COMPARATIVE EFFECT OF SILYMARIN AND ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITOR ON BIOCHEMICAL AND MORPHOLOGICAL ALTERATIONS OF REGENERATING LIVER

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BY

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CERTIFICATE

This is to certify that this dissertation, submitted by **AYSHA AMBREEN,** is accepted in the present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad as satisfying the requirements for the degree of Master of Philosophy (Developmental Biology).

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In the name of Allah, Most Gracious, Most Merciful.

Praise be to Allah, the Cherisher and Sustainer of the worlds

Most Gracious, Most Merciful;

Master of the Day of Judgment.

Thee do we worship, and Thine aid we seek.

Show us the straight way,

The way of those on whom Thou hast bestowed Thy Grace, those whose (portion) is not wrath, and who go not astray.

DEDICATED

TO

MY PARENTS

AND MY FRIENDS

UZMA AND SHUMAILA

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Abstract

Liver diseases are becoming more and more common and often require surgical resection. Liver, the vital body organ has the remarkable capacity to regenerate after partial hepatectomy (PHx) or toxic insult. However, fibrotic liver shows impaired regeneration. Therefore there is need for such drugs that can enhance and improve regeneration of fibrotic liver after PHx. In current study a comparison was made to evaluate the efficacy of the two drugs i.e. silymarin and angiotensin converting enzyme (ACE) inhibitor on the morphological and biochemical parameters of regenerating fibrotic liver. Female Sprague –Dawley rats were made fibrotic by the administration of carbon tetrachloride (CCl_4) dissolved in olive oil (1:7) at the dose of 1.5ml/kg i.p for seven weeks while the vehicle group received 1.5ml/kg i.p olive oil for the same duration. Then the animals received saline (1ml), ACE-inhibitor (2.5mg/kg) and silymarin (70mg/kg) orally for one week before 55% PHx. 24hours after surgery the rats were dissected. The result showed that liver regeneration rate (LRR) of fibrotic animals and even that of drug treated ones was significantly less than vehicle. It was observed that both ACE inhibitor and silymarin treatment did not improve LRR significantly as compared to fibrotic control. Relative liver weight (RLW) of different groups showed no significant difference.

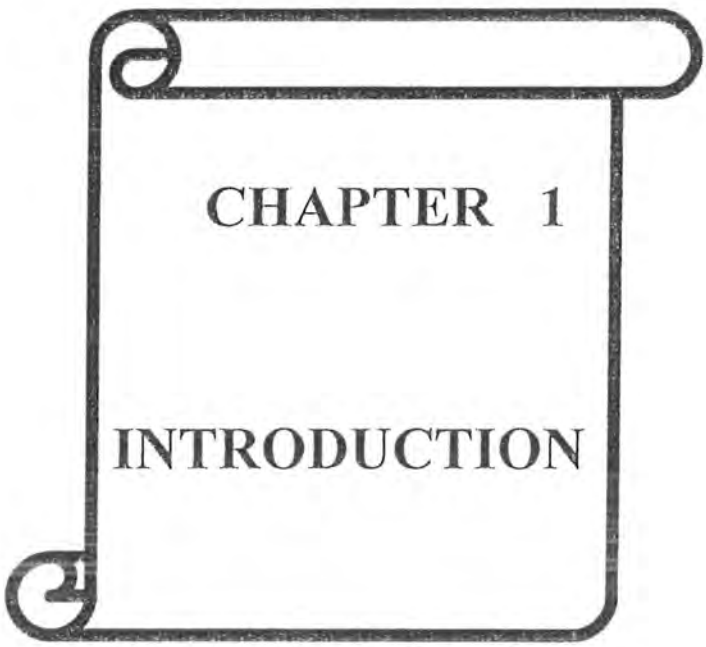
Biochemical parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST) of vehicle were significantly low than all other groups having fibrotic rats. Bilirubin level, however, was significantly less in ACE inhibitor treated rats as compared to vehicle. A comparison of the liver enzymes of fibrotic animals after PHx showed that their level was significantly improved in ACE-Inhibitor group as compared to that of both fibrotic control and silymarin receiving rats.

No significant difference in hepatic cell plate width (HCPW) of untreated fibrotic and normal livers after the surgery was observed. In drug treated groups silymarin showed no significant variation from both vehicle and fibrotic control, however, ACE inhibitor given rats had significantly less HPCW than all other groups. Sinusoidal width (SW) of vehicle was significantly more than fibrotic control and silymarin treated rats however, this difference was not observed in the case of ACE inhibitor group. ACE inhibitor treatment

resulted in significantly more SW than both fibrotic control and silymarin group. Both drug treated and non-treated fibrotic regenerating rat livers had significantly more necrotic and apoptotic nuclei as compared to vehicle. ACE inhibitor treatment had significantly reduced the number of such nuclei in regenerating fibrotic livers after PHx. These results suggest that ACE-Inhibitor (2.5mg/kg) can improve the biochemical parameters of regenerating fibrotic livers more effectively than the silymarin (70mg/kg) after PHx. However, as far as the histological parameters are concerned neither drug proved to be effectively improving all the histological markers of liver regeneration. ACE inhibitor improves certain histological parameters more effectively than silymarin while certain others are effectively improved by silymarin.

ABBREVIATION

%	Percentage
AC-HSC	Activated Hepatic Stellate Cells
ACE-inhibitor	Angiotensin Converting Enzyme Inhibitor
ALT	Alanine Aminotransferase
ARBS	Angiotensin- II Receptor Blockers
AST	Aspartate Aminotransferase
AT-II	Angiotensin -II
CCl_4	Carbon Tetrachloride
DNA	Deoxyribose Nucleic Acid
ECM	Extracellular Matrix
HCC	Hepatocellular Carcinoma
HCPW	Hepatic Cell Plate Width
HCV	Hepatitis C virus
HGF	Hepatocyte Growth Factor
IFN	Interferon
iNOS	Inducible Nitric Oxide Synthase
IL-6	Interleukin-6
LDH	Lactate Dehydrogenase
LRR	Liver Regeneration Rate
MHD	Malate Dehydrogenase
NADH	Nicotine Amide Dehydrogenase
NIH	National Institute Health
PH	Partial Hepatectomy
RAS	Renin Angiotensin System
RLW	Relative Liver Weight
RNA	Ribonucleic Acid
SW	Sinusoidal Width
TGFβ	Transforming Growth Factorβ
TIMP	Tissue Inhibitor of Metalloproteinase
VEGF	Vascular Endothelial Growth Factor
VK	Vitamin K



CHAPTER 1

INTRODUCTION

INTRODUCTION

Liver is a vital body organ. It is the largest gland in the body and performs an astonishingly large number of tasks that impact all body systems. One consequence of this complexity is that hepatic disease has widespread effects on virtually all other organ systems. Despite its large metabolic load, the liver is a largely quiescent organ in terms of cell proliferation with only 0.01-0.001% of hepatocytes undergoing mitosis at any given time (Starzl *et al.*, 1977; Fausto *et al.*, 1994; Diehl *et al.*, 1996; Michalopoulos *et al.*, 1997) This low cell turnover in healthy liver tissue, however, can be altered by toxic liver injury or surgical resection, which results in sudden, massive hepatocyte proliferation, producing recovery of functional liver mass within two weeks after the loss of up to two-thirds of the liver (Holt *et al.*,2000).

In humans (Nagino *et al.*, 2001) and other species (Higgins and Anderson, 1931) surgical resection of the liver is followed by rapid regeneration. In rats after partial hepatectomy (PH) the remnant liver rapidly proliferates and returns to the original mass within 7 to 10 days (Michalopoulos *et al.*, 1997; Higgins and Anderson, 1931; Bucher and Swaffeld, 1964). This process involves a variety of growth factors and cytokines (Michalopoulos *et al.*, 1997; Higgins and Anderson, 1931; Bucher and Swaffeld, 1964; Fausto *et al.*, 1995) The regenerative response is typically mediated by proliferation of surviving hepatocytes within the acinar architecture of the remnant liver (Holt et *al.*, 2000).

The interactions between hepatocytes, non-parenchymal cells (e.g. endothelial cells) and the extracellular matrix are important for the regulation of liver regeneration (LaBrecque, 1994; Michalopoulos and DeFrances, 1997). Immediately after partial hepatectomy, numerous cytokines, growth factors and proteases are upregulated and facilitate matrix remodeling and proliferation of the different hepatic cell populations as the liver remnant regenerates. Among hepatocyte growth factor (HGF) is the most pluripotent growth factor displaying a remarkable ability to promote tissue repair and organ regeneration after injury (Ishiki *et al.*, 1992). HGF is a potent agent for acceleration of tissue

regeneration following an acute insult, as well as amelioration of tissue fibrosis and dysfunction in chronic conditions (Ueki *et al.*, 1999; Fujimoto, 1999; Shiota *et al.*, 2000).

Liver tissue remodeling after surgery includes development of new hepatic microvasculature. Therefore angiogenesis has become a new field of focus in the effort to shed more light on hepatic regenerative mechanisms. Several important angiogenic growth factors, including vascular endothelial growth factor (Mochida *et al.*, 1996) and angiopoietins (Sato *et al.*, 2001) are upregulated after partial hepatectomy (PH) and are thought to constitute a proliferative signal for sinusoidal endothelial cells (Yamane *et al.*, 1994; Taniguchi *et al.*, 2001). Eugenio *et al.*, (1998) had investigated rat liver ultrastructure after partial hepatectomy. Their study showed sinusoid dilatation and disappearance of the sieve-plate arrangement of small endothelial pores, thus leaving the parenchymal liver cell surface directly exposed to portal blood. Widening of sinusoids, endothelial fenestrations, intercellular spaces and spaces of Disse, was accompanied by dilatation of bile canaliculi.

Exchange of oxygen and nutrients between hepatocytes and the blood is crucial for the preservation of metabolic function, especially in conditions of reduced functional liver mass, such as directly after PH. A key determinant of microenvironment oxygenation is microvascular permeability, which in turn is dependent on vascular surface area (Yuan, *et al.*, 1993) Research findings indicate that in conditions of hepatic tissue loss, the rapid development of a sufficiently large exchange interface between hepatocytes and the blood may be a prerequisite for adequate regenerative capacity. It appears that the vascular changes seen in regenerating liver activate a most efficient repair mechanism to compensate functional tissue loss, which leads to rapid and complete restoration of normal hepatic architecture (Vogten *et al.*, 2003)

Vogten (2003) presented the dynamics of functionality in the regenerating mouse liver. He found a transient increase in hepatic cell plate width in the regenerating liver during the first 14 days post PH. This finding corresponds to earlier reports of increased plate

width in rats as determined by fixed-tissue studies (Martinez. and Amenta, 1995; Wack. *et al*, 2001).

The liver has the ability to precisely regulate its growth and mass. Apoptosis is a type of cell death that serves to eliminate excessive or unwanted cells during the remodeling process in liver regeneration, as well as in embryonic development and wound healing (Lockshin *et al.*, 1990). Surgical resection of hepatic lobes or hepatocytes loss caused by viral or chemical injury triggers hepatocyte replication, while enlarged liver mass is corrected by apoptosis (Cruise et *al.*, 1987). During successful liver regeneration, the expression and activity of pro-apoptotic pathways is inhibited through increased expression of anti-apoptotic Bcl-2 family members (Kaplowitz, 2002; Neuman, 2001). Numerous factors have been implicated in activating anti-apoptotic signals in the liver generally most mitogens have a concomitant anti-apoptotic activity and examination of knock-out mice have demonstrated critical roles for inducible nitric oxide synthase and IL-6. In genetically altered mice, absence of either iNOS or IL-6 will result in elevated expression of pro-apoptotic signals, increased hepatocyte death, and reduced animal survival, Rai *et al* ., 1998; Kovalovich *et al.*,2001). The study of Helling *et al.*, (2004) showed that apoptosis, except for occasional sporadic bursts, is effectively suppressed after PHx and is not likely to contribute in depletion of functional hepatocytes after partial hepatecomy. Yahya *et al.*, (2005) in their study showed a significant increase in Necrotic cell count in 70% and 85% hepatectomy groups was higher than sham. Same was the result in case of apoptotic cell count. The role of apoptosis in liver regeneration is still not clear.

Hepatic resection is often required nowadays, as the rate of liver diseases has become more common with time. The most common liver disorders are hepatic fibrosis and cirrhosis. Liver fibrosis is the excessive accumulation of extracellular matrix proteins including collagen that occurs in most types of chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation. Now it is known that activated hepatic stellate cells, portal fibroblasts, and myofibroblasts of bone marrow origin have been identified as major collagen-

producing cells in the injured liver. These cells are activated by fibrogenic cytokines such as transforming growth factor β1 (TGF-ß1), angiotensin II, and leptin. Emerging antifibrotic therapies are aimed at inhibiting the accumulation of fibrogenic cells and/or preventing the deposition of extracellular matrix proteins. (Bataller *et al.*, 2003).

Cirrhosis is the end stage of chronic liver injury caused by viral hepatitis or alcohol intake, and effective treatment capable of reversing cirrhosis has not been developed. Two main factors are responsible for the irreversibility of cirrhosis. One involves the increased and continuous deposition of extracellular matrix (ECM), the result of increased collagen synthesis accompanied by insufficient breakdown of collagen (Bickel *et al.*, 1998). The other factor is the impaired capability of liver regeneration, which predisposes to postoperative dysfunction or liver failure (Andiran *et al.*, 2000).

As far as microvasculature of abnormal tissue such as tumor growth the microvessels in them are highly abnormal and impede normal perfusion dynamics (Jain, 2001). Hepatic fibrosis and cirrhosis are also characterized by decreased sinusoidal density, microvascular shunting and disturbances in diffusion and permeability (Varin and Huet, 1985; Vollmar *et al.*, 1998; Rappaport *et al.*, 1983).

Hepatic injuries are usually resulted in necrosis and apoptosis of hepatocytes. Carbon tetrachloride (CCl_4) is a well-known toxic chemical to produce hepatic injury. CCl_4 induced toxicity resulted in severe necrosis as well as apoptosis .Shi *et al.*, (1998) re-examined the hepatic injury evoked by CCl4 in rats and explored the possibility that apoptosis may also contribute to its pathogenesis. The results of the study indicated that apoptosis occurs in the ballooned and injured hepatocytes of the centrilobular area. Such findings are important in reducing inflammation and preventing the development of fibrosis. CCl_4 toxicity induces apoptosis in hepatic stellate cells too (Lee *et al.*, 2003).

There are conflicting data regarding the ability of the liver to regenerate after partial hepatectomy in animals and humans with cirrhosis. However most of the previous studies have shown that a fibrotic liver following hepatic resection regenerates very slowly in

comparison with the normal liver (Andiran *et al.*, 2000; Kawasaki *et al.*, 1992; Hashimoto and Watanabe, 1999).

Chen and Hwang in 1994 conducted the study to document liver regeneration after partial hepatectomy in a carbon tetrachloride rat model of cirrhosis. Results indicated that live regeneration did occur after partial hepatectomy in carbon tetrachloride-treated rats but was impaired in comparison with that in vehicle-treated control rat. Restitution of liver mass after partial hepatectomy was significantly decreased in carbon tetrachloride-treated rats at 1 and 7 days compared with the vehicle-treated control rats. Similar result was shown by Chijiwa *et al.*, (1994) in the thioacetamide induced liver cirrhotic rats.

Hashimoto and Watanabe (2005) evaluated the functional capacity of the rat liver rendered cirrhotic by orally administered thioacetamide, and examined the correlation between morphological and functional restoration after 2/3 hepatectomy in comparison with hepatectomized normal rats and sham-operated cirrhotic rats. Cirrhotic rats were functionally deteriorated in comparison with the normal rats. Morphological restoration in cirrhotic rats was delayed in comparison with normal rats. Functional restoration after 2/3 hepatectomy was advanced in comparison with morphological restoration. In comparison with sham-operated cirrhotic rats, functional restoration of the cirrhotic liver was accelerated by partial hepatectomy.

The study in which patients with hepatic tumors associated with liver disorders of various severity had underwent hepatectomy, it was observed that severe fibrosis of the liver parenchyma is associated with poorer regeneration of the remnant liver leading to poor restoration of post-operative liver function. The regeneration rates of the remnant liver indicated a significant decline in patients with severe fibrosis. While in the normal and mild fibrosis patients, an increased removal rate was associated with increased regeneration rate. However, in the moderate fibrosis and severe fibrosis groups, an increased removal rate was not associated with increased regeneration rate (Miyazaki *et al.*, 1999).

It is observed that certain disease lead to an impaired ability to down regulate

apoptotic pathways in the liver. Steatosis, for example, has been shown in animal models to result in increased apoptotic signaling with decreased anti-apoptotic NF-KB signaling following endotoxin challenge (Deaciuc *et al.*, 2001). These laboratory observations correlate with an increased death rate seen in patients with steatosis following liver resection. Similarly, cirrhosis leads to markedly impaired control of apoptotic pathways. Masson and coworkers (2000) compared normal and cirrhotic livers in a mouse model and found mRNA levels of pro-apoptotic genes were significantly elevated in cirrhotic liver. Furthermore, when these animals underwent 2/3 PH induction of protective antiapoptotic mRNA levels were both decreased and their expression delayed, relative to non-cirrhotic animals undergoing 2/3 PH. In humans fulminant hepatic failure following liver resection occurs at a markedly increased rate in cirrhotic livers; evidence for excess apoptosis and impaired anti-apoptosis are observed and human studies in decreasing liver injury following hepatic resection (YI *et al.*, 2002; Li *et al.*,1993; Jung *et al* .,1999).

There are many substances that not only reduce liver fibrosis but also enhance regeneration of normal liver. Silymarin and ACE-Inhibitor are among them. Silymarin is being used by patients with different liver disorders, however, ACE- inhibitor is one of the emerging therapies which is not used currently for liver diseases but have shown that it not only reduce liver fibrosis but also enhance liver regeneration. There are studies that show their antifibrotic and regenerative abilities.

SILYMARIN

Silymarin, a standardized extract from the milk thistle pla a hepatoprotective drug (Bosiso *et al.*, 1992; Carola *et al.*, 1996). It significantly enhanced liver regeneration rate after partial hepatectomy (Magliulo, *et al.*, 1973). It has been reported to stimulate enzymatic activity of DNA-dependent RNA polymerase 1 and subsequent biosynthesis of RNA and protein, resulting in DNA biosynthesis and cell proliferation (Sonnenbichler and Zetl, 1986□). Srivastava *et al.*, (1994) had observed the effect of silymarin on partially hepatectomized liver of rats utilising macromolecular levels, DNA, RNA synthesis and mitotic figure as indices of regeneration. The levels of DNA, RNA, protein

and cholesterol increased in the regenerating liver of rats treated with silymarin. Later on mitotic figure also showed an increasing trend in the residual liver. These results clearly suggest that silymarin stimulate liver regeneration after partial hepatectomy.

The effects of silybin, an active constituent of silymarin, upon the biological activities of Kupffer's cells in regenerating livers of rats subjected to partial hepatectomy showed that proliferative activity of Kupffer's cells was appreciably increased in rats treated with silybin as compared with control. The increased mitotic activity induced on Kupffer's cells by silybin was interpreted as a further expression of the effectiveness of silybin on the cellular components of the liver and its positive role in liver regeneration (Magliulo *et al.*, 1979)

Kropachova and Mihurova (1992) examined the effect of flavobion (silymarin) and a single whole-body irradiation on the regeneration of rat liver. Liver regeneration was estimated on the basis of chosen morphological parameters on hour 30 after partial hepatectomy. Radiation-induced latent injury to intact rat liver 30 min before partial hepatectomy manifested in remaining regenerating liver by slowing-down of the increase in liver weight, cellularity and inhibition of the mitotic activity and in more frequent chromosome aberrations.this hepatoprotective agent, lessen the manifestations of latent injury as indicated by an increase in cellularity and mitotic index as compared with unprotected animals. Furthermore, the preparations tested decreased the frequency of radiation-induced chromosome aberrations.

Silymarin not only enhance liver regeneration but also reduced the liver fibrosis as evident from the research studies. Silymarin has clinical applications in the treatment of cirrhosis, ischemic injury, and toxic hepatitis induced by various toxins such as ethanol, carbon tetrachloride, acetaminophen, organic solvents, and toxic mushroom (Saller *et al.*, 2001).

Carbon tetrachloride is used in laboratory tests to assess the ability of a substance to actually protect the liver from any potentially damaging compound. Increasing the dosages of carbon tetrachloride takes the liver through fatty infiltration, fibrosis and

eventually cirrhosis. During these tests, administering Milk Thistle extract (silymarin) resulted in effective protection of liver tissue from the toxic effects of the chemical (Daniel and Mowre, 1988). Jeong *et al.*, (2005) showed anti-fibrotic and antiinflammatory effects of silymarin against carbon tetrachloride induced liver fibrosis They also showed that silymarin prevent hepatic fibrosis through suppression of inflammation and hypoxia in the hepatic fibrogenesis.

Silymarin reduces hepatic collagen accumulation by 35% in rats with secondary biliary cirrhosis. It has been demonstrated that silymarin improves hepatic fibrosis *in vivo* in rats subjected to complete occlusion of the biliary duct, a manoeuvre that causes progressive hepatic fibrosis without inflammation. Silymarin, administered at a dosage of 50 mg/kg/day for 6 weeks, is able to reduce fibrosis by 30 to 35% as compared with controls (Boigk *et al.*, 1997). Elevated procollagen alpha1, metalloproteinases-1 (TIMP-1) and transforming growth factor β1 (TGF β1) mRNA levels are downregulated by 40-60% in rats treated with silyamrin and subjected to biliary cirrhosis (Jia *et al.*, 2001).

Salmi *et al.*, (1982) conducted a study in which one hundred and six consecutive patients with liver disease were selected on the basis of elevated serum transaminase levels. The patients were randomly allocated into a group treated with silymarin (treated) and a group receiving placebo (controls). Ninety-seven patients complete the 4-week trial-47 treated and 50 controls. In general, the series represented a relatively slight acute and subacute liver disease, mostly induced by alcohol abuse. There was a statistically highly significant decrease of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the treated group than in controls. Serum total and conjugated bilirubin decreased more in the treated than in controls, but the differences were not statistically significant. Normalization of histological changes occurred significantly more often in the treated than in controls.

Two interesting studies are reported in the review by Flora *et al.*, (1998). The first study was performed in 2637 patients with chronic liver disease, treated with high doses of silymarin (560 mg/day) for 8 weeks. Resolution of subjective symptoms was achieved in 63% of cases; AST diminished on average by 36% and ALT by 34% Furthermore,

the investigators reported a reduction in hepatomegaly upon palpation. The second study was performed under double-blind conditions in patients with persistent or aggressive chronic hepatitis, with or without cirrhosis, monitored for 3 to 12 months and treated with silymarin. Treatment did not produce any signs of improvement in liver function; however, histological examination revealed an improvement in portal inflammation, parenchymal alterations and necrosis.

The radioprotective effect of silymarin using different modes of treatment against radiation induced hepatotoxicity 1, 3 and 7 days post-irradiation was studied. Whole-body gamma-irradiation revealed an increase in serum alkaline phosphatase (AP) activity as well as liver glutathione reductase and glutathione peroxidase activities Administration of silymarin as single (70 mg / kg), fractionated (490 mg / kg) oral doses or as intravenous (i.v.) injection (50 mg / kg), caused significant protection. Intravenous treatment showed the most pronounced protection. The protective effect of silymarin was attributed to its antioxidant and free radicals scavenging properties (Ramadan *et al.*, 2002).

Hepatoprotective effects of silymarin in patients with alcoholic liver disease are controversial. Lieber (2003) conducted a study in which the effect of silymarin on the progression of alcohol induced hepatic fibrosis in baboons was determined. The authors concluded that Silymarin retards the development of alcohol-induced hepatic fibrosis in baboons, consistent with several positive clinical trials.

Silymarin is also known to protect mice from Fumonisin B_1 induced liver injury, which is a hepatic carcinogen in rats, and mice. The activities of circulating alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also reduced by silymarin treatment. Silymarin had significantly diminished the number of apoptotic hepatocytes. It also augmented hepatocyte proliferation indicated by an increase in proliferating cells. (Quanren *et al.*, 2004). Shalan *et al.*, (2005) investigated the impact of combined administration of Vitamin C and silymarin on lead toxicity in rat. Lead administration resulted in portal inflammation, steatosis, apoptosis and mild fibrosis of liver. Silymarin along with Vitamin C showed protective effect against lead toxicity.

The combined treatment markedly improved the biochemical, molecular and histopathological findings.

ACE-Inhibitor

ACE-Inhibitors are well known drugs against heart diseases. They work by blocking the action of angiotensin (AT) a substance that causes blood vessels to narrow. Since ACE inhibitors are used widely in clinical practice without serious side effects, therefore in recent years effect of these drugs on other physiological phenomenon is also studied.

Liver regeneration is a unique phenomenon in which the loss of hepatic tissue rapidly induces an orchestrated response involving sequential changes in gene expression, growth factor production, and morphologic structure (Michalopoulos and DeFrances (1997; LaBrecque, 1994). Several substances with potentially important roles in liver regeneration have been recently identified (LaBrecque, 1994). The kallikrein-kinin system exerts a variety of biological effects, including vasodilatation, increased vascular permeability and smooth muscle relaxation or contraction (Regoli and Barabe, 1980; Ito *et al.*, 1997; Coutant *et al.*, 1996). Ramalho *et al.*, (2001) reported that bradykinin augments liver regeneration after partial hepatectomy in rats. Angiotensin-converting enzyme (ACE) is also a powerful bradykinin-degrading enzyme. They investigated the effect of ACE inhibition by lisinopril, on liver regeneration after partial hepatectomy. Adult male Wistar rats underwent 70% partial hepatectomy (PH) in their study. The animals received lisinopril at a dose of 1 mg / kg intraperitoneally, for 5 days before hepatectomy, and daily after surgery. The value for the lisinopril-treated group was three-fold above the corresponding control at 12 h after PH, remaining elevated at approximately two-fold above control values at 24, 36, 48, and at 72 h after PH. The present study shows that plasma ACE inhibition enhances liver regeneration after PH in rats. Since it was reported that bradykinin also augments liver regeneration after PH, this may explain the liver growth stimulating effect of ACE inhibitors.

signaling cascade, either by the clinically used angiotensin-converting enzyme inhibitors (ACE-Is) or by AT-II type 1 receptor blockers (ARBs) attenuates the liver fibrosis development, and these agents produce regression even in developed fibrosis in experimental studies, and in clinical practice as well (Terui *et al.*, 2002; Yoshiji *et al.*, 2001).

Yoshiji *et al.*, (2004) studied the combined effect of an ACE inhibitor, perindopril, and interferon (IFN) on liver fibrosis markers in patients with chronic hepatitis C. They found that neither the fibrosis makers nor the ALT level changed significantly with the IFN only treatment in several virologic nonreponders. On the other hand, all fibrosis markers in the IFN and perindopril treated group improved significantly without alteration of the HCV-RNA or serum ALT levels.

Angiotensin converting enzyme (ACE) and angiotensin II (AT-II) have been suggested to play a vital role in liver fibrogenesis. There is a significant relationship between inheritance of hightened expression of transforming growth factor β1 (TGF-β1) and AT-II and the development of progressive hepatic fibrosis. The study to investigate the effects of captopril, an ACE inhibitor and candesartan cilexetil, an AT-II type 1 receptor (AT1-R) blocker, on liver fibrosis induced in rats by carbon tetrachloride (CCl_4) administration showed that candesartan cilexetil treatment significantly reduced the fibrosis development. These inhibitory effects were not observed in the captopril-treated group. The mean fibrosis score was significantly lower in the CCl_4/candesartan group compared with the group applied to CCl_4 alone and the group applied to CCl_4/captopril. Similarly, the number of α-smooth muscle actin positive cells was markedly suppressed by candesartan treatment (Tuncer *et al.*,2003).

The renin-angiotensin system (RAS) is frequently activated in patients with chronic liver diseases. Angiotensin-II (AT-II) has many physiological effects, including strong pro-angiogenic activity. AT-II induces the potent angiogenic factor, vascular endothelial growth factor (VEGF). Recent studies have revealed that angiogenesis is an essential process in many pathological events, such as tumor growth including hepatocellular

It has been reported that ACE inhibition increases renal hepatocyte growth factor (HGF) mRNA and cardiac HGF concentration in experimentally hypertensive rats, and that angiotensin II downregulates HGF production in mesangial cells in a cultured model (Nakano *et al.*, 1997; Matsumoto *et al.*, 1999). HGF was initially identified as the most potent growth factor for hepatocytes (Nakamura *et al.*, 1989). Another interpretation of these data is that angiotensin II may also downregulate hepatocellular HGF production, and that hepatic HGF concentration might be elevated in response to ACE inhibition, potentially resulting in the improvement of liver regeneration.

Although most studies have focused on the hepatocytes, all the hepatic cells participate in the regenerative process, among them the stellate cells. The stellate cells are mesenchymal cells involved in local neurotransmission and paracrine regulation of several liver functions. Acute hepatic tissue loss promotes the proliferation and activation of stellate cells from a quiescent state to myofibroblast-like cells. Acute hepatic tissue loss promotes the proliferation and activation of stellate cells from a quiescent state to myofibroblast-like cells (Knittel *et al.*, 1999). It was found that tissue loss induced by partial hepatectomy determined the highest population of activated stellate cells at 36 hours after surgery. The activation of these cells enabled them to produce several growth factors that can promote hepatocellular proliferation, as well as HGF (Boros and Miller, 1995). In addition to being the major suppliers of hepatic HGF, activated stellate cells participate in the hepatic synthesis of extracellular matrix which performs and organizes hepatocellular proliferation (Jiang *et al.*, 1993). ACE inhibition by lisinopril stimulated the stellate cell population. The improvement of stellate cell population in response to ACE inhibition may also increase hepatocellular HGF production. The main effect of ACE inhibition on the stellate cell population is not the reduction of angiotensin II synthesis, but the lower hepatic degradation of bradykinin (Ramalho *et al.*, 2003)

ACE inhibitors are also effective in reducing fibrosis. It has been shown that angiotensin-II (AT-II) which is produced by angiotensin-converting enzyme (ACE), plays an important role in liver fibrosis development (Bataller *et al.*, 2003). Blockade of the AT-II

carcinoma (HCC), and even in liver fibrogenesis. ACE inhibitors are currently widely used as anti-hypertensive agents in clinical practice. Studies have found that the ACE inhibitor, perindopril (PE), which is a potent inhibitor of experimental HCC growth and angiogenesis, is associated with the suppression of VEGF at a clinically comparable dose. PE also markedly suppressed the hepatocarcinogenesis step. In liver fibrogenesis, AT-II is known to stimulate proliferation and production of tissue inhibitor of metalloproteinases-1 (TIMP-1) in activated hepatic stellate cells (Ac-HSC), which play a pivotal role in liver fibrosis development. PE markedly inhibited liver fibrogenesis associated with suppression of Ac-HSC proliferation and TIMP-1 expression. As ACE inhibitor is used widely in clinical practice without serious side effects, it may provide an alternative new strategy for the treatment of liver fibrosis and HCC (Yoshiji *et al.*, 1992)

Studies have revealed that angiogenesis plays a pivotal role in carcinogenesis and tumor growth. It was reported the clinically used vitamin K (VK) and ACE-I exerted potent antiangiogenic activities. Both these substances at clinically comparable low doses exerted significant inhibitory effects on tumor development in the liver. The combined effect of VK and ACE-I on hepatocarcinogenesis induced by diethyl-nitrosamine, and orthotopic hepatocellular carcinoma (HCC) growth in rats showed a more potent suppressive effect against hepatocarcinogenesis. Neovascularization increased during hepatocarcinogenesis, and VK and ACE-I significantly attenuated angiogenesis in the tumor. In orthotopic HCC transplantation, VK and ACE-I also showed marked suppressive effects against HCC development similar to those against hepatocarcinogenesis. In the orthotopic model, VK and ACE-I treatment resulted in a marked increase of apoptosis in the tumor, whereas tumor cell proliferation itself was not altered (Yoshiji *et al.*, 2006).

Hepatitis C recurrence after liver transplantation is often associated with accelerated graft fibrosis and progression to cirrhosis. As drugs blocking the action of angiotensin-II can reduce fibrosis in different human and experimental models it was concluded that administration of angiotensin-blocking agents might be beneficial to reduce the development of graft fibrosis in hepatitis C recurrence after liver transplantation (Rimola *et al.*, 2004).

As is evident from the above studies that both silymarin and ACE-Inhibitor enhance liver regeneration and reduce fibrosis too so the present study is conducted to evaluate and compare the effect of both these drugs on morphological and biochemical alterations of those livers that were fibrotic and had undergone partial hepatectomy.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHOD

Chemicals and Drugs

Carbon tetrachloride CCl4 was obtained from Merck K24447505, Germany. All other chemicals were purchased from local sources. The description of drugs used in the study is given below:

1-Lisinopril, an ACE- Inhibitor, under the brand name Coarce (Bosch Pharmaceuticals) was used in the study. It was used in tablet form. Each tablet is of 5mg.

2-The second drug used in the study was Silymarin that was purchased under the brand name Silimarin (Amson Vaccines & Pharma, PVT, Limited). Its suspension form was used. Each 5.0ml of Silimarin contains 100mg of silymarin.

3-Olive oil under the brand name Consul was used in the study.

Animals

Six-week-old female Sprague-Dawley rats weighing about 180-200g were obtained from National Institute of Health (NIH), Islamabad. Animals were housed five each cage. They were acclimated for 1 week before dosing under controlled environmental conditions at 25°C ±2 with a 12-h light/dark cycle. All animals were fed rat chow and water *ad libitum*.

Induction of fibrosis

The CCl4 model described by Ozbek *et al.*, (2004) was used for scheduling the dose regimen. Briefly 1.5ml/kg of carbon tetrachloride diluted in olive oil (1:7 dilution) was intraperitoneally (i.p.) administered to rats for inducing liver fibrosis.

Experimental procedure

Animals were first randomly divided into following two groups:

Group I: There were six animals in this group, which was given olive oil at a dose of

1.5ml /kg, i.p three times a week for seven weeks.

Group II: This group contained 18 animals and they received CCl4:olive oil (1:7)

1.5ml/kg, i.p three times a week for seven weeks.

At the end of seven weeks rats in the two groups were further divided into different sub-groups:

Group I included only one subgroup known as vehicle group. The animals in this group received 1ml saline orally for one week.

The animals in Group II also known as fibrotic group was randomly divided into following three groups:

1- Fibrotic control the animals of which received 1 ml saline orally for one week.

2- ACE-Inhibitor group in which the animals received an ACE-Inhibitor Lisinopril orally at a dose of 2.5ml/kg (Ruben *et al.*, 1999).

3-Silymarin group that received Silymarin at a dose of 70mg/kg orally as described by Hakova and Misurova (1996).

The experimental procedure and grouping of animals is described in Figure 1. All the animals were observed daily and any dead animals were subjected to post-mortem examination to find the cause of death.

Partial hepatectomy

After establishment of carbon tetrachloride induced fibrosis, rats underwent 55% partial hepatectomy (PHx). Animals were anesthetized with an intramuscular injection of ketamine (100mg/kg). A midline incision was made, the left and right middle lobes were

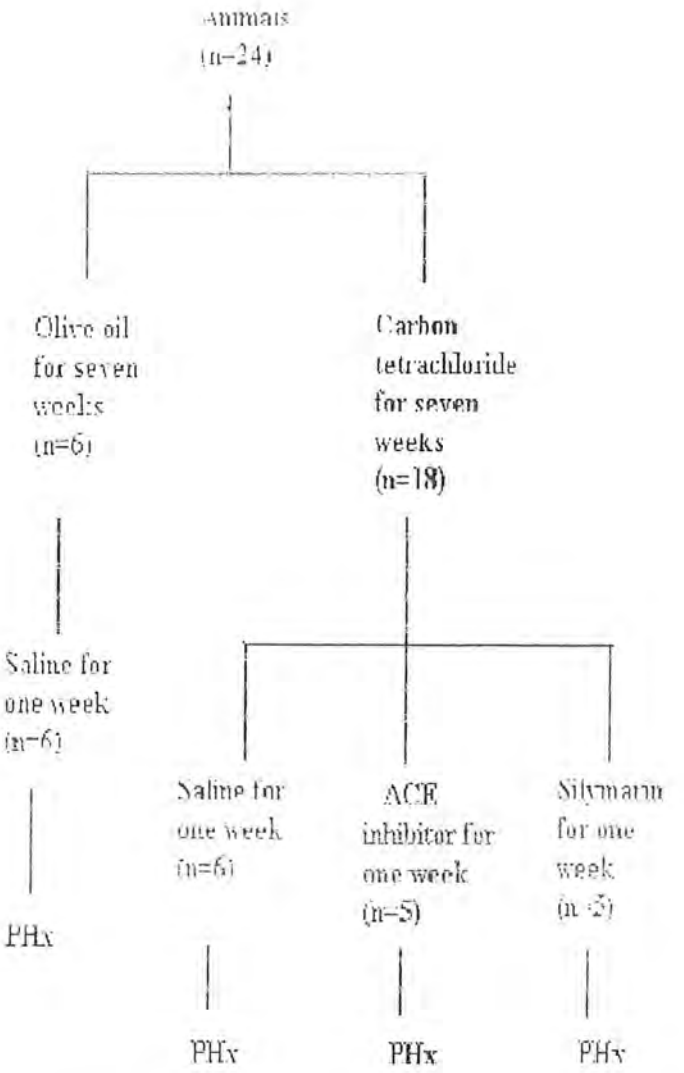


Fig 1: Summary of treatment given to animals.

ligated by a silk suture and then resected .The peritoneum was then reapproximated by catgut followed by closure of the skin with silk suture. Animals were given an intramuscular injection of Gentamycin (0.1ml) after the surgery.

Dissection

Animals were sacrificed 24 hour after hepatectomy. They were anesthetized with diethyl ether. Abdomen was cut opened and blood was aspired through cardiac puncture using 3ml syringes. Liver was separated out.

Serum separation

Serum was separated by allowing the blood to stand at room temperature for one hour and then centrifuging it at 4000 rpm for 5 minutes (Kokusan, HI 108ND Series, Ogawa Seiki Co.LTd). The serum was stored in the refrigerator at 4°C till further use.

Parameters

The following parameters were included in the study:

Rate of Increase in body weights

Initial body weight of animals at the start of experiment was determined. Then at the end of olive oil and CCl_4 treatment i.e. after seven weeks final body weight of animals was determined. The rate of increase in body weights was expressed in percentage and was calculated by the formula given below:

Final body weight– Initial body weight /Initial body weight ×100

Liver regeneration rate

Regeneration of the liver in the rats after 55%hepatectomy was assessed by means of Fishback's method (Fishback, 1929). According to this method hepatic regeneration rate under normal atmospheric conditions is calculated by the following formula:

Estimated Remnant liver weight at sacrifice/total liver weight at surgery ×100

As LRR formula requires an estimated liver weight therefore it is not a highly reliable method to deduct liver regeneration, therefore, another parameter i.e. Relative liver weight (RLW) was determined.

Relative liver weight

Since hepatic growth regulation is controlled by the ratio between liver weight and body weight rather than liver weight alone (Fausto, 2000), therefore the data were expressed as relative liver weight too. Relative liver weight defined as the ratio of liver weight to body weight was calculated by the following formula:

Liver weight /body weight ×100

Assessment of biochemical parameters

To evaluate liver biochemical parameters in different groups after PHx, plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin were assayed through visible light spectrophotometer (Schmidzu, Graphic Printer, PR-1, Spectrophotometer, Japan). Commercially available kits (FARDIAG) were used for assay.

Alanine aminotransferase (GPT/ALT)

ALT activity in U/L was determined by using a kit obtained from Fardiag .

Principle

The presence of alanine aminotransferase (GPT/ALT) enzyme catalyzes the transamination reaction between α-ketoglutaric acid and alanine, causing thee formation of L-ketoglumatic acid and pyruvic acid. Pyruvic acid, in the presence of lactate dehydrogenase (LHD), reacts with NADH forming lactate acid and NAD^+ in equal

quantities. Decrease in NADH absorbance, due to oxidation, is used to determine enzyme activity in the sample.

Reagent of the kit

Components of the kit were:

REAGENT 1

Tris buffer	pH 8.0
L-alanine	500 mmol/L
LDH	800 U/L

REAGENT 2 (liquid)

Carbonate Buffer	pH 10
NADH	2.8 mmol/L
α-ketoglutaric acid	12 mmol/L

.

Protocol

- Wavelength: 340 nm (334 NM-365 NM)
- Pathlength: 1 cm
- Reading: against air
- Temperature: 37 ° C
- Method: kinetic
- Reaction: 3 minutes
- Linearity: up to 260 U/L at 30 ° C
- Sample/reagent: 1/10/1

Brought the reagents to working temperature before use.

Pipetted the reagents and serum into cuvette as followed:

Reagent 1	1000µl
Sample	100µl

Mixed, incubated for 7 minutes and added:

Reagent 2	100µl

Mixed and incubated at 37 ° C temperature for one minute. Finally the absorbance was read at 340nm.

To calculate the ALT activity in U/L at 340nm Absorbance/ minute readings (ΔA/min) were substituted in the following formula:
ΔA/min × 1905 = ALT activity in U/L at 340nm.

Aspartate aminotransferase (GOT/AST)

AST activity in U/L was determined by using a kit obtained from Fardiag

Principle

The presence of aspartate aminotransferase (GOT/AST) enzyme catalyzes the transamination reaction between α-ketoglutaric acid and L-aspartic acid, causing thee formation of L-ketoglumatic acid and oxalacetic acid. Oxaloacetic acid, in the presence of malate dehydrogenase (MHD), reacts with NADH forming malic acid and NAD^+ in equal quantities. Decrease in NADH absorbance, due to oxidation, is used to determine enzyme activity in the sample.

Reagents of the kit

Components of the kit:

REAGENT 1

Tris buffer	pH 8.0
LDH	1000 U/L
MDH	1100 U/L

L-aspartate	200 mmol/L

REAGENT 2 (liquid)

Carbonate Buffer	pH 10
NADH	2.8 mmol/L
α-ketoglutaric acid	12 mmol/L

Protocol

- Wavelength: 340 nm
- Pathlength: 1 cm
- Reading: against air
- Temperature: 37° C
- Method: kinetic
- Reaction: 3 minutes
- Linearity: up to 260 U/L at 30 ° C
- Sample/reagent: 1/10/1

Brought the reagents to working temperature before use.

Pipetted the reagents and serum into cuvettes as followed:

Reagent 1	1000µl
Sample	100µl

Mixed, incubated for 7 minutes and added:

Reagent 2	100µl

Mixed and absorbance was read after 1 minute of incubation at 37° C

temperature.

To calculate the AST activity in U/L at 340nm Absorbance/ minute readings (ΔA/min) were substituted in the following formula:
ΔA/min × 1905 = AST activity in U/L at 340nm.

Total Bilirubin

Total bilirubin in mg/dL was determined by using a kit from Fardiag.

Principle

Total bilirubin an acid medium and in the presence of a cationic detergent, reacts with sulphanilic acid to form a pink diazo compound (azobilirubin). Color intensity is proportional to the concentration of direct bilirubin present in the sample
Reagent of the kit:

Components of the kit were:

REAGENT 1

Sulphanilic acid	3.5 mmol/L
Hydrochloric acid	0.06 mol/L
Detergent	7.2 g

REAGENT 2

Sodium nitrite	13 mmol/L

Protocol

- Wavelength: 546nm
- Pathlength: 1 cm

- Reading: against blank sample
- Temperature: 37 ° C.
- Method: end point
- Reaction: 10 minutes
- Sample/reagent: 1/15

Pipetted the reagents and serum into cuvette as followed:

	Blank	Sample
Reagent 1	1500µl	1500µl

Allow the reagent to reach 37 °C and added:

Reagent 2	100µl
Sample	100µl

Mixed carefully, incubated exactly for 10 minutes at 37 ° C. sample (As) absorbance was read against blank sample.

To calculate the bilirubin activity in mg/dL at 546nm Absorbance/ minute readings (ΔA/min) were substituted in the following formula:
ΔA/min × 20.4 = Bilirubin activity in mg/dL at 546nm.

Histological procedure

Liver specimens were fixed in sera the composition of which was:

Acetic acid	10ml
Formalin	30ml
Rectified alcohol	70ml

After placing the tissues for 5 hours in sera the tissues were processed in alcoholic grades:

80%	overnight
90%	2hours
100%	2hours

After alcoholic grades the tissues were placed in cedar wood oil until the tissues became transparent. After that the tissues were embedding in wax by passing them through the following steps :

Benzole I	15 min
Benzole II	15 min
Benzole +Paraplast	20 min at 63°C
Paraplast	12 hours at 63°C
Paraplast	12 hours at 63°C
Paraplast	12 hours at 63°C

After embedding the tissues in wax, blocks were made and sections of 5μm thick were cut by using microtome. Tissues were stained by the following method:

Xylene I	20 min
Xylene II	20 min
100% alcohol	5 min
90% alcohol	5 min

70% alcohol	5 min
50% alcohol	5 min
30% alcohol	5 min
Hematoxylin	1-2 dips
Tap water	10 min
30% alcohol	5 min
50% alcohol	5 min
70% alcohol	5 min
90% alcohol	5 min
Eosin	2 dips
90% alcohol	5 min
100% alcohol	5 min
Xylene	10 min

Histological measurements

Using a Nikon microscope histological study was carried out. Measurements were made by first calibrating the ocular micrometer with stage micrometer. The following histological parameters were studied in the current study.

Hepatic cell plate width

Hepatic cell plate width (HCPW), defined as the distance between two adjacent sinusoids was calculated as the mean value of at least 10 measurements per field at 400x. HCPW were expressed in μm.

Sinusoidal width

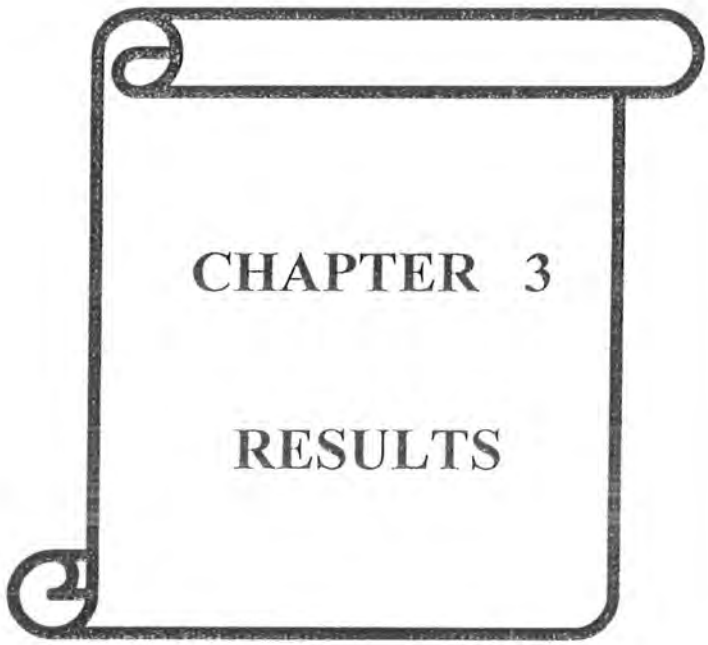
Sinusoidal width (SW), defined as the distance between two adjacent cell plates was calculated as the mean value of at least 10 measurements per field at 400x. Its values were expressed in μm.

Evaluation of Necrotic and Apoptosis nuclei

The number of necrotic and apoptotic nuclei were counted in 20 high power (x400) fields using a Nikon microscope. 100 hepatocytes were counted to determine the ratio of necrotic and apoptotic nuclei to normal nuclei. All histological evaluations were done in a blinded fashion.

Statistical analysis

Results are presented as mean ± standard error (SE). Data was analyzed by students *t*-test.



CHAPTER 3

RESULTS

RESULTS

In order to evaluate the efficacy of the two drugs in improving the regeneration of fibrotic liver after partial hepatectomy (PHx) different biochemical and histological parameters of regenerating livers was determined, the results of which are given below:

General observations

The animals receiving olive oil were normal and active during the whole treatment. Most of the animals in the fibrotic group receiving carbon tetrachloride (CCl_4) intraperitoneally appeared normal but few of them were lazy. No mortality was observed in the olive oil treated groups, however two animals from the fibrotic group died during the treatment. Autopsy showed that their livers were badly damaged (Table 1a) (Fig 2).

One animal from vehicle group with normal liver and one animal belonging to fibrotic control group died during the surgery. Similar was the case with animal mortality after the surgery i.e. one animal from vehicle group while one animal in the control group died after the surgery (Table 1b&1c).

Body weights

Initial body weights of the animals at the start of experiment was determined .No significant difference ($p=0.072, t=2.27$) was found between the body weights of animals placed in olive oil and carbon tetrachloride (CCl_4) treatment group at the start of experiment. Final body weights of rats at the end of olive oil and carbon tetrachloride treatment also showed the same result. There was no significant difference ($p=0.14, t=1.67$) in the body weights of olive oil and CCl_4 treated rats.

The rate of increase in body weights of animals was also determined at the end of olive oil and carbon tetrachloride treatment (Table 2). The rate at which body weights of rats receiving CCl_4 and olive oil, increased showed that vehicle treated animals had higher

Table 1a: Number and rate of mortality of animals treated with olive oil and carbon tetrachloride (CCl_4) for seven weeks.

Treatment Groups	No. of mortalities	Mortality rate %
Olive oil (n =6)	0	0
Carbon tetrachloride (CCl_4) (n=18)	2	11

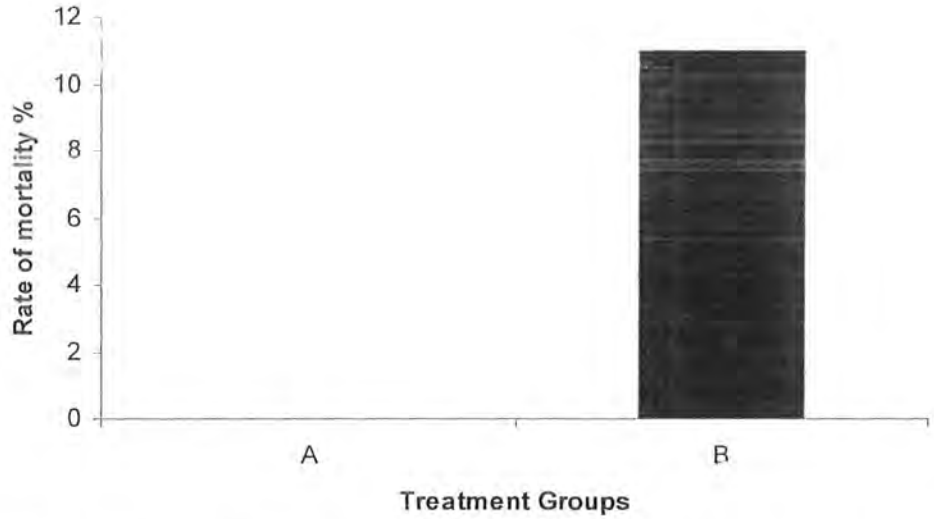


Fig 2: Rate of mortality in animals caused by seven-week olive oil and CCl_4 treatment.
A: Olive oil treated group.
B: CCl_4 treated group.

Table 1b: Number and rate of mortalities during surgery in different groups.

Treatment Groups	Number of mortalities during PHx	Rate of mortality during PHx
Olive oil +Saline + PHx	1	16.67
Fibrosis +Saline +PHx	1	16.67
Fibrosis +ACE-Inhibitor +PHx	0	0
Fibrosis +Silymarin+ PHx	0	0

Table 1c: Number and rate of mortalities after surgery in different groups.

Treatment Groups	Number of mortalities during PHx	Rate of mortality during PHx
Olive oil +Saline + PHx	1	16.67
Fibrosis +Saline +PHx	1	16.67
Fibrosis +ACE-Inhibitor +PHx	0	0
Fibrosis +Silymarin+ PHx	0	0

Table 2: Initial, final and rate of increase in body weights of animals treated with olive oil and CCl_4 for seven weeks.

Treatment Groups	Initial Body weight	Final Body weight	Rate of increase in Body weight %
Olive oil (n =6)	204.17±1.5	240.00± 5.8	17.60±2.8
Carbon tetrachloride (CCl_4) (n=18)	197.50±2.5	227.50±4.8	13.75±2.4

[a] Olive oil vs CCl_4
*p<0.05
**p<0.01
***p<0.001

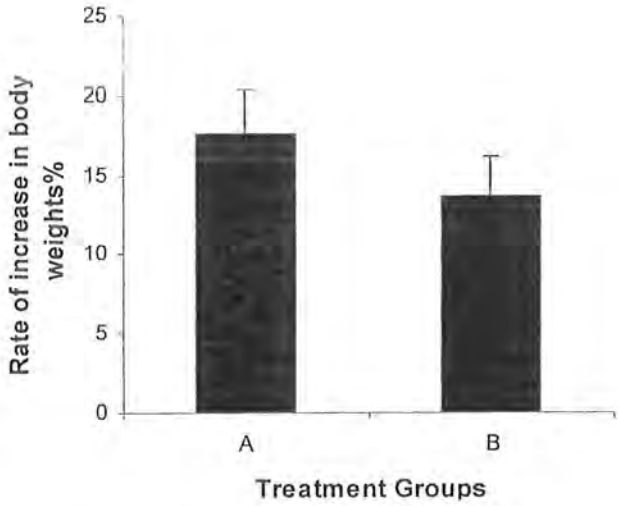


Fig 3:Rate of increase in body weights of animals administered wit oral treatment of olive oil (1.5ml/kg) and CCl_4 (1.5ml/kg).
[a] Olive oil vs CCl_4
*p<0.05
**p<0.01
***p<0.001

rate of increase in body weights as compared to fibrotic ones, however, this difference was not significant (p=0.33,t=1.04) (Fig 3).

Liver regeneration rate (LRR)

The liver regeneration rate is determined by Fishback (Fishback, 1929) formula and expressed in percentages. LRR values of different groups are shown in Table 4. Vehicle group had significantly higher LRR values than all other groups.

A comparison between olive oil treated animals and CCl_4 treated fibrotic animals showed that the former had highly significantly more (p=0.0051,t=4.76) LRR after PHx than vehicle. Drug treated fibrotic rats had also significantly lower (p=0.011,t=3.91 for ACE inhibitor; p=0.0047,t=2.62 for silymarin) LRR values than vehicle group.

It was observed that both ACE inhibitor and silymarin treatment did not produce any significant increase in the LRR of fibrotic rats after PHx. LRR of both drug treated groups was higher than fibrotic control but this increase was not significant at $p<0.05$(p=0.13,t=1.82 for ACE inhibitor; p=0.089,t=2.10 for silymarin). No significant difference (p=0.56,t=0.62) was found when comparison of the LRR values of two drug treated groups was made (Fig 4).

Relative liver weight (RLW)

Relative liver weight of different groups is given in Table. There was no significant difference (p= 0.54,t=0.67) between RLW of vehicle and control fibrotic animals. Similar is the result when vehicle group was compared with drug treated groups. No significant difference (p=0.37,t=1.02) was observed between ACE inhibitor treated fibrotic animals and vehicle. Fibrotic rats receiving silymarin had also shown no significant difference (p=0.56,t=0.62) from the vehicle.

Table 3: LRR and RLW of regenerating livers.

Treatment Groups	LRR %	RLW
Olive oil +Saline + PHx	10.57±0.57	0.035±0.0023
Fibrosis +Saline +PHx	5.65±0.86 [a**]	0.037± 0.0011
Fibrosis +ACE-Inhibitor +PHx	7.49±0.54 [b*]	0.032±0.0018
Fibrosis +Silymarin+ PHx	8.06±0.76 [c*]	0.037±0.0022

LRR: Liver Regeneration rate.
RLW: Relative liver weight.
[a] Vehicle vs Control
[b] Vehicle vs ACE -Inhibitor
[c] Vehicle vs Silymarin
[d] Control vs ACE-Inhibitor
[e] Control vs Silymarin
[f] ACE-Inhibitor vs Silymarin
*p<0.05
**p<0.01
***p<0.001

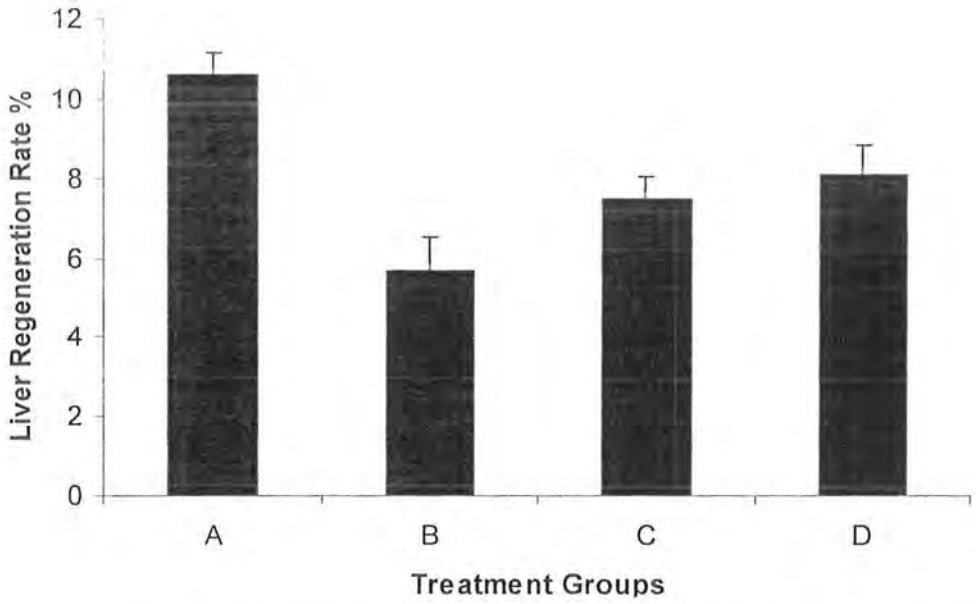


Fig 4:LRR of different groups 24 hours post PHx. Data is expressed as Mean ± S.E

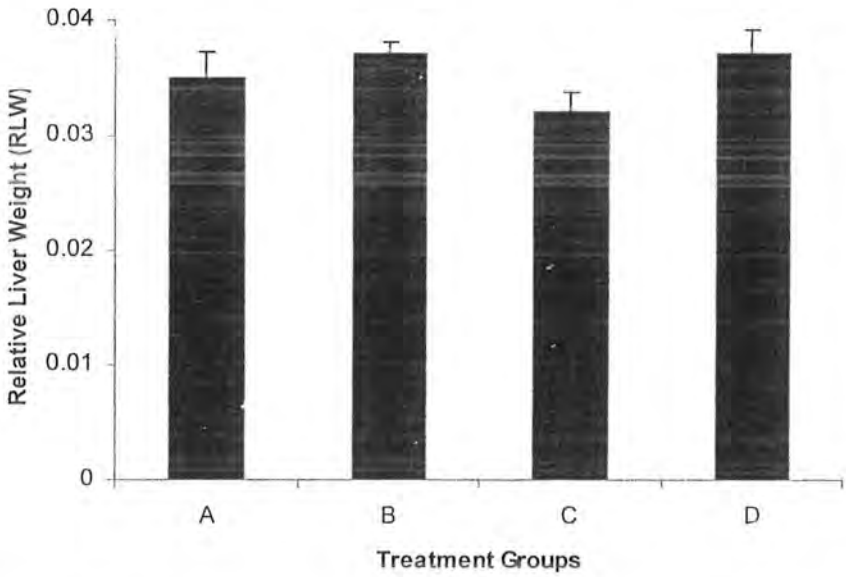


Fig 5:RLW of different groups 24 hours post PHx. Data is expressed as Mean ± S.E

A: Olive oil +Saline + PHx
B:Fibrosis +Saline +PHx
C:Fibrosis +ACE-Inhibitor +PHx
D:Fibrosis +Silymarin+ PHx

[a]Fibrotic Control vs ACE-Inhibitor
[b]Fibrotic Control vs Silymarin
[c]ACE- Inhibitor vs Silymarin

*p<0.05, **p<0.01, ***p<0.001

The comparison of RLW of untreated fibrotic and drug treated fibrotic animals showed no significant difference (p=0.065,t=2.26 for ACE inhibitor; p=0.92,t=0.1for silymarin). There was no significant difference (p=0.12,t=1.75) between the RLW of the two drug groups as was evident from their p-value (Table 4, Fig 5).

Biochemical parameters

To evaluate the liver function of regenerating livers after partial hepatectomy certain biochemical parameters like ALT, AST and bilirubin were assessed.

Alanine aminotransferase (ALT)

After PHx ALT levels of vehicle, control , silymarin and ACE inhibitor treated animals were 537.4±16, 810.4±20, 701.0±27 and 815.3±17 U/mol respectively (Table 5). The ALT of normal animals were significantly less than all other groups, which contain animals with fibrotic livers.

There was highly significant (p=0.0001,t=10.55) increase in the ALT level of fibrotic animals as compared to the normal animals. The ALT levels of drug treated groups were also significantly higher (p=0.0019,t=5.25 for ACE inhibitor group; p=0.0000,t=11.62for silymarin group) than the vehicle group.

There was significantly low (p=0.017,t=3.29) ALT levels in ACE inhibitor treated fibrotic rats and untreated fibrotic ones, showing that the drug is quite effective in improving the ALT values after the hepatectomy of fibrotic liver. However there was no significant difference (p=0.86,t=0.19) in the ALT levels of fibrotic control and silymarin treated fibrotic animals after PHx indicating that the drug has no effect on the ALT values of fibrotic liver after partial hepatectomy.

The comparison of the ALT levels of the drug treated animals showed that ACE inhibitor was quite effective and better than silymarin as it had significantly (p=0.011,t=3.6) lower the enzyme levels of partially hepatectomized fibrotic liver (Fig 6).

Aspartate Aminotransferase (AST)

AST level of vehicle, control ACE- inhibitor and silymarin group after PHx was 540.9±5.1, 852.5±28, 723.9±20 and 842.0 ±16 U/mol respectively. Table 4 shows the AST levels of different groups. Animals from vehicle group having normal liver had significantly less AST levels as compared to all other groups the animals of which had fibrotic livers.

Animals in the vehicle group had significantly (p=0.0017,t=10.79) less AST levels as compared to the fibrotic animals. Drug treated fibrotic animals had also significantly higher (p=0.0009,t=8.88 for ACE inhibitor group; p=0.0001,t=17.55for silymarin group) AST levels than the vehicle after surgery.
The ACE inhibitor treated fibrotic animals had significantly (p=0.014,t=3.7) less AST levels than untreated ones thereby showing that the drug effectively improved AST levels of fibrotic liver after partial hepatectomy. However there was no significant difference (p=0.77,t=0.32) in the AST levels of untreated fibrotic and silymarin receiving animals showing that the drug had no effect on the AST levels after partial hepatectomy of fibrotic liver.

When AST levels of drug treated groups were compared then a significant difference between the two groups was observed. The enzyme in ACE-inhibitor treatment had significantly lower the enzyme (p=0.0027,t=4.57) than silymarin indicating more effectiveness of the drug than silymarin (Fig 7).

Table 4: Biochemical parameters of regenerating livers.

Treatment Groups	ALT U/L	AST U/L	Bilirubin mg/dL
Olive oil +Saline + PHx	537.4 ±16	540.9± 5.1	1.20±0.09
Fibrosis +Saline +PHx	810.4-±20[a***]	852.5± 28[a**]	2.09 ±0.26[a***]
Fibrosis +ACE-Inhibitor +PHx	701.0 ±27 [b**d*]	723.9± 20[b***d*]	0.82±0.11[b***d**]
Fibrosis +Silymarin+ PHx	815.3 ±17[c***f*]	842.0-±16[c***f**]	1.51 ±0.15[c***f*]

ALT: Alanine Aminotransferase
AST: Aspartate Aminotransferase,

[a] Vehicle vs Fibrotic Control
[b] Vehicle vs Ace -Inhibitor
[c] Vehicle vs Silymarin
[d] Fibrotic Control vs ACE-Inhibitor
[e] Fibrotic Control vs Silymarin
[f] Ace-Inhibitor vs Silymarin

*p<0.05
**p<0.01
***p<0.001

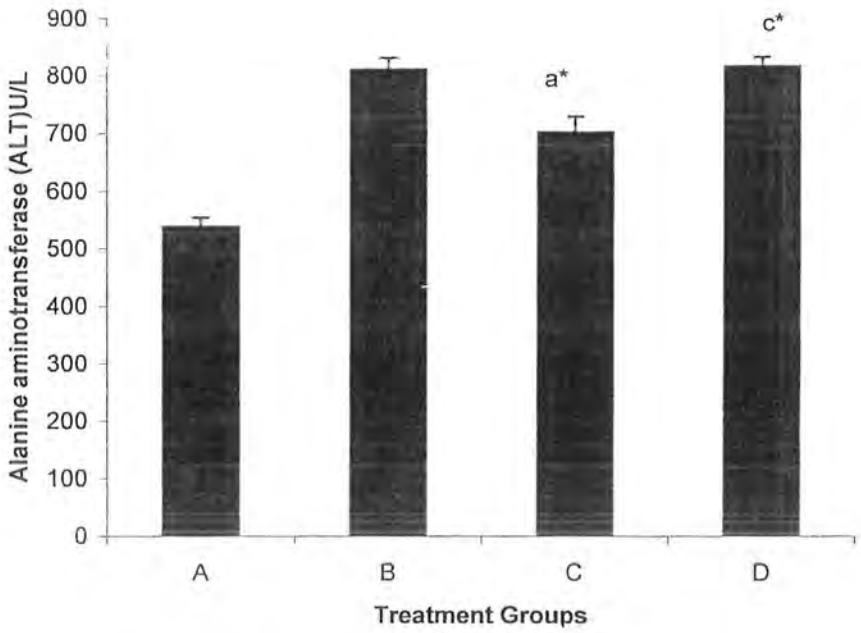


Fig 6:ALT U/L of different groups 24 hours post PHx .Data is expressed as Mean ± S.E

A: Olive oil +Saline + PHx
B:Fibrosis +Saline +PHx
C:Fibrosis +ACE-Inhibitor +PHx
D:Fibrosis +Silymarin+ PHx

[a]Fibrotic Control vs ACE-Inhibitor
[b]Fibrotic Control vs Silymarin
[c]ACE- Inhibitor vs Silymarin

*p<0.05, **p<0.01, ***p<0.001

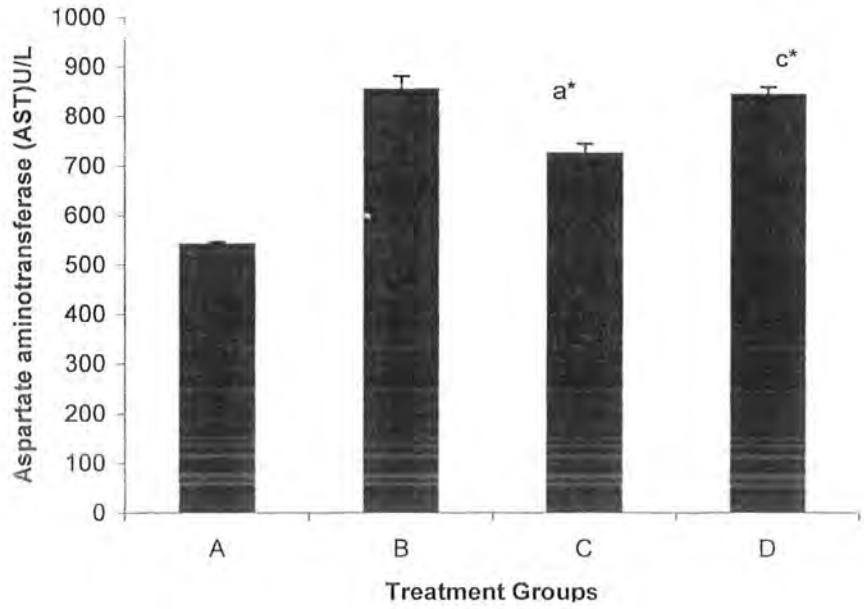


Fig 7:AST U/L of different groups 24 hours post PHx . Data is expressed as Mean ± S.E

A: Olive oil +Saline + PHx
B:Fibrosis +Saline +PHx
C:Fibrosis +ACE-Inhibitor +PHx
D:Fibrosis +Silymarin+ PHx

[a]Fibrotic Control vs ACE-Inhibitor
[b]Fibrotic Control vs Silymarin
[c]ACE- Inhibitor vs Silymarin

*p<0.05, **p<0.01, ***p<0.001

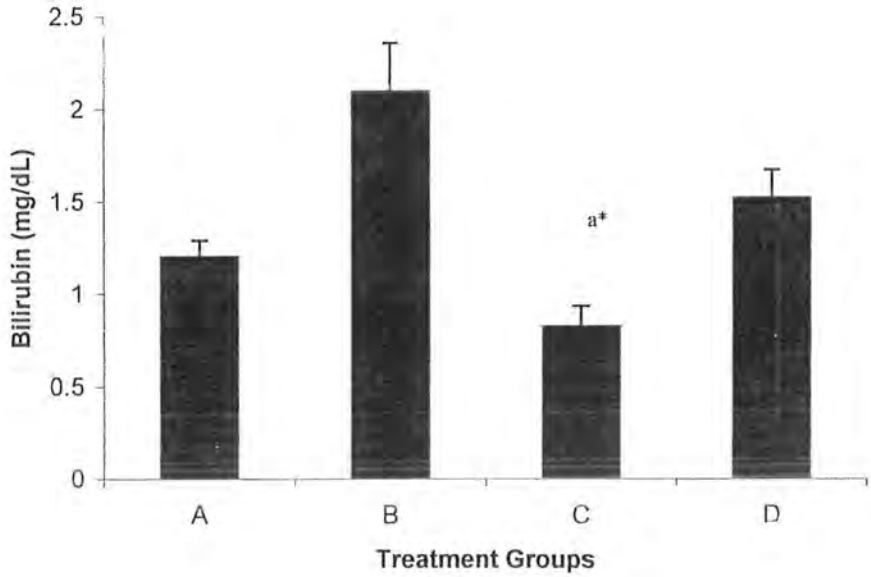


Fig 8:Bilirubin mg/dL of different groups 24 hours post PHx. Data is expressed as Mean ± S.E

A: Olive oil +Saline + PHx
B:Fibrosis +Saline +PHx
C:Fibrosis +ACE-Inhibitor +PHx
D:Fibrosis +Silymarin+ PHx

[a]Fibrotic Control vs ACE-Inhibitor
[b]Fibrotic Control vs Silymarin
[c]ACE- Inhibitor vs Silymarin

*p<0.05, **p<0.01, ***p<0.001

Bilirubin

Bilirubin levels observed after PHx of vehicle, control, ACE inhibitor and silymarin receiving rats were 1.20±0.09, 2.09±0.26, 0.82±0.11 and 1.51±0.15mg/dL respectively (Table 5). Bilirubin levels of vehicle were significantly less than all other groups that had animals with fibrotic livers.

The bilirubin of untreated fibrotic animals was highly significantly ($p=0.045, t=3.31$) elevated than the olive oil treated vehicle. There was also significant difference ($p=0.036, t=2.7$) in the bilirubin levels of vehicle and ACE inhibitor treated groups the value of latter was less than the vehicle. No significant difference ($p=0.13, t=1.77$) was observed between the bilirubin levels of silymarin receiving rats and vehicle.

As far as the comparison of untreated fibrotic and drug treated groups is concerned then it was observed that fibrotic animals had significantly higher bilirubin levels than ACE inhibitor group ($p=0.010, t=4.58$) indicating that the drug was quite effective in improving the bilirubin levels after the hepatectomy of fibrotic liver. However there was no significant difference ($p=0.11, t=1.96$) in the bilirubin levels of fibrotic control and silymarin treated fibrotic rats indicating that the drug failed to lower the bilirubin levels of fibrotic liver after partial hepatectomy.

The comparison of bilirubin levels of drug treated groups showed that ACE inhibitor is quite effective in lowering the bilirubin levels than silymarin as there is significant difference ($p=0.0081, t=3.67$) between the their bilirubin values (Fig 8).

Histological studies

The following histological parameters were included in the present study.

Hepatic cell plate width (HPCW)

The hepatic cell plate width of different groups is given in Table 5. Doubling of HCPW, a character of regenerating liver after PHx can be easily seen in vehicle. No significant difference (p=0.34,t=0.97) between HCPW of vehicle and fibrotic control was observed. HCPW of vehicle was significantly (p=0.0018,t=3.41) more than ACE inhibitor treated fibrotic rats, however, there was no significant difference (p=0.9,t=0.12) between the HCPW of vehicle and those animals, which were given silymarin.

As far as the comparison of fibrotic control with drug treated groups is concerned there was no significant difference (p=0.39,t=0.87) between the HCPW of fibrotic control and silymarin receiving animals. However, the HCPW of ACE inhibitor treated animals was significantly less (p=0.022,t=2.43) than the fibrotic control. Comparison of HCPW of ACE inhibitor and silymarin treated fibrotic rats showed that the former had significantly less (p=0.0020,t=3.41) HCPW than the latter one (Fig 9, 10.1,10.2,10.3&10.4).

Sinusoidal width (SW)

The width of sinusoidal spaces in different groups is expressed in Table 5. The SW of vehicle group was significantly more (p=0.0013,t=3.52) than the fibrotic control .The comparison of vehicle group with drug treated animals showed that there was no significant difference (p=0.82,t=0.23) between the SW of vehicle and ACE inhibitor receiving fibrotic rats, however, silymarin treated animals had significantly less (p=0.0002,t=4.31) SW than the vehicle.

Vehicle and ACE inhibitor receiving animals showed significantly more (p=0.0017,t=3.39) SW than the fibrotic control while silymarin group showed no significant (p=0.34,t=0.97) difference from control. The comparison of SW values of the two drug groups showed that silymarin group had significantly less (p=0.0002,t=4.21) SW than ACE inhibitor group (Fig 10.1,10.2,10.3,10.4&11).

Table 5: Histological parameters of regenerating livers.

Treatment Groups	HCPW μm	SW μm	Necrotic and Apoptotic nuclei/100 nuclei
Olive oil +Saline + PHx	16.97±1.5	9.46±0.77	34.87±0.9
Fibrosis +Saline +PHx	14.92±1.5	6.0±0.61[a**]	53.49 ±1.6 [a***]
Fibrosis +ACE-Inhibitor +PHx	10.33±1.2 [b**d*]	9.23±0.73[d**]	44.57±1.0 [b***d**]
Fibrosis +Silymarin+ PHx	16.72±1.4	5.14±0.64[c***f***]	49.31±1.3[c***f*]

SW: Sinusoidal width
HCPW: Hepatic Cell Plate Width.

[a] Vehicle vs Control
[b] Vehicle vs ACE -Inhibitor
[c] Vehicle vs Silymarin
[d] Control vs ACE-Inhibitor
[e] Control vs Silymarin
[f] ACE-Inhibitor vs Silymarin
*p<0.05
**p<0.01

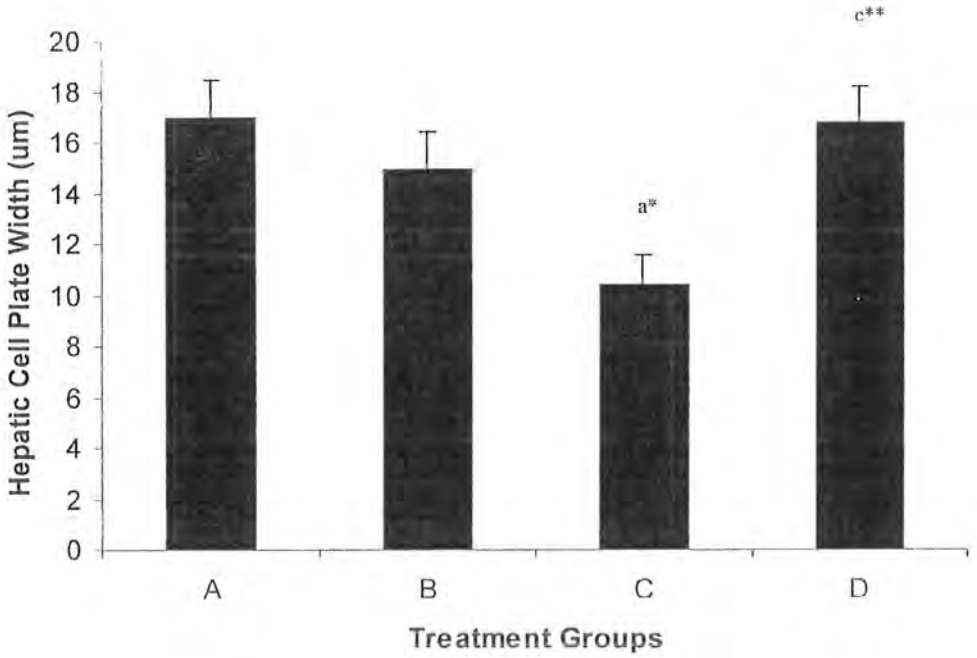


Fig 9:Hepatic Cell Plate Width found in regenerated livers of different groups 24 hours post PHx. Data is expressed as Mean ± S.E

A: Olive oil +Saline + PHx
B:Fibrosis +Saline +PHx
C:Fibrosis +ACE-Inhibitor +PHx
D:Fibrosis +Silymarin+ PHx

[a]Fibrotic Control vs ACE-Inhibitor
[b]Fibrotic Control vs Silymarin
[c]ACE- Inhibitor vs Silymarin

*p<0.05, **p<0.01, ***p<0.001

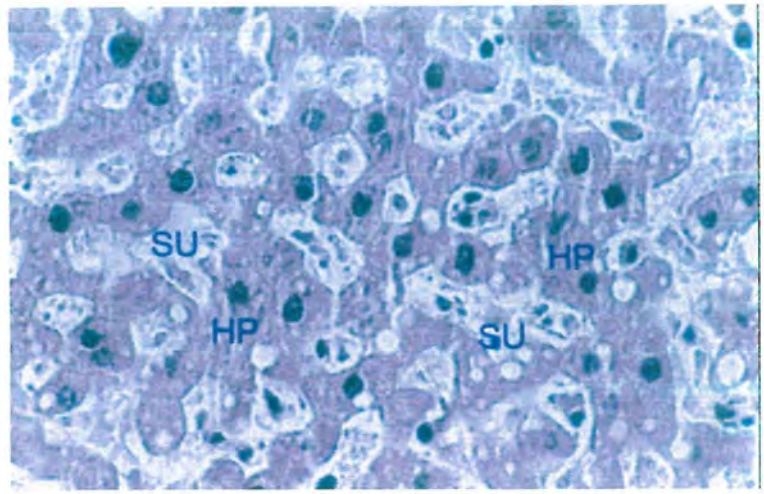


Fig 10.1:Photomicrograph of regenerated rat liver section 24 hours post PHx. Animal in this group were treated with olive oil for seven weeks + Saline for one week +PHx. Widened sinusoidal width (SU) can be seen, doubling of hepatic cell plate (HP) is also in view which is a characteristic of regenerating liver after PHx ×400. HE.

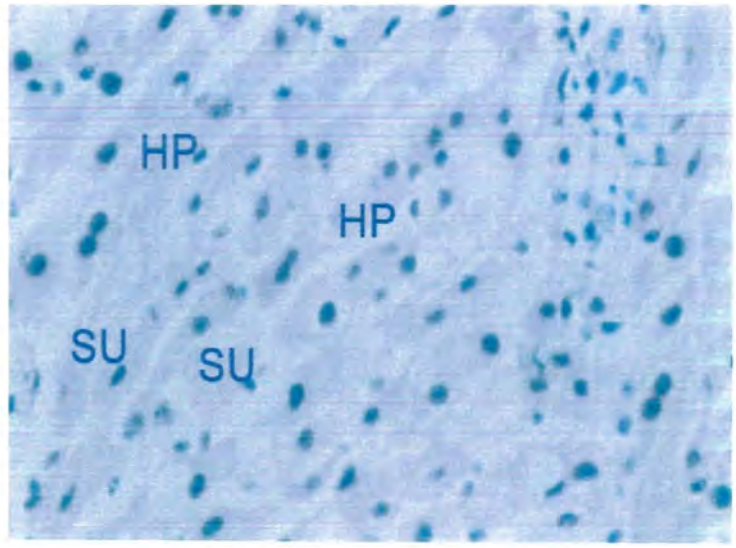


Fig 10.2:Photomicrograph of regenerated rat liver section 24 hours post PHx. Animal in this group were treated with CCl_4 for seven weeks + Saline for one week +PHx. Narrow sinusoids (SU) can be seen, hepatic cell plate width (HP) is also in view ×400. HE.

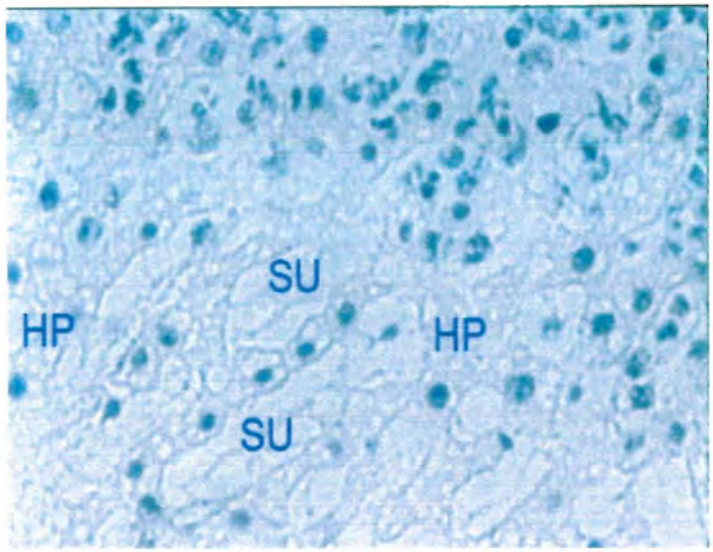


Fig 10.3:Photomicrograph of regenerated rat liver section 24 hours post PHx. Animals in this group were treated with CCl_4 for seven weeks +ACE inhibitor for one week+PHx. Much wider sinusoids (SU) can be seen, hepatic cell plate width (HP) is significantly less than all other groups×400. HE

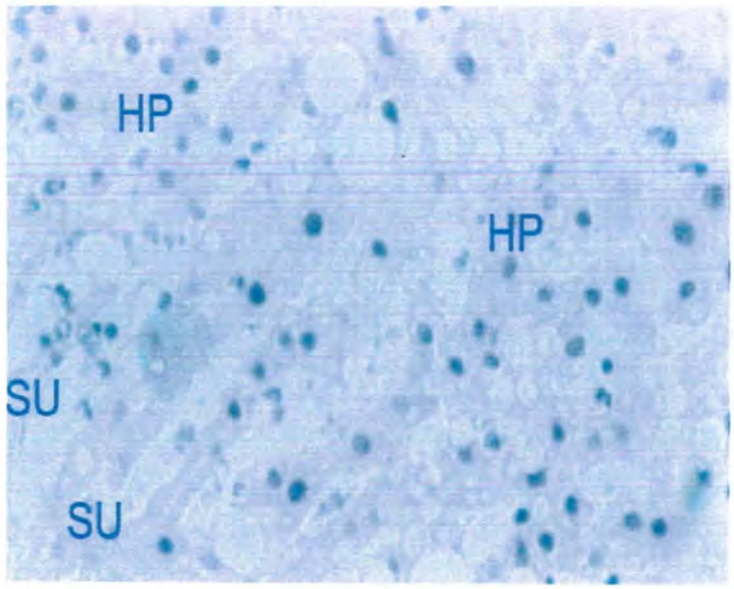


Fig 10.4:Photomicrograph of regenerated rat liver section 24 hours post PHx. Animal in this group were treated with CCl_4 for seven weeks + Silymarin for one week +PHx. sinusoids (SU) are narrow compared to vehicle and ACE inhibitor treatment, hepatic cell

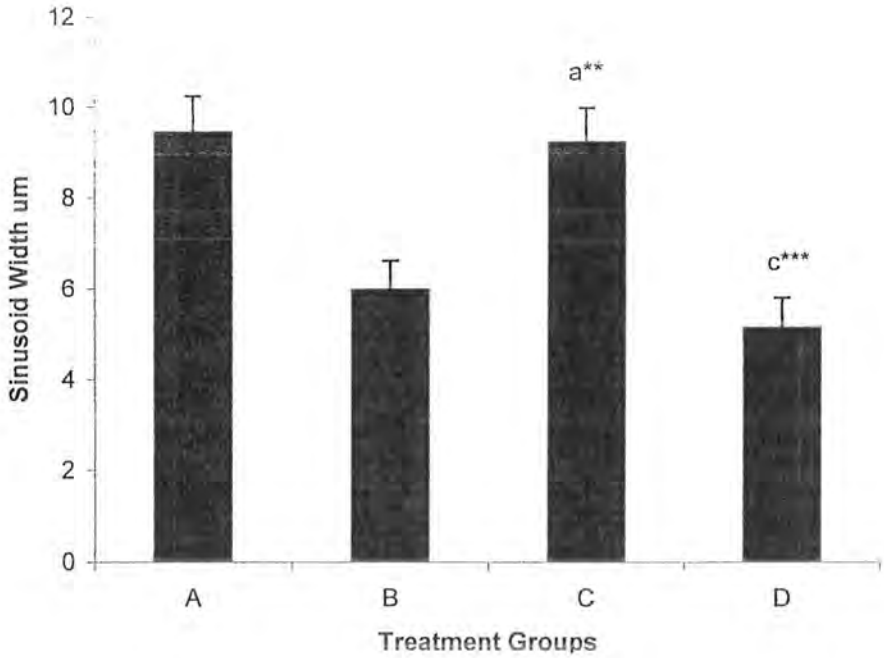


Fig 11:Sinusoidal width (SW) found in regenerated livers of different groups 24 hours post PHx .Data is expressed as Mean ± S.E

A: Olive oil +Saline + PHx
B:Fibrosis +Saline +PHx
C:Fibrosis +ACE-Inhibitor +PHx
D:Fibrosis +Silymarin+ PHx

[a]Fibrotic Control vs ACE-Inhibitor
[b]Fibrotic Control vs Silymarin
[c]ACE- Inhibitor vs Silymarin

*p<0.05, **p<0.01, ***p<0.001

Necrotic and Apoptotic nuclei

The number of necrotic and apoptotic nuclei in different groups is expressed in Table 5. Necrotic and apoptotic nuclei were highly significantly more ($p=0.0005, t=10.39$) than the fibrotic control. Similar result was observed when the number of necrotic and apoptotic nuclei found in vehicle and drug treated fibrotic rats was compared. Necrotic and apoptotic nuclei were highly significantly more ($p=0.0008, t=7.17$for ACE inhibitor group; $p=0.0002, t=9.44$for silymarin group) in both drug treated groups than vehicle.

ACE inhibitor receiving animals showed less number of necrotic and apoptotic nuclei than that of fibrotic control and this reduction was highly significant ($p=0.0051, t=4.76$). Silymarin treated group had almost the same number of necrotic and apoptotic nuclei as was found in fibrotic control because no significant ($p=0.092, t=2.08$) difference from control. The comparison of number of necrotic and apoptotic nuclei found in two drug treated groups showed that ACE inhibitor group had significantly less ($p=0.033, t=2.92$) necrotic and apoptotic nuclei than silymarin group (Fig12 &13.1, 13.2,13.3&13.4).

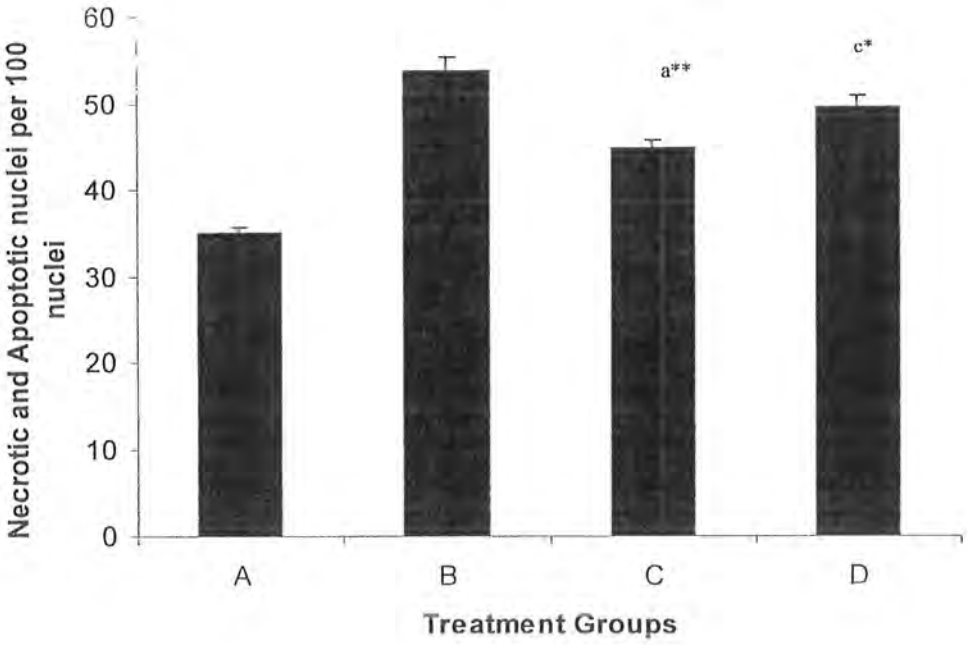
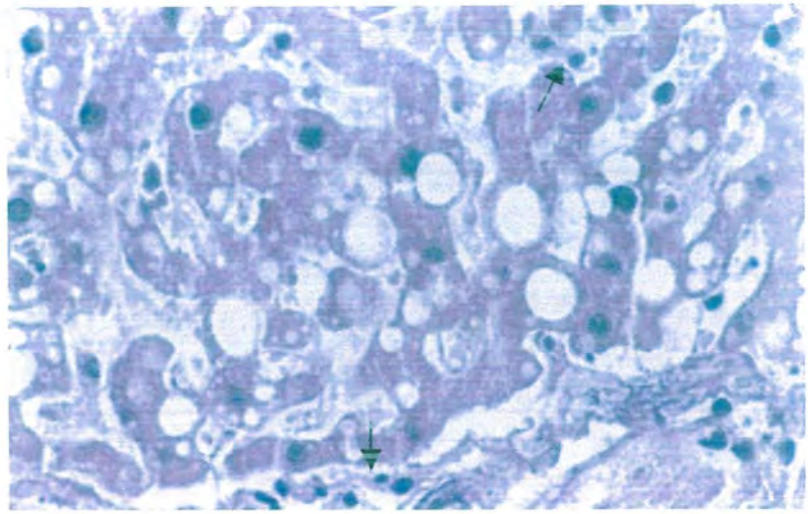


Fig 12:Necrotic and apoptotic nuclei found in regenerated livers of different groups 24 hours post PHx. Data is expressed as Mean ± S.E

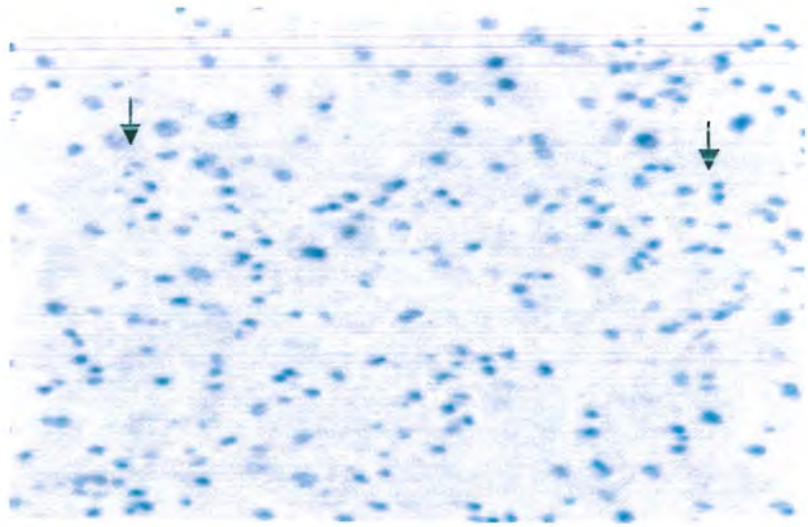
A: Olive oil +Saline + PHx
B:Fibrosis +Saline +PHx
C:Fibrosis +ACE-Inhibitor +PHx
D:Fibrosis +Silymarin+ PHx

[a]Fibrotic Control vs ACE-Inhibitor
[b]Fibrotic Control vs Silymarin
[c]ACE- Inhibitor vs Silymarin

*p<0.05, **p<0.01, ***p<0.001



Photomicrograph of regenerated rat liver section 24 hours post PHx. Animal in this group were treated with olive oil for seven weeks + Saline for one week +PHx. Necrotic and apoptotic nuclei can be seen ×400. HE



Photomicrograph of regenerated rat liver section 24 hours post PHx. Animal in this group were treated with CCl_4 for seven weeks + Saline for one week +PHx. A large area occupied by necrotic and apoptotic nuclei can be seen ×400. HE

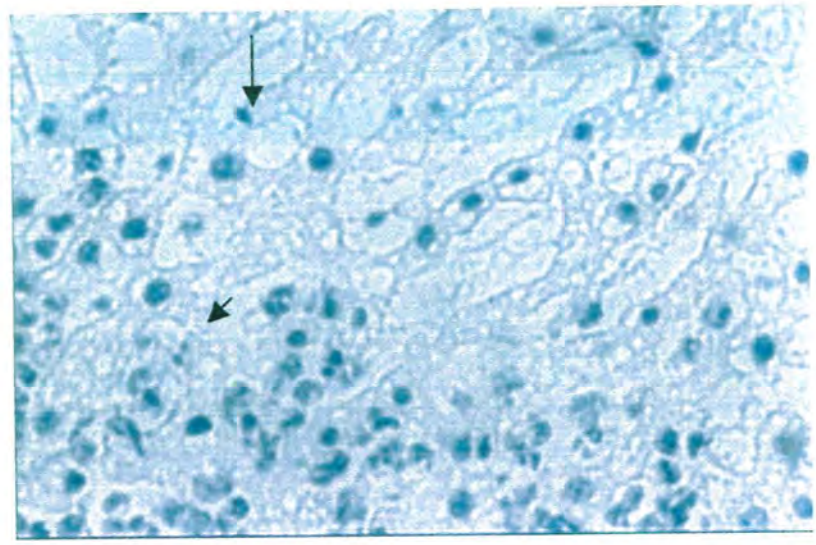


Fig 13.3:Photomicrograph of regenerated rat liver section 24 hours post PHx. Animals in this group were treated with CCl_4 for seven weeks +ACE inhibitor for one week+PHx. Marked necrosis and apoptosis is in view×400. HE

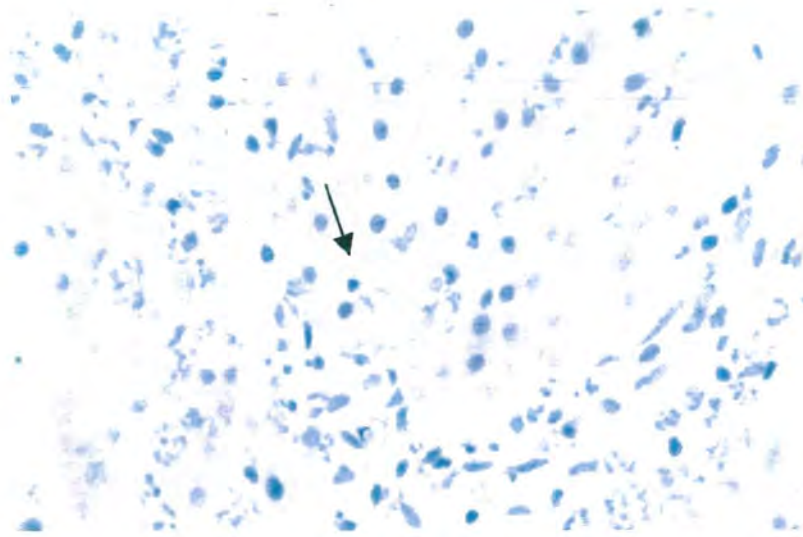
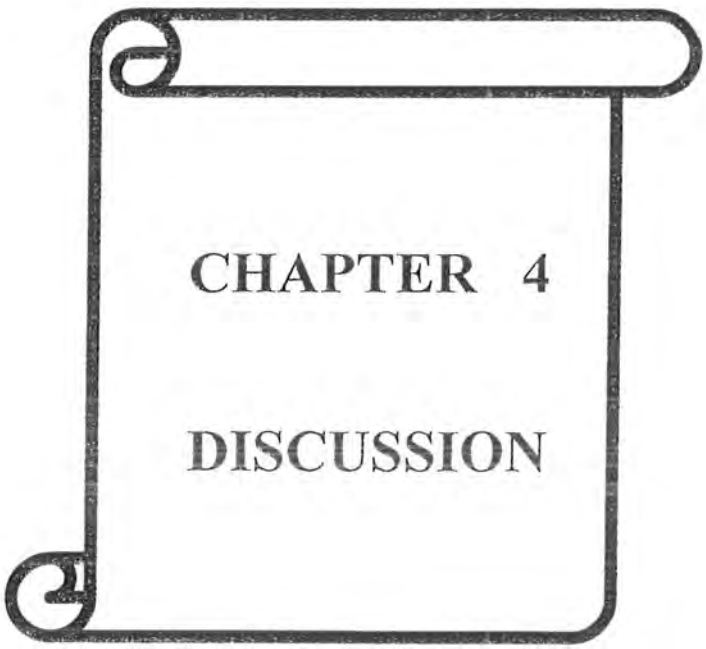


Fig 13.4:Photomicrograph of regenerated rat liver section 24 hours post PHx. Animal in this group were treated with CCl_4 for seven weeks + Silymarin for one week +PHx. sinusoids (SU) are narrow compared to vehicle and ACE inhibitor treatment. Extreme necrosis and apoptosis can be clearly seen. Fibrotic scar is also in view×400. HE.



CHAPTER 4

DISCUSSION

DISCUSSION

In this study the comparative efficacy of silymarin and angiotensin converting enzyme (ACE) inhibitor in improving the fibrotic liver regeneration is evaluated. Liver diseases have become more and more common now and surgical resection is often required. Normal liver has a remarkable capacity to regenerate (Nagino *et al.*, 2001; Higgins and Anderson, 1931) but the fibrotic and cirrhotic liver has impaired and slow liver regeneration as compared to control (Andiran *et al.*, 2000; Kawasaki *et al.*, 1992; Hashimoto and Watanabe, 1999) Severe fibrosis of the liver parenchyma is associated with poorer regeneration of the remnant liver leading to poor restoration of post-operative liver function (Miyazaki, 1999). Impaired regeneration and dysfunction of the cirrhotic liver following partial hepatectomy (PHx) are the most serious risk factors for postoperative liver failure (Xue, *et al.*, 2002).

There are certain therapies that not only enhance liver regeneration but also reduce fibrosis. Silymarin and ACE inhibitor are among them .The former is being clinically used for the treatment of liver diseases while the latter is not currently in use for hepatic disorders. Therefore the present study is conducted to assess the effect of these two drugs on regeneration of fibrotic liver.

Carbon tetrachloride (CCl_4) has been extensively used to induce fibrosis. In the present study same chemical is used for fibrosis induction. Rajesh and Latha (2004) showed that feeding CCl_4 to rats for two months resulted in significant body weight loss. A reduction in body weights of CCl_4 treated fibrotic rats was observed than that of olive oil treated vehicle in the current study. However this reduction in body weight was not significant ($p>0.05$).

Liver regeneration rate (LRR) determined at 0, 1, 2, 3 and 7 days after partial (70%) hepatectomy in normal and thioacetamide-treated cirrhotic rats showed that liver regeneration of cirrhotic liver was impaired and LRR of cirrhotic liver was significantly

low than the normal (Nakano, 1994) . The current study showed the same result as the LRR of fibrotic animals was highly significantly ($p<0.01$) reduced than vehicle. This result of present study is consistent with what Chijiiwa *et al.*, (1994) had showed in their study. They concluded that liver regeneration rate as expressed by percent of initial liver weight was impaired in the cirrhotic liver, and significantly lower regeneration rate was observed on days 3 and 7 after hepatectomy in the cirrhotic rats as compared with controls. ACE inhibitor, lisinopril administered orally (2.5mg/kg) for one week before PHx had significantly ($p<0.01$) improved the LRR of fibrotic liver after PHx. Ramalho *et al*, (2001) had previously shown that lisinopril an ACE inhibitor given to rat at a dose of 1 mg kg body weight^{-1} day^{-1}, intraperitoneally, for 5 days before hepatectomy, and daily after surgery enhances regeneration in normal liver. The current study showed that ACE inhibitor treatment did not improve LRR of fibrotic liver significantly ($p>0.05$) after PHx. Taken together it is concluded that it only enhances normal liver regeneration and no improvement was observed in case of fibrotic liver.

Silymarin also enhance normal liver regeneration after partial hepatectomy (Savita *et al.*, 1994; Kropachova and Mihurova 1992). Male Sprague-Dawley rats receiving a single dose (140.5mg/kg) of silymarin intravenously had significantly high liver regeneration rate (Magliulo, *et al.*, 1973). However the results of present study indicated that it improved LRR of fibrotic liver but this improvement was not significant ($p>0.05$). The comparison of the LRR values of two drug treatments showed no significant difference ($p>0.05$).

As LRR is determined by the formula containing estimated weight of residual liver at PHx so there are chances of misleading results, therefore, another parameter liver to body weight ratios known as relative liver weight (RLW) was also determined. According to Xue *et al.*, (2002) significant differences in the liver to body weight ratio (%) between cirrhotic and HGF treated groups were found as early as day 2 and peaked on day 10 after PHx. Significantly decreased relative liver weight (RLW) was observed in plasminogen knockout mice as compared to control on 2 and 7 days post PHx (Vogten *et al.*, 2003). In

the present study, which was limited to 24 hours, the relative liver weight of different groups did not show any significant difference ($p>0.05$) after PHx.

Liver enzymes elevate after Partial hepatectomy (Alexandra *et al.*, 2004). The study in which rats had undergone 70% and 85% hepatectomy and their liver enzymes had measured found that liver ALT and AST had significantly elevated levels in both 70% and 85% hepatectomized rats as compared to sham. However bilirubin was highly increased only in rats with 85% liver resection, 70% hepatectomized rats show increased bilirubin but the difference was not significant from that of sham operated rats (Yahya *et al.*, 2005). Carbon tetrachloride induced liver fibrosis also result in increased biochemical markers of liver function (Jung-Chou *et al.*, 2000). Sprague Dawley rats when given subcutaneous injection of 40% CCl_4 (0.3 mL/100 g, every 3 days for 6 weeks) showed significantly increased ALT and AST in fibrotic rats as compared to the rats of control group that recieve injection of same dose of olive oil (Hong Shan *et al.*, 2001). Silymarin significantly lower the liver enzymes especially ALT and AST in fibrotic patients however bilirubin levels were not significantly reduced by it but it was less than that of control (Salmi and Sarna, 1982). Same is the case with ACE inhibitor (Hongshan *et al.*, 2001). Interferon- when given with an ACE inhibitor, perindopril significantly improved all the fibrosis markers in chronic hepatitis C patients. Present study showed that the liver enzymes i.e. ALT and AST were highly significantly elevated in untreated fibrotic rats ($p<0.001$for ALT and $p<0.01$ for AST) than that of normal liver after partially hepatectomy. Bilirubin levels, however, showed some astonishing results as its levels were significantly ($p<0.05$) less in ACE inhibitor treated fibrotic rats as compared to vehicle. There found no significant ($p>0.05$) difference in the bilirubin levels of vehicle and silymarin receiving fibrotic rats. Fibrotic control had significantly high ($p<0.05$) bilirubin levels than vehicle. ACE inhibitor was successful in significantly ($p<0.05$ for ALT and AST, $p<0.01$ for bilirubin) improving all the biochemical parameters of regenerating fibrotic liver studied in present research. However silymarin failed to significantly ($p>0.05$) lower the liver enzymes after PHx. The comparison of the two drugs showed that ACE inhibitor showed much better results as compared to silymarin as far as decrease in liver enzyme levels are concerned as it had significantly ($p<0.05$ for

ALT, $p<0.01$ AST and for bilirubin) lowered these enzymes than silymarin in fibrotic rats 24 hours after PHx.

Another important feature of liver after PHx is that the hepatic cell plate width (HCPW) is increased (Martinez. and Amenta, 1995; Wack, K. *et al.*, 2001). HCPW of rats undergoing partial hepatectomy was significantly more than sham on 1, 2, 4 and 7 days post PHx. In present study no significant ($p>0.05$) in HCPW of fibrotic and normal livers after the surgery was observed. ACE inhibitor had highly significantly less ($p<0.01$ ACE inhibitor vs vehicle; $p<0.05$ ACE inhibitor vs fibrotic control) HCPW than vehicle and fibrotic control. However silymarin showed no significant ($p>0.05$) difference from both that of vehicle and fibrotic control but it must be noted that its HCPW was more than fibrotic control but this difference is not significant. HCPW of silymarin treated rats was highly significantly ($p<0.01$) more than that of ACE inhibitor treated ones.

An astonishing feature of PHx is the widening of liver sinusoidal spaces (Eugenio *et al.*, 1998). 24 hours after PH, increased distance between sinusoids was observed in mice subjected to 70% partial hepatectomy (Vogten *et al.*, 2003). Following partial hepatectomy blood flow-to-liver mass ratio reached maximal values 24 hrs post resection (Braet, 2004). It must be noted that in tumor growth microvessels are highly abnormal and impede normal perfusion dynamics (Jain, 2001). Hepatic fibrosis and cirrhosis is also characterized by decreased sinusoidal density, microvascular shunting and disturbances in diffusion and permeability (Varin and Huet, 1985; Vollmar *et al.*, 1998; Rappaport *et al.*, 1983). The present study gave the result that normal animals had significantly ($p<0.05$) wider sinusoidal spaces as compared to fibrotic animals after PHx. Silymarin could not widen the sinusoidal width (SW) of fibrotic animals significantly ($p>0.05$). However ACE inhibitor that work by blocking the action of angiotensin, a substance that causes the blood vessels to narrow had significantly widened the sinusoidal spaces as compared to control and silymarin treated animals. ACE inhibitor at comparable at clinically comparable low doses exerted significant inhibitory effects on tumor development in the liver. ACE-I in combination with Vitamin K (VK) showed a more potent suppressive effect against hepatocarcinogenesis. Neovascularization increased

during hepatocarcinogenesis, and VK and ACE-I significantly attenuated angiogenesis in the tumor (Yoshiji et al., 2006).

There are certain diseases i.e.steasois and cirrhosis, which enhance apoptosis in liver after partial hepatectomy. (Deaciuc *et al.*, 2001). When mRNA levels of pro-apoptotic genes in normal and cirrhotic livers in a mouse model were compared then it was found that their levels were significantly elevated in cirrhotic liver. Furthermore, when these animals underwent 2/3 PH induction of protective antiapoptotic mRNA levels were both decreased and their expression delayed, relative to non-cirrhotic animals undergoing 2/3 PH (Masson *et al.*, 2000). In current study too regenerated fibrotic livers even that of drug treated ones had highly significantly ($p<0.001$) more necrotic and apoptotic nuclei as compared to vehicle. ACE inhibitor treatment had significantly lessened ($p<0.01$) the apoptotic and necrotic nuclei as compared to fibrotic control while silymarin treatment could not produce any significant ($p>0.05$) effect. ACE inhibitors are also known to reduce apoptosis in myocardial ischemia-reperfusion injury, which involves necrosis and apoptosis. The inhibition of angiotensin-converting enzyme (ACE) has been reported to suppress infarct size. Kobara *et al.*, (2003) investigated the effect of ACE inhibitor on myocardial apoptosis and apoptosis-related proteins in rats with experimental myocardial infarction. Quinapril, an ACE inhibitor had significantly reduced the ratio of myocardial infarct size in the ischemic are at a risk. In addition, it had significantly suppressed the incidence of apoptotic myocytes around the necrotic region, the intensity of DNA ladder formation, and the activation of caspase-3. In short inhibition of ACE reduces myocardial infarction and apoptosis .In contrast to this ACE inhibitor along with VK markedly increase the apoptosis in orthotopic hepatocellular carcinoma.the tumor whereas tumor cell proliferation itself was not altered (Yoshiji *et al.*, 2006). Silymarin also induces apoptosis in certain carcinomas .Its active constituent Silibinin significantly induces growth inhibition, a moderate cell cycle arrest and a strong apoptotic death in both small cell and non-small cell human lung carcinoma cells (Sharma *et al.*, 2003). A comparison of the two drugs showed that ACE inhibitor had significantly less ($p<0.05$) necrotic and apoptotic nuclei than silymarin.

In summary it is concluded that ACE-inhibitor has effectively improve the biochemical markers of fibrotic liver 24 hours after partial hepatectomy. ACE inhibitor treatment has widened the sinusoidal spaces but the hepatic cell palate is significantly reduced in its treatment .To study its elaborative effect on biochemical and specially on morphological parameters of fibrotic liver after PHx a more comprehensive study is required. Present study has indicated that silymarin filed to improve the biochemical parameters of regenerating fibrotic liver 24 hours post-PHx but histologically specially in case of HCPW its effect was a positive one. However to evaluate its effect more comprehensively a wider study is needed.

CHAPTER 5

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