至少有 10 種蛋白質參與細胞凋亡過程。許多研究均針對如何刺激及促進肝細胞再生的速度以維持肝細胞功能，然而只有少數文獻注意到肝細胞凋亡與肝細胞再生之相關性。至於脂肪肝病患在肝損傷後，肝細胞凋亡與肝細胞再生的變化，則目前尚未明瞭。

本計劃以重約 200 公克之 Wistar 雄性大鼠做實驗，先以高脂肪食物攝取 4 週引發脂肪肝，並另分組，以再攝取 4 週低脂肪及高纖維飲食方式，觀察脂肪肝是否為可逆性。然後將各組大鼠分別施行百分之七十部份肝臟切除術，並於術後 6、24、48、72 小時犧牲取樣，測定(1)脂肪肝各項指標；(2)肝細胞經由 agarose 測定細胞凋亡數目；(3)肝細胞 in situ DNA fragmentation (TUNEL)，做為肝細胞凋亡指標；(4)肝細胞內 5-Bromo-2-Deoxyuridine (BrdU)活性；(5)肝細胞內 Ornithine decarboxylase 含量作為肝細胞再生指標。結果發現(1)高脂肪飲食可引發明顯脂肪肝現象；(2)脂肪肝在肝臟部分切除後細胞凋亡數目明顯增加($p<0.05$)；(3) 脂肪肝在肝臟部分切除後肝細胞再生能力(BrdU 及 ODC 含量)明顯較差($p<0.05$)；(4)恢復正常飲食後，細胞凋亡數目及肝細胞再生能力均可稍為恢復；(5)高纖維飲食可使細胞凋亡數目及肝細胞再生能力有意義恢復($p<0.05$)。

關鍵詞：脂肪肝，肝細胞凋亡，肝細胞再生，肝臟部份切除

ABSTRACT

Fatty liver could deter the liver function through the interfere of enzyme activity and biochemical reaction in cytoplasm of hepatocytes due to deposition of much lipid droplets. Fatty liver may occur from unbalant nutritional intake, especially high fat diet feeding. High fiber diet can stimulate the bile flow and then increase the metabolism of fat. However, whether fatty liver can be reversed by low fat diet and high fiber diet is still be conversial. The incidence of fatty liver is more than 35% of population in Taiwan, which highly threaten the health of Taiwanese. Malignancy is number one disease of top ten causes of death in Taiwan. Hepatoma is the first in male and second in female patients. Chronic hepatitis is the

sixth of top ten causes of death. The prognosis would be worse if fatty liver is combined with these patients.

Apoptosis (programmed cell death) is a morphology of cell death characterized by shrinkage, nuclear condensation, membrane blebbing, and membrane changes that lead to phagocytosis of the affected cell. Apoptosis and cell proliferation are also complementary and account for the maintenance, growth or involution of liver tissue. Many papers concerned their focus in how to stimulate and increase the degree of liver regeneration. However, only few reports could be reviewed about the relationship between apoptosis and liver regeneration. As for the effect of fatty liver on apoptosis and regeneration is still be unknown.

The main purpose of this project is to find out the effect of fatty liver on the hepatocytes apoptosis and liver regeneration after partial hepatectomy. Male Wistar rats around 200g were used as subject. Fatty liver was induced by high fat diet feeding for 4 weeks. The reversibility by low fat diet or high fiber diet feeding for another 4 weeks will be observed. Partial hepatectomy (around 70%) will be performed and they will be sacrificed at 6 、 24 、 48 and 72 hours after hepatectomy. We measured: (1) indicators of fatty liver; (2) cell death through agarose and gel electrophoresis of hepatocyte; (3) in situ cell death detection (TUNEL) of DNA fragmentation as indicators of apoptosis; (4) 5-Bromo-2-Deoxyuridine (BrdU) activities; (5) ornithine decarboxylase contents in remnant liver as markers of regeneration. The results were: (1) high fat diet could induce fatty liver;(2) the apoptotic hepatocytes increased in fatty liver after partial hepatectomy ($p<0.05$) ; (3) the liver regeneration capacity (BrdU and ODC contents) decreased significantly in fatty liver after partial hepatectomy ($p<0.05$) ; (4) normal diet could mildly recover the apoptotic situation and regeneration capacity ; (5) high fiber diet could significantly recover the apoptotic number and improved the liver regenerating capacity ($p<0.05$).

Key words: fatty liver 、 apoptosis 、 liver regeneration 、 partial hepatectomy

INTRODUCTION

Fatty liver may occur due to lacking lipotropic factors, injury by hepototoxic agents, or too much intake of fatty acid[1]. In Taiwan area, fatty liver occurred in more than 35% of population because of obesity, hyperlipidemia, diabetes mellitus, drug (eg.steroid) abuse, and unbalance of diet intake[2]. Because of the high incidence of hepatitis B carrier and so as to many hepatoma patients, the mortality rate of hepatic disease (including hepatoma cases) is one of the tope ten causes of death in Taiwan. If the patient combined with fatty liver, the treating course will be more complicated, and the prognosis may became worse.

Apoptosis (programmed cell death) is a morphology of cell death characterized by shrinkage, nuclear condensation, membrane blebbing, and membrane changes that lead to phagocytosis of the affected cell. Apoptosis is a common form of cell death that commonly occurs in normal healthy adult tissue and under both physiological and pathological conditions. Apoptosis and cell proliferation are complementary and account for the maintenance, growth or involution of a tissue[3]. However, only few studies about apoptosis and liver regeneration were reported. It is believed that regulation of apoptosis should plays an important role in hepatic cell regeneration. We have performed serial studies about the DNA synthetic rate, mitosis situation of remnant hepatocytes, nutritional and hormonal factors, immunological responses which affecting the regenerating liver after partial hepatectomy in rats. Lee PH et al also did the study about apoptosis in the ischemic liver. However, the role of apoptosis and liver regeneration in fatty liver is still not clear. The purpose for this study is to find out the effects of fatty liver on hepatocyte apoptosis and liver regeneration after partial hepatectomy.

MATERIALS AND METHODS

(1) Animals:

Male Wistar rats (purchased from Charles River, Osaka, Japan) weighing about 200 g will be used as subjects. All the rats (N=288) will be randomly assigned to the following different groups.

(2) Grouping:

A. Evaluation of fatty liver situation:

Group A1 (N=8), Feeding with normal diet (AIN-93G) for 4 weeks as control group (C)

Group A2 (N=8), Feeding with high fat diet for 4 weeks (HF)

Group A3 (N=8), Feeding with high fat diet for 4 weeks, then transfered to general diet (FN)

Group A4 (N=8), Feeding with high fat diet for 4 weeks then transfered to high fiber diet (Ff)

Composition of various diets are as Table 1:

Table 1. Composition of Various Diets in Weight

Contents\Diets	Normal	High Fat	High Fiber
Carbohydrate	61%	53%	55%
Protein	20%	20%	20%
Fat	7%	16%	7%
Fiber	0%	0%	10%
Vitamin	1%	1%	1%
Mineral	3.5%	3.5%	3.5%
Others	7.5%	6.5%	3.5%
Total	100%	100%	100%

*Normal diet is AIN-93G Diet

**Fat or fiber were added in AIN-93G Diet as High Fat Diet or High Fiber Diet

B. Effect of fatty liver on hepatocytes apoptosis and regeneration:

Group C (N=32), Partial hepatectomy done on normal (AIN-93G) diet feeding rats.

Group HF (N=32), Partial hepatectomy done on HF diet feeding rats.

Group FN (N=32), Partial hepatectomy done on FN diet feeding rats.

Group Ff (N=32), Partial hepatectomy done on Ff diet feeding rats.

Each group is divided in to four subgroups (N=8 in each): sacrificed on 6、24、48 and 72 hour after partial hepatectomy. Then indicators of apoptosis, including isolation of DNA and gel electrophoresis, in situ assay for DNA fragmentation (TUNEL), BrdU Index, and ODC content were measured.

(3) Surgical Procedures

All rats are anesthetized by intraperitoneal pentobarbital (10mg/Kg) injection. A midline laparotomy was performed. Partial hepatectomy was then carried out by means of aseptic extirpation of the median and left lateral lobes, according to the procedure of Higgins and Anderson[4]. The removed liver sample was immediately weighed. All of the surgeries were performed only between 8 am and 11 am to reduce the influence of diurnal variation. Eight rats from each group were sacrificed at 6、24、48 and 72 hours after the operations, and the livers were removed immediately.

(4) Measurements

A. Evaluation of fatty liver situation:

(a) Weighing the liver immediately after sacrifice, and the ratio of liver weight/body weight was calculated.

(b) Observation of liver color

(c) Observating the sharp degree of liver edge

(d) Histological examination of liver

B. The degree of apoptosis

The liver was divided into two parts, one stores in 10% neutral buffered formalin for in situ assay for DNA fragmentation, the other is frozend for DNA fragmentation and gel electrophoresis. In situ cell death detection by TUNEL was also measured for apoptotic number counting[5,6].

C. 5-Bromo-2-Deoxyuridine (BrdU) Incorporated Hepatocytes

Small pieces of liver tissue for histopathological examination were fixed in 10 % neutral formalin, embedded in paraffin and sectioned. Then the BrdU incorporated hepatocytes were detected by immunocytochemical system[7] for monitoring cell proliferation using monoclonal anti-BrdU cell proliferation Kit (Code RPN20, Amersham International plc., Amersham, UK). The BrdU incorporated index were calculated by counting the numbers of BrdU incorporated hepatocytes in 50 randomly-selected fields statistics under magnification×400.

D. Ornithine Decarboxylase (ODC) Contents in Remnant Liver

The ODC contents were measured by ELISA Kit(ODC Ab-1, Clone MP16-2, # MS-464-PO, Purified, Neomarkers, CA, U.S.A.). Briefly, the remnant livers were cut in pieces. Then they were homogenized and centrifuged at 105.000g for 1h at 4 ℃ with a Beckman model L5-75 ultracentrifuge. The supernatant fractions were obtained and ODC activities were measured by the method described by Schipper et al [8]

Statistical analysis

All experimental data were expressed as mean ±SD. The significance of difference among all groups was analyzed using one-way ANOVA. A p value of less than 0.05 was considered statistically significant.

RESULTS

All rats survived after all kinds of diets and lived well after partial hepatectomy during the experimental periods. Fatty liver with yellowish colored and greezy appearance observed in group HF rats. The liver margine became round in group HF instead of sharp in group C rats. The changes of liver weight/ body weight ratio could be seen in Fig 1. The ratio increased markedly after Hf diet for 4 weeks. It continued increased at 8 weeks stage, but decreased in FN and Ff groups rats. In situ apoptosis measured by TUNEL of control group rats could be seen in Fig 2. The apoptotic number after partial hepatectomy in each group rats were shown in Table 2. The apoptotic number increase markedly at 6h after partial hepatectomy. The increased numbers were significantly more in group HF、FN and Ff rats before and at 6、24、48、72h after partial hepatectomy ($p<0.05$). When comparing with group HF rats, the number decreased significantly at 6、24、48h stages in groups FN and Ff rats. The BrdU incorporated index was detected at the mitotic stage as shown in Table 3. The index number were almost zero preoperative, but increased at 6h and ever more at 24 stage, the decreased gradually at 48h and 72h stages. The index were significantly less in all experimental groups rats. However, the decrease of BrdU index in group HF rats was significantly more when compare with groups FN and Ff rats ($p<0.05$). The ornithine decarboxylase contents in the remnant liver increased significantly from 255± 26 mg/dL preoperatively to 656± 46, 604± 48, 460± 42 and 399± 40 mg/dL at 6h、24h、48h and 72h, respectively, after partial hepatectomy in group C rats as shown in Table 4. The increase were significantly lower in experimental groups rats. When comparing with group HF rats, the ODC contents were higher in groups FN and Ff rats significantly at 6h and 48h after partial hepatectomy ($p<0.05$).

DISCUSSION

Liver is a core organ in our body. Although it contains good regeneration ability, hepatic failure can still occur after massive hepatic injury or hepatectomy, especially in fatty liver. The capacity of the mammalian liver to regenerate in response to numerous stimuli has been well documented[9-11]. Although there are much controversy

continues on the initiation, regulation, metabolic changes, and termination of liver regeneration after partial hepatectomy that well initiate proliferation of the remaining hepatocytes, several factors, such as hormones, growth factors, nutritional components, and pharmacological agents, have been demonstrated to directly or indirectly affect liver regeneration [12-14]. However, the regenerative capacity of fatty liver after major tissue loss is still unclear. It is highly suspected that hepatic resection or transplantation in patients with fatty liver should be associated with morbidity and mortality. It will even increase the threaton of our people's health and lives.

Apoptosis and cell proliferation are complementary and account for the maintenance, growth or involution of a tissue[3]. However, only few reports about apoptosis and liver regeneration could be reviewed. Baroni GS et al reported that in chronic ethanol-treated rats apoptosis and mitosis were increased four and fivefold respectively, suggesting a physiological equilibrium between the two phenomena after exposure to ethanol[15]. Melchiorri C et al found a large increase in tetraploid and octoploid mononucleate hepatocytes with almost complete disappearance of binucleate cells at 3 days after partial hepatectomy and implicated apoptosis of regenerated mononucleate cells during 3 to 14 days[16]. In our previous study, we have proved that apoptosis is coexist with liver regeneration at early period after partial hepatectomy. However, the phenomenon of apoptosis and liver regeneration in fatty liver after partial hepatectomy is still unknown.

Through this study, we deotected that fatty liver could be markedly induced by high fat (16%) diet when compared with normal diet (AIN-93G, with 7% fat). The effect of fatty liver on liver regeneration after partial hepatectomy when compared with control group(AIN-93G diet feeding) rats was significantly worse by BrdU Index and ODC contents measurements. The effect of fatty liver on apoptosis after partial hepatectomy when compared with control group rats was also significantly higher by TUNEL and apoptotic number counting.

As for the effect of changing diet pattern from high fat to normal or high fiber diets, it's indeed could reverse the apoptotic situation and the regenerating capacity after partial hepatectomy. The high fiber diet (10%) could ever have significant better results in decreasing apoptosis situation and increasing liver regeneration when compared with high fat diet feeding rats.

The results of this study suggested that fatty liver can be induced by high fat diet, and the fatty liver can increase the apoptosis and decrease the liver regenerating capacity of remnant liver after partial hepatectomy. Fortunately, the fatty liver and it's disadvantage effects could be reversed when changing diets into normal or especially high fiber diets. Further study for the mechanism of high fiber diets effects should be needed.

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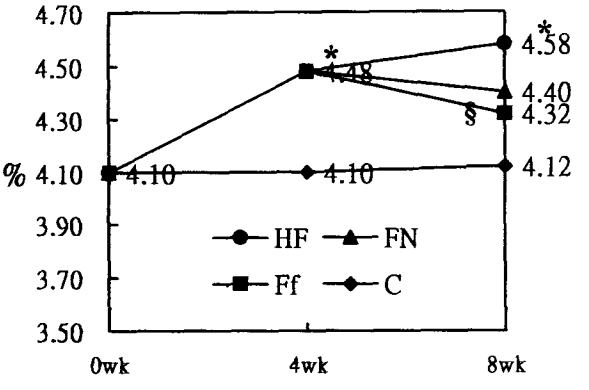

Fig 1. Liver Weight / Body Weight Ratio (* vs group C: p<0.05, § vs group HF: p<0.05)

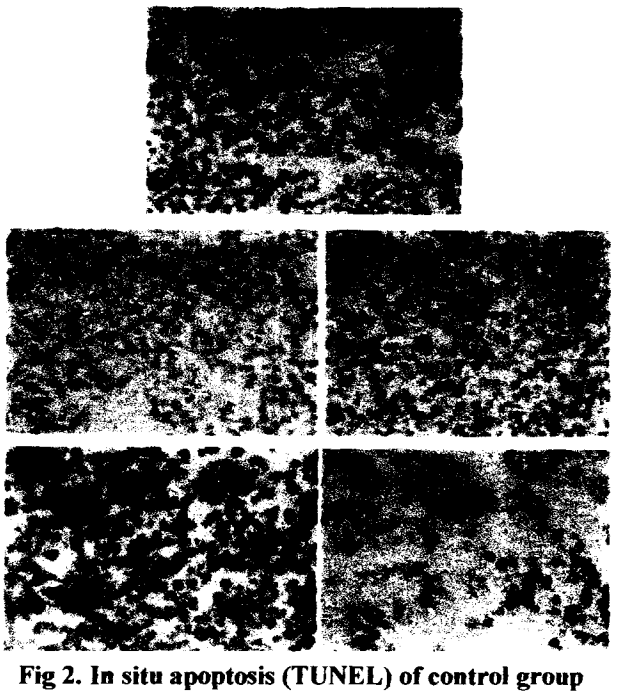

Fig 2. In situ apoptosis (TUNEL) of control group rats before and at 6, 24, 48, 72 hours after partial hepatectomy.

Table 2. Apoptotic Number after Partial Hepatectomy in Each Group Rats

GR \ Time	B	6h	24h	48h	72h
C	15±3	36±7$^{\#}$	22±5	20±5	20±5
HF	60±11*	98±19$^{\#*}$	150±23$^{\#*}$	102±21$^{\#*}$	72±15*
FN	40±9*	58±10$^{\#*\S}$	90±17$^{\#*\S}$	60±12$^{\#*\S}$	50±10*
Ff	20±5§	46±9$^{\#\S}$	70$^{\#*\S}$	52±10$^{\#*\S}$	38±8$^{\#*\S}$

B: before, C: control, HF: high fat, FN: high fat to normal diet, Ff: high fat to high fiber diet,
vs Time B: $p<0.05$, * vs Gr C: $p<0.05$, § vs Gr HF: $p<0.05$

Table 3. BrdU Index after Partial Hepatectomy in Each Group Rats

GR \ Time	B	6h	24h	48h	72h
C	1±2	10±2	30±5	20±4	10±2
HF	1±1	4±1	12±3*	10±2*	5±1*
FN	1±2	6±1	18±4*	12±3*	6±1*
Ff	1±2	8±2	24±4 §	15±3* §	8±2 §

B: before, C: control, HF: high fat, FN: high fat to normal diet, Ff: high fat to high fiber diet,
* vs Gr C: $p<0.05$, § vs Gr HF: $p<0.05$

Table 4. Ornithine Decarboxylase Content (mg/dL) after Partial Hepatectomy in Each Group Rats

GR \ Time	B	6h	24h	48h	72h
C	255±26	656±46$^{\#}$	604±48$^{\#}$	460±42$^{\#}$	399±40$^{\#}$
HF	190±20	444±45$^{\#*}$	390±42$^{\#*}$	380±37$^{\#*}$	330±32$^{\#}$
FN	222±22	550±50$^{\#*\S}$	404±42$^{\#*}$	412±40$^{\#}$	338±34$^{\#}$
Ff	276±29	650±60$^{\#\S}$	534±49$^{\#\S}$	438±45$^{\#}$	392±38$^{\#}$

B: before, C: control, HF: high fat, FN: high fat to normal diet, Ff: high fat to high fiber diet,
vs Time B: $p<0.05$, * vs Gr C: $p<0.05$, § vs Gr HF: $p<0.05$