Effect of Oophorectomy and Exogenous Estrogen Replacement on Liver Injury in Experimental Obstructive Jaundice

Ooforektomi ve Eksojen Östrojen Replasmanının Deneysel Tıkanma Sarılığı ile Oluşmuş Karaciğer Hasarı Üzerine Etkisi

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Abstract

The aim of this study was to investigate the role of estrogen on liver injury in an experimental obstructive jaundice (bile duct obstruction) model, commonly faced with in the surgical practice. Three groups of female rats were constituted; group 1 was oophorectomized and given E2 (n=14), group 2 was oophorectomized and given placebo (n=14), and group 3 was sham operated to keep blood estrogen level in the normal range (n=14). In all groups, 14 days following constitution of bile duct ligation, serum tests and the histopathologic parameters were assessed and tissue levels of IFN-gamma and IL-6 were measured by ELISA method. Then all groups were compared. Serum GGT and ALT levels were significantly higher in group 1 and 3 than group 2 ($p<0.05$). It is found that, the parameters representing both the injury and/or the reactive response and healing were more pronounced in groups 1 and 2 ($p<0.05$). In the sham operated or E2 given groups significantly lower tissue levels of IFN-gamma and higher IL-6 levels were found, contrary to this, high IFN-gamma and low IL-6 tissue levels were found in the oophorectomized and placebo given group ($p<0.001$). The Kupffer cell alterations were observed to be more pronounced in the groups 1 and 3 ($p<0.05$). Our study indicates that E2 impaired the liver functions, accelerated both the liver damage and healing. In the conditions of bile duct obstruction, estrogen significantly changed the cytokine milieu in the liver and also might change liver injury through the Kupffer cells that could modify the cytokine milieu.

Key Words: Estrogen, Liver injury, Oophorectomy, Obstructive Jaundice, Experimental

Özet

Bu deneysel çalışmanın amacı, cerrahi uygulamalarda sıkça karşılaştığımız tıkanma sarılığına (safra yolu tıkanıklığı) bağlı karaciğer hasarı üzerine estrojenin etkilerini araştırmaktır. Dişi sıçanlar üç gruba ayrıldı; grup 1 overleri alınmış ve E2 verilmiş (n=14), grup 2 overleri alınmış ve plasebo verilmiş (n=14), grup 3 ise kan östrojen seviyelerinin normal sınırlarda kalması için yalnızca laparotomi yapılmış (n=14) sıçanlardan oluşmaktaydı. Safra yolunun bağlanmasından sonra tüm sıçanlarda serum testleri ve histopatolojik incelemeler yapıldı. Ayrıca ELISA metodu ile doku IFN-gamma ve IL-6 seviyeleri ölçüldü. Daha sonra gruplar karşılaştırıldı. Serum GGT ve ALT seviyeleri grup 2'ye göre grup 1 ve 3'te anlamlı ölçüde daha yüksek bulundu. ($p<0.05$). Hasar ve/veya reaktif cevap ile iyileşmeyi temsil eden göstergelerin grup 1 ve 2'de çok daha belirgin olduğu gözlendi ($p<0.05$). Sadece laparotomi yapılan veya E2 verilen gruplarda düşük IFN-gamma ve yüksek IL-6 seviyeleri tespit edilirken, bu durumun tersine overleri alınmış ve plasebo verilmiş grupta yüksek IFN-gamma ve düşük IL-6 doku seviyeleri saptandı ($p<0.001$). Kupffer hücre değişikliklerinin grup 1 ve 3'te daha şiddetli olduğu tespit edildi ($p<0.05$). Çalışmamız E2'nin karaciğer fonksiyonlarını bozduğu, karaciğerde hem iyileşme hem de hasarı hızlandırdığını göstermiştir. Safra yolu tıkanıklığının olduğu durumlarda, östrojen karaciğerdeki sitokin tablosunu belirgin olarak değiştirmekte ve muhtemelen sitokin ortamını değiştirme yeteneği olan Kupffer hücreleri aracılığı ile karaciğerdeki hasarı etkileyebilmektedir.

Anahtar Kelimeler: Östrojen, Karaciğer hasarı, Ooforektomi, Tıkanma sarılığı, Deneysel

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INTRODUCTION

It has long been known that there are differences between the two sexes in the occurrence of some diseases or the response that they give to the same disease entity (1), such as cardiovascular disease (2), sepsis, (3), various autoimmune and collagen diseases including autoimmune thyroid disease (4), or liver diseases (5). These findings suggest that, the gender may determine the susceptibility to some diseases whereas it may be preventive to others, which is a fact that is probably attributed to estrogen(s).

The recent identification of estrogen receptor (ER) in many tissues beside the target organs of estrogen, which mainly possess ER (6,7) has suggested that a larger tissue area than previously known is affected by estrogen through its specific receptors (8,9). The demonstration of ER(s) in the liver (10) has led the studies to focus on the influences of steroid hormones, particularly estrogens, on liver disorders and regeneration (11). In rats, liver injury induced by alcohol (12, 13) or carbontetrachloride (14) is more frequently seen in females. In transplanted liver (15) and hepatectomy models (16,17), estrogen significantly increases regeneration and proliferation, and reverses inhibitory effect of the estrogen receptor antagonist, tamoxifen, on hepatocyte proliferation. These findings demonstrate that estrogens may aggravate injury or potentiate regeneration, depending on the model used.

The influence of estrogen on liver injury has not been studied in obstructive jaundice before. Biliary stones are found in 10% of adult population, and the risk of occurrence increases in women on oral contraceptives and with pregnancy. Each year 3-5% of patients with symptomatic biliary stones become complicated (18). In patients with chronic extra-hepatic bile duct obstructions, endotoxemia has been demonstrated and prolonged bile duct obstruction is harmful for fagocytic activity of reticuloendothelial system and increases the risk of mortality in the presence of endotoxemia (19,20).

In this experimental study we have searched the effects of estrogens on liver injury and regeneration with the associated cytokine production using the bile duct ligation model. The aim of the study is to determine the differences, in terms of biochemical and histopathological parameters of liver injury in obstructive jaundice, between the groups of rats that represent women who are postmenopausal or oophorectomized with or without estrogen replacement therapy, and premenopausal; and to demonstrate the possible role of cytokines in these differences; and to asses the clinical importance of these data.

METHODS

This study was conducted in the experimental research laboratory at the Gazi University Medical School and completely funded by Gazi University Research Fund. Local ethics committee approval was obtained prior starting the study.

Female Wistar albino rats, 12-16 weeks old and 180-200 grams, were used. They were kept in temperature-controlled environment, and they had free access to standard rat food and water.

The study design and administration of estrogen Forty-two female rats were randomly assigned to three groups of 14 each. In order to nullify the effect of ovarian estrogen, the first two groups, group 1 and 2 (n=14), underwent bilateral oophorectomy, as previously described (22) while group 3 (n=14) was sham operated to maintain physiological estrogen level. Fourteen days after surgeries, during which effects of ovarian estrogen had disappeared, Group 1 was given subcutaneous 5mg/ml 17b-estradiol (Sigma Chemical Co., Steinheim, Germany) dissolved in sesame oil (Sigma). Group 2 and group 3 were given 0.1ml sesame oil subcutaneously(SC) as a placebo. This procedure was repeated three times, 1 and 5 days after the first.

The bile ducts were ligated twenty-four hours after administration of the last dose of estrogen (or placebo), as previously described (20). The animals were anesthetized by an intramuscular injection of ketamine hydrochloride (50 mg/kg of body weight) under semi-sterile conditions and through a 3 cm long upper midline abdominal incision, the common bile ducts were isolated, double ligated with 6-0 vicryl (Ethicon, Birmingham, UK) and transected between the sutures. Meticulous attention was paid not to damage the blood supply and neighboring organs.

Table 1. Study design

Day	Group 1 (n=14)	Group 2 (n=14)	Group 3 (n=14)
15, 16, and 22	Bilateral oophorectomy	Bilateral oophorectomy	Sham operation
23	5 mg/0.1 ml E2 in sesame oil, (SC)	0.1 ml sesame oil, (SC)	0.1 ml sesame oil, (SC)
27	Killed, blood and tissue samples obtained	Killed, blood and tissue samples obtained	Killed, blood and tissue samples obtained

Table 2. Histopathologic scoring of liver injury

Parameters	Score
Necrosis	0,1,2,3
Regenerative activity	0,1,2,3
Portal PMNL infiltration	0,1,2,3
Portal MNL infiltration	0,1,2,3
Ductular proliferation	0,1,2,3
Fibroblastic activity	0,1,2,3
Kuppffer cell abnormalities	0,2
Sinusoidal PMNL infiltration	0,2
Sinusoidal MNL infiltration	0,2
Portal vascular congestion	0,2
Sinusoidal vascular congestion	0,2
Portal vascular thrombosis	0,2
Sinusoidal vascular thrombosis	0,2
Portal and central venous phlebitis	0,2
Arterial vall changes	0,1,2,3
Hydropic degeneration	0,1,2,3
Decrease in hepatocyte glycogen content	0,2

PMNL: Polymorphonuclear leukocytes, MNL: Mononuclear leukocytes

Fourteen days after bile duct ligation, the abdominal incision was reopened following ketamine anesthesia. The animals were killed by cardiac puncture and blood withdrawn for the measurements of estrogen level and biochemical parameters. Then hepatectomy was performed and the liver was splitted into two; one piece was kept in 10% formaldehyde solution for histopathological evaluation and the other sample was sent to the laboratory immediately as a fresh tissue for cytokine level measurements by ELISA (Table 1). Measurements of blood biochemical parameters, estrogen and tissue cytokine levels. Blood estrogen levels were measured by chemiluminescence method in pg/ml with Access Immunoassay System (Beckman Coulter, Inc., CA, USA) analyzer. To show altered liver functions, blood levels of bilirubin in mg/dl, alanin tansaminase (ALT), and g-glutamyl transpeptidase (GGT) in U/L were measured by Synchron CX-7 Clinical System (Beckman).

To assess the effect of estrogen on cytokines, liver IFN-g and IL-6 levels were measured by ELISA in pg/ml, because of their significant role on liver injury and regeneration. For the measurements, 0.5 g of the fresh liver tissue was harvested and prepared for ELISA by homogenization as described previously (22). Briefly, each specimen was homogenized for 60 seconds in 10 ml of phosphate buffered solution containing a cocktail of protease inhibitors including 2mM phenylmethylsulphonyl floride and 2 mg/ml aprotinin, leupeptin and pepstatin A (Sigma) to inhibit proteolysis of cytokines.

Then homogenates were obtained from homogenizator (Jencons Scientific Ltd., Bedfordshire, UK), ultra-centrifuged with 10.000 rpm at 4°C for 45 minutes and the supernatants were sampled by micropipettes and stored at -70 °C. Cytokines were measured by ELISA with rat IFN-g and rat IL-6 kits (BioSource Int.Inc.,CA, USA) as recommended. Histopathological evaluation. Liver fragments were kept in 10% formaldehyde solution for 6-48 hours. Following treatment with various concentrations of alcohol and xylol, the specimens were embedded in paraffin blocks. After the preparation of sections they were stained with Hematoxylene&Eosin and Masson's trichrome dyes. Evaluations were made by a pathologist blindly using the parameters described previously (20). Each of the histopathological parameter was evaluated and scored for each slide in terms of the degree of change such that zero was given for no change, 1 for slight, 2 for moderate, and three for severe changes. For some of the parameters that could not be so scored, zero or two was assigned for absence or presence of the pathology, respectively (Table 2).

Statistical analysis. For all parameters the means and the standard deviations (SD) were calculated. Kruskall-Wallis variance analysis and chi-squared tests were used for all nonparametric comparisons.

RESULTS

Blood estrogen levels were significantly different in all groups ($p<0.001$). The highest values were in the group 1 in which oophorectomy and E2 replacement had been done, whereas the lowest values were obtained in placebo given group 2 (Table 3).

Serum bilirubin, ALT, and GGT levels increased in all groups as evidences of obstructive jaundice and liver damage. Serum bilirubin levels were not found to be different among the groups ($p>0.05$). Blood ALT and GGT levels generally were lower in the placebo given group 2 than in the groups 1 and 3 in which blood estrogen levels were normal or high. The significant differences emerged with its lowest value for ALT in group 2 and its higher values for GGT only between groups 1 and 2 ($p<0.05$, Table 4).

Table 3. Blood estrogen levels of the groups

Groups	Estrogen levels (pg/ml)
Group 1	580±124.01
Group 2	61.50±15.97
Group 3	208±35.02

$p<0.001$ for all groups

Liver specimens of all rats were examined histopathologically according to the parameters mentioned in table 2 and groups were then compared with chi-squared test. Among 17 parameters necrosis, regenerative activity, ductular proliferation, fibroblastic activity, Kupffer cell abnormalities, sinusoidal congestion and portal-central venous phlebitis showed statistical difference among groups ($p<0.05$, Table 5).

In terms of portal PMNL and MNL infiltration, sinusoidal PMNL and MNL infiltration, portal vascular congestion, portal and sinusoidal vascular thrombosis, arteriolar wall changes, hydropic degeneration, and hepatocyte glycogen content there was no significant difference among groups ($p>0.05$, data not shown).

When necrosis was considered there appeared a significant difference, which was due to the lower values in group 2 ($c2=17.2$, $p<0.05$, table 5). Group 2 was also found to be significantly different with its lower regenerative activity ($c2=10.22$, $p<0.05$, table 5).

Ductular proliferation, a reactive response of liver to bile duct obstruction, was statistically more in groups 1 and 3 than in group 2 ($c2=12.43$, $p<0.05$, table 5).

In groups 1 and 3 with higher estrogen levels, fibroblastic activity was also significantly more pronounced ($c2=31.06$, $p<0.001$, table 5). There was a significant difference in terms of Kupffer cell abnormalities between groups 1 and 2 ($c2=6.13$, $p<0.05$, table 5).

Sinusoidal congestion and portal venous phlebitis were significantly lower in group 2 than in group 3 ($c2=8.17$, $p<0.05$, $c2=7.636$, $p<0.05$, respectively, table 5).

To summarize, in all groups, bile duct ligation produced histopathological changes in liver and there appeared a significant difference between group 2 with the lowest estrogen level and the others in terms of at least some of the parameters.

The groups were also compared according to the tissue cytokine measurements of IFN-g and IL-6, and were found to be statistically different. While IFN-g was significantly higher, IL-6 was lower in group 2 with the lowest estrogen level ($p<0.001$, table 5, figure 1).

With Pearson's correlation analysis, IFN-g and IL-6 levels were tested and found to be inversely related to each other when group 2 was compared with groups 1 and 3 ($r= -5.4$, figure 2).

Table 4. Comparisons of liver function tests of the groups

Groups	ALT (U/L)	GGT (U/L)	Bilirubin level (mg/dl)
Group 1	347.85±61.12	65.35±20.98*	6.83±1.32
Group 2	256.57±54.19*	46.50±12.0	5.9±2.12
Group 3	360.50±65.22	58.78±7.47	6.30±1.57

*There are significant difference when compared with other groups ($p<0.05$)

Table 5. Comparision of groups by categories

Categories	Group 1	Group 1	Group 1	c2 / p*
Necrosis				
No change	5 (35.7%)	12 (85.7%)	7 (50%)	c2=17.20
Slight	7 (50%)	0 (0%)	1 (7.1%)	p<0.05
Moderate	2 (14.3%)	2 (14.3%)	6 (42.9%)	
Regenerative activity				
Slight	6 (42.9%)	13 (92.9%)	6 (42.9%)	c2=10.22
Moderate	5 (35.7%)	1 (7.1%)	6 (42.9%)	p < 0.05
Severe	3 (21.4%)	0 (0%)	2 (14.2%)	
Ductular proliferation				
Slight	0 (0%)	6 (42.9%)	1 (7.1%)	c2=12.43
Moderate	5 (35.7%)	5 (35.7%)	4 (28.6%)	p < 0.05
Severe	9 (64.3%)	3 (21.4%)	9 (64.3%)	
Fibroblastic activity				
Slight	1 (7.1%)	9 (64.3%)	1 (7.1%)	c2=31.06
Moderate	9 (64.3%)	4 (28.6%)	11 (78.6%)	p<0.001
Severe	4 (28.6%)	1 (7.1%)	2 (14.3%)	
Kupffer cell abnormalities				
Abscence	6 (42.9%)	12 (85.7%)	7 (50%)	c2=6.13
Presence	8 (57.1%)	2 (14.3%)	7 (50%)	p < 0.05
Sinusoidal congestion				
Abscence	13 (92.9%)	9 (64.3%)	14 (100%)	c2=8.17
Presence	1 (7.1%)	5 (35.7%)	0 (0%)	p < 0.05
Portal central venous phlebitis				
Abscence	3 (21.4%)	6 (42.9%)	0 (0%)	c2=7.64
Presence	11 (78.6%)	8 (57.1%)	14 (100%)	p < 0.05
IFN-gamma (pg/ml)	349.46±125.15	764.61±191.95	450.21±98.25	P<0.001
IL-6 (pg/ml)	839.84±245.05	466.5±106.86	710.02±167.75	p<0.001

DISCUSSION

Differences exist between the two sexes of humans and animals (1). It is known that in premenopausal period, female gender is a preventive factor against cardiovascular disorders (23), peptic ulcer disease (24) and some others (3, 25); on the other hand the risk of occurrence of autoimmune and collagen tissue disorders is more frequent in women (26). It has been proposed that this fact may be due to the differences between two sexes in hormone levels and tissue distribution of hormone receptors.

Because estrogen has influences on many tissues other than its main target tissues, it has been imagined and shown that estrogen has a larger field of distribution than previously believed (7,27). After the existence of estrogen receptor in liver was shown, studies on interaction of estrogen and its receptor in liver have accumulated (28,29). Estrogen is thought to enhance regeneration after hepatectomy, due to its potentiative effect on proliferation and to existence of its receptor in liver, and studies have shown that estrogen increases regeneration (17) and mitotic activity of hepatocytes (15) significantly.

On the other hand, it potentiates liver injury with alcohol and carbontetrachloride administration (5,13,14, and 30).

Previous literature comprises data about influences of estrogen on various types of liver injury other than obstructive jaundice. In this experimental study, effects of estrogen on liver injury in bile duct ligation model were investigated.

Bile stones, 85% of which is cholesterol type, are frequent in surgical practice. Women are affected twice as much as men, and oral contraceptives and pregnancy increase the risk for biliary stone formation. Each year, 3-5% of symptomatic biliary stones become complicated (18,31). In general, benign and malignant bile duct obstructions are mostly seen in elderly.

In our study, blood estrogen levels of groups were significantly different, as intended (p<0.001, table 3). While oophorectomized groups 1 and 2 had estrogen levels higher and lower than normal respectively, group 3 kept its physiological level.

By this way, groups representing postmenopausal or bilaterally oophorectomized women with estrogen replacement (group 1), without estrogen replacement (group 2) and premenopausal normal women (group 3) were constituted.

Increased serum bilirubin and liver enzyme levels after 14 days of bile duct obstruction proved that bile duct ligation had been successfully performed. The fact that the group with the lowest estrogen level (group 2) had significantly lower levels of liver enzymes than groups 1 and 3 suggested that estrogen enhanced liver injury. To test this, previously described parameters of liver injury were used for the comparison of groups (table 2).

Histopathological changes were to a significantly lesser extent in group 2 with the lowest estrogen level than in the other groups, considering the seven parameters yielding statistical difference among groups (table 5).

This finding has shown that estrogen has influence on liver injury. Reactive changes secondary to bile duct obstruction, like necrosis, ductular proliferation, Kupffer cell abnormalities, sinusoidal congestion, were significantly more pronounced in groups 1 and 3 than in group 2, which meant both physiological and supraphysiological levels of estrogen potentiated not only liver injury but also reactive responses (table 5).

These data correlates well with the results of studies reporting that estrogen accelerates liver injury caused by alcohol and CCl4 (13,15, and 17).

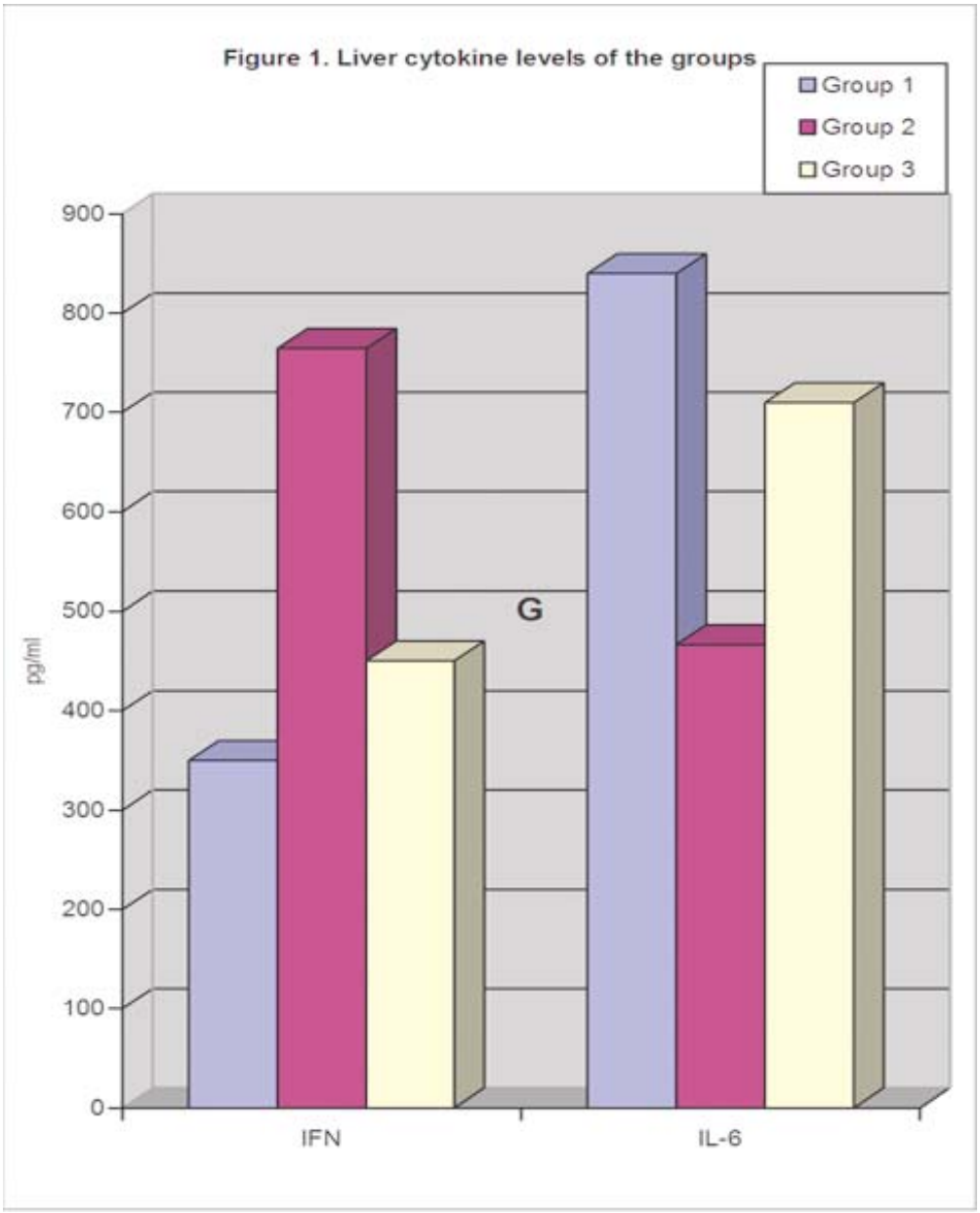
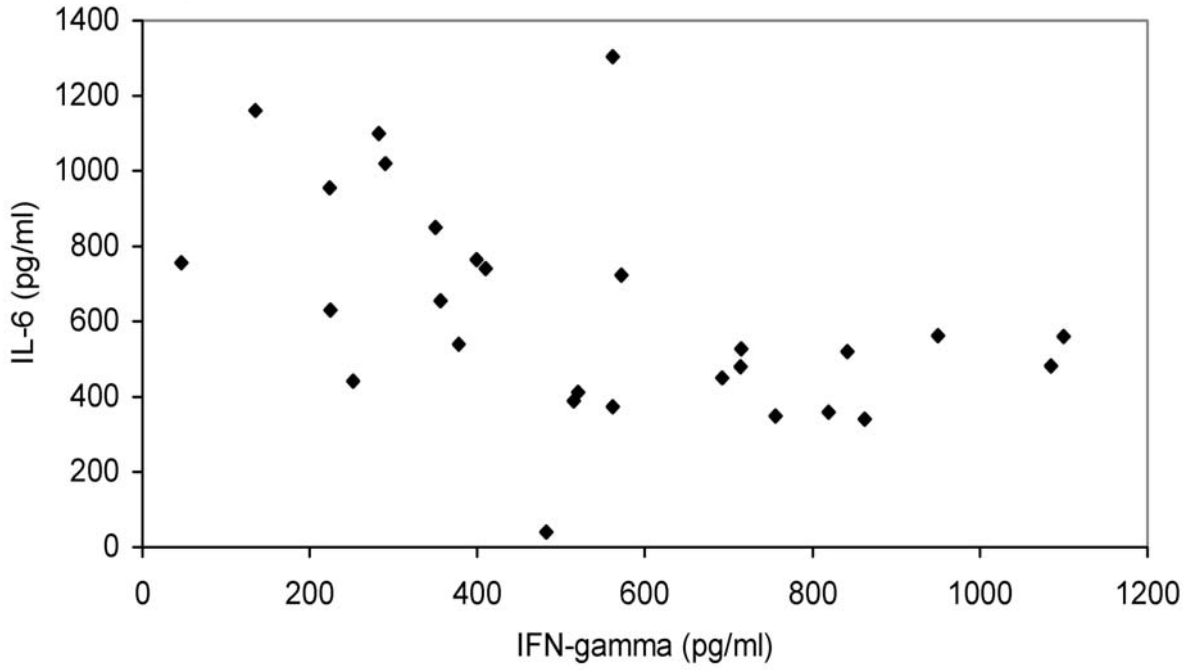


Figure 2. Correlation between liver IFN-gamma and IL-6 levels



The fact that portal central venous phlebitis was more in both group 1 and 3, with statistical significance in the latter only, suggested that the occurrence of this change might be due to the influence of estrogen on coagulation and venous thromboemboli (32,33).

Another striking result of this study has been the statistically significant difference in cytokine levels among groups. There has been an inverse correlation between IFN-g and IL-6 ($r < -0.5$). In group 2 with the lowest estrogen level, IL-6 was significantly lower ($p<0.001$) while IFN-g was significantly higher ($p<0.001$) than in the other groups in which IL-6 was higher while IFN-g was lower (table 5, figure 1). This finding showed the possible strong effect of estrogen on liver cytokine pattern. It has been proposed that for regeneration, hepatocyte needs growth factors like HGF (hepatocyte growth factor), TGF-a (transforming growth factor alpha), and EGF (epidermal growth factor), however, to be responsive to these growth factors, it must be sensitized with TNF (tumor necrosis factor) and IL-6 (34).

Kupffer cells have important effects on liver regeneration and in one of the studies a decrease in that cell population, and hence in the production and the levels of many cytokines including IL-6, have been shown to slow down regeneration (35).

It has been accepted that IL-6 accelerates regeneration (34,36, and 37) and the most important synthesizers of IL-6 are Kupffer cells and macrophages infiltrating liver (38).

Besides, it has been suggested that IL-6 increases fibrosis because TGF-b, that enhances fibrosis as a response to liver injury, seems to decrease in rats deprived of IL-6 (39). In this study, group 2 with low serum estrogen and liver IL-6 levels showed decreased regeneration and fibroblastic activity unlike the other groups with higher IL-6 levels, and this supported the data about IL-6 in the literature.

IFN-g increases fibrosis in liver and in the absence of lymphocytes responsible for the production of this cytokine more intense fibrosis is resulted (40, 41). In our study IFN-g was found to be high in group 2 with the lowest estrogen level and in that group regeneration and fibroblastic activity were decreased unlike the other groups with low IFN-g levels. This suggested that IFN-g had a role opposite to IL-6.

It has been reported that, estrogen suppresses (42-44), stimulates (45) or is ineffective (3) on the secretion of IL-6 from various tissues or cells. The mentioned different effects of estrogen on IL-6 secretion probably are due to the distinct features of different tissues. Although it has been shown that, liver (46) and serum (47) IL-6 level is increased transiently after obstructive jaundice, the effects of estrogen on liver tissue cytokine environment is not well understood. Influence of estrogen on IFN-g secretion is also contradictory; some reports have suggested suppression (48,49), while others have claimed inhibition (50,51).

In this study we showed for the first time, even after 14 days of bile duct ligation, secretion of IL-6 was found to be increased while IFN-g was decreased in a reverse correlation under the influence of estrogen. Increased IL-6 and decreased IFN-g, together with the demonstration of accelerated regeneration and fibroblastic activity in liver, suggested the role of estrogen on liver injury might be mediated by these cytokines. Furthermore, determination of significantly more Kuppfer cell changes in group 1 and 3 with high level of estrogen, reminded us changing of liver cytokine level might be controlled by these cells. However, demonstration of higher degree of necrosis and other injury parameters in the groups with high level of estrogen, suggests that beside augmentation of regeneration, estrogen also increases existing injury.

As a result, our study indicates that in liver injury that is caused by bile duct obstruction, medications that contain estrogens should be avoided until the underlying cause of the liver damage is eliminated but thereafter they may be used because of possible effect of estrogen on liver regeneration. The existence and the considerable effects of estrogen receptors in liver may give rise to experiments studying on specific estrogen receptor modulators, at least, in some liver diseases.

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