The Efficacy of Antioxidative Therapy in Hepatic Fibrosis Induced Experimentally by Bile Duct Ligation in Rats

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Abstract: Liver fibrosis which is a common result of chronic hepatic injury of diverse origins might be related to the occurrence of oxidative stress and accumulation of free radicals. It is characterized essentially by increased secretion and accumulation of different components Extracellular Matrix proteins (ECM). To investigate the beneficial effect of combined supplementation of vitamin E and selenium on hepatic fibrosis induced experimentally in bile duct-ligated rats. Forty albino rats were randomly assigned to 4 groups (10 rats in each), control group, sham-operated group Bile Duct-Ligated (BDL) group where the bile ducts of rats were ligated and BDL group co-treated with vitamin E and selenium where the rats underwent bile duct ligation and were fed with chow supplemented with vitamin E (250 mg kg⁻¹) and selenium (0.2 mg kg⁻¹ diet) starting from three days before the operation. At the end of 3rd week, the rats were sacrificed where blood was taken for biochemical estimations of serum enzymes and bilirubin. Fresh liver tissue was taken for determination of hydroxyproline content. Also, other liver samples of 5 mm³ were immediately fixed and processed for immunohistochemical demonstration of collagen type I and IV, fibronectin and laminin using the indirect immunoperoxidase method. Bile duct ligation resulted in a marked elevation in the levels of serum enzymes, bilirubin and hydroxyproline together with extensive bile duct proliferation. Strong staining was found for collagens I and IV, fibronectin and laminin in enlarged portal spaces around the newly formed bile ductless. Co-treatment with vitamin E and selenium resulted in a shift in the serum enzymes, bilirubin and hydroxyproline towards the normal values. Also, a remarkable decrease of bile duct proliferation and in the intensity of staining for different ECM components in the portal spaces was observed. Antioxidants vitamins E and selenium combination attenuate the development of hepatic fibrosis in bile duct-ligated rats. Long-term, prospective studies in humans with chronic cholestatic liver diseases may be helpful to evaluate the beneficial effects of these elements.

Key words: Liver fibrosis, chronic, hepatic injury, supplement, selenium, Saudi Arabia

INTRODUCTION

Hepatic fibrosis which is one feature of liver cirrhosis is a highly integrated cellular response to tissue injury (Friedman, 2000). It is essentially characterized by secretion and accumulation of Extracellular Matrix proteins (ECM) (Schuppan et al., 2001). The ECM of the liver comprises three sets of protein macromolecules, collagens, non-collagenous glycoproteins and glycosaminoglycans (Rojkind and Ponzo-Noyola, 1982). Collagens form the major matrix component and include at least four genetically distinct subtypes which have been identified immunohistochemically and immunohistochemically (Rojkind et al., 1979; Grimald et al., 1980; Voss et al., 1980). The distribution of various matrix components varies in normal and pathological livers. Some elements, such as laminin, collagen type IV and fibronectin are concentrated in the basement membranes whereas others such as collagens types I, pro III and III are present in intercellular spaces (Martin and Timpl, 1987).

It is generally accepted that once it is established, fibrosis is irreversible (Ramachandran and Iredale, 2009). A few observations however, in man and studies with animals have led to the hypothesis that experimental hepatic fibrosis is a reversible process (Williams et al., 1969; Yeong et al., 1982). Animal models have been proposed for studying the diminishing of hepatic fibrosis. These include Schistosomiasis mansoni in mice (Perez-Tamayo, 1979; Mehlhorn et al., 1982; Morcos et al., 1985) and chemicals, e.g., CCl₄, and ethionine (Perez-Tamayo, 1979). Duration of the treatment and the difficulty of identifying the causes of fibrosis, however had made these models matter in debate.

Extrahepatic cholestasis is another model to investigate the formation of hepatic fibrosis. Prolonged obstruction of bile flow or hepatic diseases that lead to anatomic destruction of the biliary tree results in morphologic and biochemical changes and the development of secondary biliary cirrhosis (Popper and Schaffner, 1985). Changes induced by experimental bile
duct ligation in the rat have been partially analyzed (Carpino et al., 1981; Kountouras et al., 1984). They include an extensive proliferation of bile ducts in enlarged portal spaces with slight inflammation and necrosis and the formation of periportal fibrosis in <2 weeks after obstruction of the biliary tree (Aronson et al., 1988). The possibility that cholestasis-induced fibrosis is a preventable phenomenon has not been explored.

Studies have shown that oxidative stress and accumulation of free radicals might contribute to the development of hepatic fibrosis in biliary obstruction (Poli et al., 1987; Poli, 2000; Parola and Robino, 2001). Cholestasis was found to reduce antioxidative capacities in liver mitochondria in bile duct-ligated rats (Krabenbuhl et al., 1995; Huang et al., 2003). Accumulation of bile acids and inflammatory cells in the liver tissue may cause increased production of free radicals in biliary obstruction (Sokol et al., 1993; Poli and Parola, 1997). Bile acids especially, enhance reactive oxygen species released by polymorpho-nuclear leukocytes (Dahm et al., 1988; Singh et al., 1992).

Many studies have shown that antioxidative nutrients such as vitamin E and selenium can have protective effect against chronic liver damage and cirrhosis. Both antioxidants were found to decrease significantly hepatocellular damage and elevation in the plasma enzymatic activities caused by hepatocellular damage (Geetha et al., 1990; Lee and Clemers, 1992; Nagamatsu and Hasegawa, 1993; Naziroglu et al., 1999).

In addition, some previous studies have confirmed that dietary supplementation with vitamin E and selenium can protect hepatocytes and prevent liver fibrosis induced by CCl4 in rat model (Lee et al., 2001; Svegliati-Baroni et al., 2001; Nieto et al., 2002; Zhan et al., 2003). However, the mechanism of the effects is still not clear (Li et al., 2003). Therefore, in the present study, it was aimed to investigate the beneficial effect of combined supplementation of proper doses of vitamin E and selenium on liver fibrosis induced experimentally in bile duct-ligated rats.

**MATERIALS AND METHODS**

**Animals and experimental design:** Forty adult male Sprague-Dawley albino rats (weighing 220-250 g at the beginning of the experiment) were used in this study. They were obtained from the Animal House of King Fahd Medical Research Center. At the beginning of the experiment the animals were kept in separate metallic cages under standard temperature (24±2°C), humidity (55±5%) and lighting (12 h: 12 h Light: Dark) conditions.

Food formed of Purina rat chow and drinking water was supplied ad libitum. This study was approved and registered by the Committee of Animal Investigations in Department of Anatomy, Faculty of Medicine, King Abdul-Aziz University during all the steps of the study, the animals were cared in King Fahd Center for Medical Research. The rats were randomly assigned to 4 groups (10 rats in each).

**Control group:** Rats were left without any treatment or operation.

**Bile Duct-Ligated (BDL) group:** The bile ducts of rats were ligated and transected.

**Sham-operated group:** The operation was performed to the rats in the same way as the previous group but without bile duct ligation or transection.

**BDL group co-treated with vitamin E and selenium:** The rats underwent bile duct ligation and transection and were fed with chow supplemented with vitamin E (250 mg kg⁻¹) and selenium (0.2 mg kg⁻¹ diet) starting from 3 days before the operation.

**Operative procedure:** Rats were anesthetized with ketamin and diazepam. Laparatomy was performed under antiseptic conditions. A mid-line incision in the abdomen was made from the xiphisternum to the pubis, exposing the muscle layers and the linea alba which was then incised over a length corresponding to the skin incision. The edge of the liver was then raised and the duodermum pulled down to expose the common bile duct which pursues an almost straight course of about 3 cm from the hilum of the liver to its opening into the duodenum. There was no gall bladder and the duct was embedded for the greater part of its length in the pancreas which opens into it by numerous small ducts. A blunt needle was passed under the part of the duct selected and the duct was double ligated with 5.0 polypropylene sutures and divided in between. The peritoneum and the muscle layers as well as the skin wound were closed with cotton stitches (Kountouras et al., 1984).

**Biochemical assay:** At the end of the study period (3 weeks), the rats were decapitated and blood was withdrawn from all groups of rats by puncturing retroorbital plexus. The blood samples were allowed to coagulate at room temperature. Serum was separated by centrifugation at 3,000 rpm at room temperature for 20 min and subjected to biochemical estimations of serum...
transaminases, alkaline phosphatase, lactate dehydrogenase and bilirubin. Determination of serum total and direct bilirubin was done by using COBAS INTEGRA® 400 plus analyzer.

**Determination of hydroxyproline content in the liver:** At the end of the study period, fresh liver tissue (10 mg) was taken and homogenized in 1 mL of 10 N HCl with a homogenizer. This homogenate was used directly for hydroxyproline analysis. Hydroxyprolines attached to strong acid cations were exchanged with resins. Then the resins were washed with distilled water to remove substances which may have caused interference. Peptide bonds in the resins were broken at 100°C for 16 h.

The hydroxyprolines were then oxidized with a piroli derivat. Following incubation at 60°C for 25 min, the samples were kept at room temperature for 30 min to stabilize the color and the optical density of the developed color was read at 560 nm with a Schimadzu UV 1201 (Schimadzu Corp., Japan) spectrophotometer. The results were expressed as mg g⁻¹ tissue (Stegemann and Stalder, 1967).

**Fixation and immunohistochemical procedure:** At the end of the study period, fresh liver samples of 5 mm³ were cut and immediately immersed in a 4% paraformaldehyde solution buffered with 0.1 M sodium cacodylate, pH 7.4 at 4°C for 4 h. Then, the samples were extensively washed in 0.1 M phosphate buffered saline at 4°C for 18 h followed by soaking in 10% glycerol in phosphate buffered saline for 1 h.

Then, the fragments were frozen in liquid nitrogen-cooled isopentane and stored at −70°C. Extracellular matrix components were localized in situ using the indirect immunoperoxidase technique (Clement et al., 1985). Briefly, 5 μm cryostat sections were prepared from different frozen liver fragments and incubated in 0.05% saponin in phosphate buffered saline with 10% fetal calf serum for 1 h before a further another 1 h incubation with the specific primary antibodies.

After three phosphate buffered saline washes, sections were incubated with sheep anti-rabbit immunoglobulin labelled with peroxidase enzyme. The peroxidase activity was revealed by using 3, 3'-diaminobenzidine/H₂O₂ solution for 20 min. Then, the sections were mounted on glass and examined by Seize light microscope (the positive reaction was visualized as a brown staining). Control liver sections were first incubated with normal immunoglobulins or directly with the medium employed to reveal peroxidase activity.

**Source of antibodies:** The specific primary antibodies against collagen type I and IV, laminin and fibronectin were bought from Sigma Agency. These antibodies were raised in New Zealand white rabbits and purified by affinity chromatography.

**Statistical analysis:** Data are expressed as mean±Standard Deviation (SD). For all variables, the statistical differences between treated groups and the control group were analyzed statistically by student t-test, p<0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Biochemical findings:** Table 1 and 2 showed the activities of various liver enzymes, total and conjugated bilirubin measurements in control, bile duct ligated and bile duct ligated co-treated with vitamin E and selenium groups. In bile duct ligated group there was significant and dramatic increase in serum enzyme levels, compared to control, while vitamin E and selenium co-treated groups showed a decrease in all enzyme levels towards the normal levels. Also, there was significant rise in total and conjugated bilirubin measurements which returned back towards the normal values in the bile duct ligated rats co-treated with vitamin E and selenium.

**Liver collagen content:** As shown in Table 2, hepatic injury induced by bile duct ligation caused a significant rise in the hydroxyproline content whereas vitamin E and selenium co-treatment resulted in a decrease towards the normal values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Aspartate transaminase (IU/L)</th>
<th>Alanine transaminase (IU/L)</th>
<th>Alkaline phosphatase (IU/L)</th>
<th>Dehydrogenase (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>67±3.3</td>
<td>48±4.4</td>
<td>175±7.8</td>
<td>150±6.7</td>
</tr>
<tr>
<td>Sham operated</td>
<td>64±3.2</td>
<td>54±4.8</td>
<td>179±8.1</td>
<td>145±5.6</td>
</tr>
<tr>
<td>Bile duct ligated</td>
<td>157±3.4</td>
<td>128±11.8</td>
<td>424±26.8</td>
<td>331±15.9</td>
</tr>
<tr>
<td>Bile duct ligated co-treated with vitamin E and selenium</td>
<td>82±5.1</td>
<td>71±5.3</td>
<td>198±9.3</td>
<td>178±8.1</td>
</tr>
</tbody>
</table>

*p<0.01, **p<0.0001 when compared to control; values are expressed as mean±SD

<table>
<thead>
<tr>
<th>Group</th>
<th>Total bilirubin (mg dl⁻¹)</th>
<th>Direct bilirubin (mg dl⁻¹)</th>
<th>Hep (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.23±0.022</td>
<td>0.17±0.019</td>
<td>4.8±0.950</td>
</tr>
<tr>
<td>Sham operated</td>
<td>0.24±0.023</td>
<td>0.18±0.022</td>
<td>5.1±0.820</td>
</tr>
<tr>
<td>Bile duct ligated</td>
<td>0.81±0.076***</td>
<td>0.23±0.021**</td>
<td>13.8±0.129***</td>
</tr>
<tr>
<td>Bile duct ligated co-treated with vitamin E and selenium</td>
<td>0.29±0.025*</td>
<td>0.26±0.020*</td>
<td>7.4±0.570***</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.001, ***p<0.0001, when compared to control; values are expressed as mean±SD
Immunolocalization of collagen, fibronectin and laminin

Livers of normal rats: As shown in the Fig. 1-4 and the major sites of deposition of the different components were in the portal spaces (around the basement membranes of the bile ducts and blood vessels) and around the central veins in the hepatic lobules. All the components were visualized although the intensity of staining and the manner of distribution varied from one component to
bile ducts. In addition, in the hepatic lobules, liver sinusoids showed a moderate and discontinuous sinusooidal staining with Col IV. Regarding the fibronectin, it was the most prominent component, which showed very strong staining in the portal spaces and a continuous sinusoidal labeling in the hepatic lobules with slight cytoplasmic staining. The laminin showed a moderate staining, especially around the bile ducts and blood vessels while in the hepatic lobules, only faint and continuous sinusoidal staining could be detected.

**Livers of bile-duct ligated rats:** As shown in the Fig. 1-4, the biliary obstruction resulted in a rapid extensive bile duct proliferation and fibrogenesis in enlarged portal areas. Infiltration of the portal tract with polymorphonuclear inflammatory cells was occasional and hepatocyte necrosis was nearly absent. Central vein areas remained unchanged. By the immunostaining technique, extensive fibrous septa were stained for collagens I and IV, fibronectin and laminin. The most intense staining was found in portal spaces. Intense staining for collagen IV and laminin was observed around bile duct proliferations. In the sinusoids, collagen 4 was continuously distributed along hepatocyte cords. Laminin and fibronectin were deposited mainly within the perportal areas and in the borders of septa. Regarding the fibronectin, a strong staining was seen in the portal spaces around the basement membranes of bile ducts and blood vessels, with strong sinusoidal staining in the hepatic lobules.

**Livers of bile-duct ligated rats co-treated with vitamin E and selenium:** As shown in the Fig. 1-4, the co-administration of vitamin E and selenium resulted in much regression of bile duct proliferation and fibrogenesis in enlarged portal areas. The distribution of collagens, fibronectin and laminin was in some extent similar to that observed in normal liver. In portal spaces, the remaining clusters of neo-formed bile ducts were still surrounded by laminin and collagen IV. In the sinusoids, the distribution of collagens I, laminin and fibronectin was similar to that in normal liver. Only collagen IV remained abundant in the sinusoids and continuously deposited all along hepatocyte cords.

Liver fibrosis is the common result of chronic hepatic injury of diverse origins such as chronic viral infections, metabolic/storage diseases (hemochromatosis), helminthic infections (schistosomiasis), chronic toxic exposure (alcohol and environmental poisons) and biliary obstruction (biliary cirrhosis). In end stage liver fibrosis or cirrhosis, the liver Extracellular Matrix (ECM) may contain up to 6-10 times more collagen and proteoglycans than in the normal state (Gressner *et al.*, 2007; Wells, 2007).
regulation of the turnover of its components and identification of liver cells responsible for the synthesis of matrix proteins are especially crucial for the ultimate design of effective therapies for liver fibrosis (Schuppan, 1990).

The use of animal models of liver fibrosis such as the administration of liver toxins CCl₄ (Rojkind et al., 1977), dimethyl nitrosamine (Risteli et al., 1976), alcohol (Mezey et al., 1976) have greatly helped in the characterization of the temporal expression of various components of the ECM during fibrogenesis. It was reported that there is increase in the amounts of collagens types I-VI (Wu et al., 1982, Isaka et al., 1996). It was reported that in early fibrosis, the amounts of types III and IV collagens increase relative to other collagens. In late fibrosis, type I collagen predominates (Schuppan, 1990). Other components of liver ECM such as laminin, fibronectin and proteoglycans are also increased in fibrosis (Rojkind et al., 1979; Geerts et al., 1986; Reid et al., 1992).

The reversibility of hepatic fibrosis remains the subject of controversy. In this study, it was decided to investigate fibrogenesis in cholestatic rat liver experimentally induced by common bile duct ligation. This model is morphologically characterized by marked proliferation of bile ducts and formation of enlarged fibrous septa with only limited inflammation and necrosis. Dramatic and early increase in the serum concentration of bilirubin and enzymes indicated that extrahepatic cholestasis was induced rapidly in this experimental model. Moreover, the bile duct ligation model of rats mimics best the clinical and histopathological aspects of hepatic fibrosis secondary to extrahepatic biliary obstruction in humans. This model allows researchers to study in detail all the developmental stages of hepatic fibrosis, ultimately, terminating in cirrhosis within about 4 weeks (Moritz and Snodgrass, 1972; Kountouras et al., 1984).

Elevated tissue collagen levels can be measured directly or be indirectly evaluated by the measurement of hydroxyproline levels (Stegmann and Stalder, 1967). In this study, the bile duct ligation resulted in highly significant rise in serum hydroxyproline levels when compared to the control values. It was demonstrated that liver fibrosis is accompanied by a significant increase in collagen content in this organ (Bankowskic, 1994; Matsuda et al., 1997). Collagen is involved in wound healing via formation of hydroxylation. However, the balance of collagen is important in wound healing. If an uncontrolled accumulation of collagen cannot be degraded by collagenases, it will result in fibrosis.

Fig. 4. Light photomicrographs of liver sections showing immunolocalization of laminin. (Ps = Portal space, CV = Central Vein); A) Control group: moderate staining was seen in the portal spaces around the basement membranes of bile ducts and blood vessels with faint continuous sinusoidal staining in the hepatic lobules. B) BDL group: strong staining of basement membranes around the proliferated bile ductules in the dilated portal spaces with moderate sinusoidal staining in the hepatic lobule. C) BDL group co-treated with vitamin E and selenium: decreased positive staining around mild proliferated bile ductules in the portal spaces with negative sinusoidal staining in the hepatic lobule.

Because the ECM support of the liver parenchyma is particularly critical to its function, research that emphasizes the nature of liver ECM, the molecular
formation instead of normal healthy hepatocytes. The immunohistochemical study in this research demonstrated that fibrosis is formed by interstitial collagen types I, fibronectin and basement membrane proteins. In agreement, it was reported that as bile duct cells actively proliferated, it can be expected that they were the producers of basement membrane components, i.e., collagen IV and laminin and were responsible for the accumulation of these proteins in portal areas (Miyabayashi et al., 1987; Clement et al., 1988).

Studies have shown that oxidative stress (lipid peroxides) is one of the mechanisms in the development of liver fibrosis (Lee et al., 2001; Veglia et al., 2001; Nieto et al., 2002; Zhan et al., 2003); it is worth investigating the therapeutic role of antioxidative nutrients in hepatic fibrosis. Over the years, many compounds have been studied as possible protectors for liver fibrosis, like antioxidants such as flavonoids and other phenolic derivatives. These compounds belong to a group of a bigger family known as phytochemicals. They are widely found in fruits and vegetables and while not having an energetic value are an important part of the human diet. Flavonoids exhibit a wide variety of biological properties, including hepatoprotection and inhibition of fibrosis (Middleton et al., 2000). Studies demonstrating the beneficial effect of vitamin E supplementation in prevention of both enhancement of lipid peroxidation and synthesis of collagens might support this suggestion (Hong et al., 1991; Parola et al., 1992; Chojciar et al., 1998). However, it has been reported that treatment of bile duct-ligated rats with vitamin E completely prevented the increase in lipid peroxidation in liver and plasma but failed to prevent tissue injury histologically.

Thus, the current controversies between the performed studies to evaluate the role of oxidative stress in tissue injury induced by bile duct ligation make newer studies to be performed necessary (Baron and Muriel, 1999; Friedman, 2000; Schuppan et al., 2001). Selenium is a trace element which has antioxidant properties because it is an essential component of some oxidoreductase enzymes. Selenium deficiency is associated with higher levels of ROS and lipid peroxidation which can be reversed by selenium supplementation (Luoma et al., 1984; Bedwal et al., 1993). In one study, administration of vitamin E together with Se and zinc resulted in a lowered 1 month mortality in patients with alcoholic hepatitis (Wenzel et al., 1993). In other studies, however, administration of Se together with vitamins A and E did not affect survival of patients with alcoholic hepatitis (Stewart et al., 2007).

Selenium-enriched lactobacillus decreased liver injury and lipid peroxidation induced by CCl4 administration in mice (Chen et al., 2005) while supplementation of the diet with vitamin E and Se decreased hepatic fibrosis produced in rats by acute and chronic CCl4 administration (Shen et al., 2005). In this study, co-treatment with vitamin E and selenium resulted in an evident shift to normal liver architecture and functions including normal values in serum enzyme activities and bilirubin levels suggesting that massive and selective disruption of bile ducts and death of the newly formed bile duct epithelial cells rapidly occurred.

The dosage of vitamin E and selenium used in the study was selected as previously described (Tsukamoto et al., 1990, Zhang et al., 1996, Li et al., 2003). In accordance, it was reported that several antioxidants were scavenged free radicals (Sies et al., 1992; McPherson, 1994) and these radicals in hepatic cellular structures may increase the cellular degeneration. This process may be able to affect the levels of liver enzymes due to damage of cellular membrane.

Thus, abnormal levels of the liver enzymes in plasma are usually indicative of the hepatic cellular injury in experimental animals (Albano et al., 1989; Miao et al., 1990; Biasi et al., 1991; Parola et al., 1992; Knock et al., 1995). Also, the results from histology and serum enzymes showed that vitamin E and selenium reduced liver damage during liver injury and had the effect of decreasing collagen fibers thus preventing liver fibrosis. Immunostaining of different component of ECM showed that the amount of collagen fibers decreased markedly in vitamin E and selenium co-treated group when compared to the bile duct ligated group, suggesting that these elements can prevent liver damage during liver injury, by affecting cellular sources of ECM (Iredale et al., 1998). In accordance, a recently published study, the combination of vitamins E and C was associated with decreased ethanol-induced hepatic glutathione peroxidase activity and hepatic fibrosis in protein-deficient rats fed with a high-fat liquid diet (Soylu et al., 2006).

**CONCLUSION**

In the study, oxidative stress might contribute to the pathogenesis of secondary biliary fibrosis. Antioxidants vitamins E and selenium combination attenuate the development of hepatic fibrosis in bile duct-ligated rats. Long-term, prospective studies in humans with chronic cholestatic liver diseases may be helpful to evaluate the beneficial effects of these elements.
REFERENCES


