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CLINICAL RESEARCH

Effect of oophorectomy and exogenous estrogen replacement on liver injury in experimental obstructive jaundice

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Abstract

AIM: To investigate the role of estrogen on liver injury in an experimental obstructive jaundice model.

METHODS: 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 (n = 14). Fourteen days following constitution of bile duct ligation, all groups were compared in terms of serum tests, histopathologic parameters, and tissue levels of IFN-γ and IL-6.

RESULTS: The parameters representing both the injury and/or the reactive response and healing were more pronounced in groups 1 and 2 (χ^2 = 17.2, χ^2 = 10.20; χ^2 = 12.4, $P < 0.05$). In the sham operated or E2 administered groups significantly lower tissue levels of IFN-γ and higher IL-6 levels were found. In contrast, high IFN-γ and low IL-6 tissue levels were found in the oophorectomized and placebo group ($P < 0.001$). Kupffer cell alterations were observed to be more pronounced in the groups 1 and 3 (χ^2 = 6.13, $P < 0.05$).

CONCLUSION: Our study indicates that E2 impaired 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.

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Key words: Estrogen; Liver injury; Oophorectomy; Obstructive jaundice

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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 gender may determine the susceptibility to some diseases whereas it may be preventive to others, which is probably attributed to estrogen(s). The recent identification of estrogen receptor (ER) β in many tissues besides the target organs of estrogen, which mainly possess ERα[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 in the liver[10] has led studies to focus on the influence of steroid hormones, particularly estrogens, on liver disorders and regeneration[11]. In rats, liver injury induced by alcohol[12,13] or carbon tetrachloride[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 accelerate 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 extrahepatic bile duct obstructions, endotoxemia has been demonstrated and prolonged bile duct obstruction is harmful for phagocytic 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.

MATERIALS AND METHODS

Female Wistar albino rats, 12-16 wk old and 180-200 g, were used. They were kept in temperature-controlled environment, and they had free access to standard rat food and water. The study was approved by Gazi University ethic committee.

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 levels. Only laparotomy and ovary exploration was performed in group 3. Synthetic estrogen 17 β-estradiol (Sigma Chemical Co., Steinheim, Germany) was dissolved in sesame oil (Sigma) and 5 mg/0.1 mL injection doses were prepared. Fourteen days after surgeries, during which effects of ovarian estrogen had disappeared, group 1 was given subcutaneous 5 mg/0.1 mL 17 β-estradiol. Group 2 and group 3 were given 0.1 mL sesame oil subcutaneously as a placebo, meanwhile group 1 received 5 mg estrogen in 0.1 mL doses subcutaneous. Second and third doses were given 1 d and 5 d after the first dose.

The bile ducts were ligated 24 h after administration of the last dose of estrogen (or placebo), as previously described[20]. The animals were anesthesized by an intramuscular injection of ketamine hydrochloride (50 mg/kg of body weight) under semisterile 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.

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. The liver was excised and the liver was divided into two samples; one sample was kept in 10% formaldehyde solution for histopathological evaluation and the other was sent to the laboratory immediately as a fresh tissue for cytokine level measurements by ELISA (Table 1).

Table 1 Study design (n = 14)

Day	Group 1	Group 2	Group 3
0	Bilateral oophorectomy	Bilateral oophorectomy	Sham operation
15, 16, and 22	5 mg/0.1 mL E2 in sesame oil, SC	0.1 mL sesame oil, SC	0.1 mL sesame oil, SC
23	Bile duct ligation	Bile duct ligation	Bile duct ligation
37	Killed, blood and tissue samples obtained	Killed, blood and tissue samples obtained	Killed, blood and tissue samples obtained

SC: Subcutane.

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, alanine transaminase (ALT), and γ-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 (interferon)-γ and IL (interleukin)-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 s in 10 mL of phosphate buffered solution containing a cocktail of protease inhibitors including 2 mmol/L phenylmethyl-sulphonyl fluoride and 2 μg/mL aprotinin, leupeptin and pepstatin A (Sigma) to inhibit proteolysis of cytokines. The homogenates were collected from the homogenizer (Jencons Scientific Ltd., Bedfordshire, UK), ultra-centrifuged at 10000 r/min at 4℃ for 45 min, the supernatants aliquoted and stored at -70℃. Cytokines were measured by ELISA with rat IFN-γ 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 h. Following treatment with various concentrations of alcohol and xylene, the specimens were embedded in paraffin blocks. After the preparation, the sections were stained with Hematoxylin & Eosin and Masson's trichrome stains. Evaluations were made by a pathologist, blinded, using the parameters described previously[20]. Each of the histopathological parameters listed in Table 2 were 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 standard deviations

Table 2 Histopathologic scoring of liver injury

	Parameters	Score
1	Necrosis	0, 1, 2, 3
2	Regenerative activity	0, 1, 2, 3
3	Portal PMNL infiltration	0, 1, 2, 3
4	Portal MNL infiltration	0, 1, 2, 3
5	Ductular proliferation	0, 1, 2, 3
6	Fibroblastic activity	0, 1, 2, 3
7	Kupffer cell abnormalities	0, 2
8	Sinusoidal PMNL infiltration	0, 2
9	Sinusoidal MNL infiltration	0, 2
10	Portal vascular congestion	0, 2
11	Sinusoidal vascular congestion	0, 2
12	Portal vascular thrombosis	0, 2
13	Sinusoidal vascular thrombosis	0, 2
14	Portal and central venous phlebitis	0, 2
15	Arterial wall changes	0, 1, 2, 3
16	Hydropic degeneration	0, 1, 2, 3
17	Decrease in hepatocyte glycogen content	0, 2

PMNL: Polymorphonuclear leukocytes; MNL: Mononuclear leukocytes.

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 580 ± 124.01 in the group 1 in which oophorectomy and E2 replacement had been done, whereas the lowest values were 61.5 ± 15.97 obtained in group 2 given placebo. Blood estrogen levels of group 3 was 208 ± 35.02.

Serum bilirubin, ALT, and GGT levels increased in all groups, as evidence 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 group 2 given the placebo given group 2 than in the groups 1 and 3 in which blood estrogen levels were normal or high, respectively. 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 3).

Liver specimens of all rats were examined histopathologically according to the parameters mentioned in table 2 and groups were then compared using the 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 the groups ($P < 0.05$, Table 4). Progressive rounding, ruffling of cell surface, polarization, appearance of wormlike densities, vacuolization and degranulation were the abnormalities seen in Kupffer cells. The photos of ductal proliferation, fibrosis and sinusoidal congestion of specimens can be seen especially in group 1 and 3 (Figure 1). In terms of portal polymorphonuclear leukocyte and mononuclear leukocyte infiltration, sinusoidal polymorphonuclear leukocyte and mononuclear leukocyte infiltration, portal vascular congestion, portal and sinusoidal vascular thrombosis, arteriolar wall changes, hydropic degeneration,

Table 3 Comparison of liver function tests of the groups

Groups	ALT (U/L)	GGT (U/L)	Bilirubin level (mg/dL)
1	347.85 ± 61.12	65.35 ± 20.98[a]	6.83 ± 1.32
2	256.57 ± 54.19[a]	46.50 ± 12.0	5.90 ± 2.12
3	360.50 ± 65.22	58.78 ± 7.47	6.30 ± 1.57

There are significant differences when compared with other groups ([a]$P < 0.05$).

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 ($\chi^2 = 17.2$, $P < 0.05$, Table 4). Group 2 was also found to be significantly different with its lower regenerative activity ($\chi^2 = 10.22$, $P < 0.05$, Table 4).

Ductular proliferation, a reactive response of liver to bile duct obstruction, was statistically more in groups 1 and 3 than in group 2 ($\chi^2 = 12.43$, $P < 0.05$, Table 4).

In groups 1 and 3 with higher estrogen levels, fibroblastic activity was also significantly more pronounced ($\chi^2 = 31.06$, $P < 0.001$, Table 4). There was a significant difference in terms of Kupffer cell abnormalities between groups 1 and 2 ($\chi^2 = 6.13$, $P < 0.05$, Table 4).

Sinusoidal congestion and portal venous phlebitis were significantly lower in group 2 than in group 3 ($\chi^2 = 8.17$, $P < 0.05$, $\chi^2 = 7.636$, $P < 0.05$, respectively, Table 4).

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-γ and IL-6 and were found to be statistically different. While IFN-γ was significantly higher, IL-6 was lower in group 2 with the lowest estrogen level ($P < 0.001$, Table 4).

With Pearson's correlation analysis, IFN-γ 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 ($r = -0.36$, $P < 0.001$, Figure 2).

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[2,23], 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 the 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 speculated and shown that estrogen has a larger field of distribution than previously believed[7,27]. After the demonstration of estrogen receptors in liver, studies on the interaction of estrogen and its receptor in liver have accumulated[28,29]. Estrogen is thought to enhance regeneration after hepatectomy,

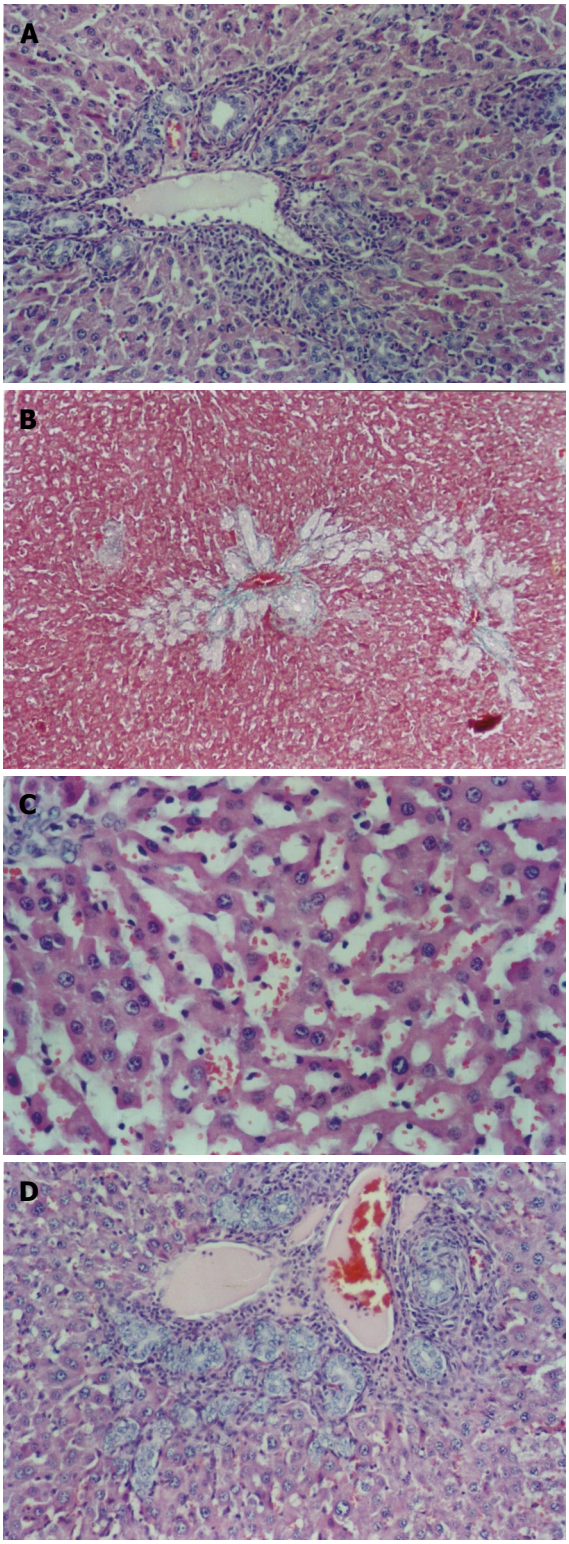


Figure 1 **A**: Ductal proliferation and inflammation (HE, × 20); **B**: Fibrosis and ductal proliferation (Masson trichrom, × 10); **C**: Sinusoidal congestion (HE, × 40); **D**: Ductal proliferation and portal vascular congestion (HE, × 20).

due to its potentiative effect on proliferation, 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 carbon tetrachloride[4] administration[5,13,14,30]. Estrogen treatment also has an effect in cholangiocyte proliferation[31].

Previous literature comprises data about the influence

Table 4 Comparison of groups by categories *n* (%)

Categories	Group 1	Group 2	Group 3	χ^2/*P*
Necrosis				
No change	5 (35.7)	12 (85.7)	7 (50)	χ^2 = 17.20
Slight	7 (50)	0 (0)	1 (7.1)	*P* < 0.050
Moderate	2 (14.3)	2 (14.3)	6 (42.9)	
Regenerative activity				
Slight	6 (42.9)	13 (92.9)	6 (42.9)	χ^2 = 10.22
Moderate	5 (35.7)	1 (7.1)	6 (42.9)	*P* < 0.050
Severe	3 (21.4)	0 (0)	2 (14.2)	
Ductular proliferation				
Slight	0 (0)	6 (42.9)	1 (7.1)	χ^2 = 12.43
Moderate	5 (35.7)	5 (35.7)	4 (28.6)	*P* < 0.050
Severe	9 (64.3)	3 (21.4)	9 (64.3)	
Fibroblastic activity				
Slight	1 (7.1)	9 (64.3)	1 (7.1)	χ^2 = 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				
Absence	6 (42.9)	12 (85.7)	7 (50)	χ^2 = 6.13
Presence	8 (57.1)	2 (14.3)	7 (50)	*P* < 0.050
Sinusoidal congestion				
Absence	13 (92.9)	9 (64.3)	14 (100)	χ^2 = 8.17
Presence	1 (7.1)	5 (35.7)	0 (0)	*P* < 0.050
Portal central venous phlebitis				
Absence	3 (21.4)	6 (42.9)	0 (0)	χ^2 = 7.64
Presence	11 (78.6)	8 (57.1)	14 (100)	*P* < 0.050
IFN-γ (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

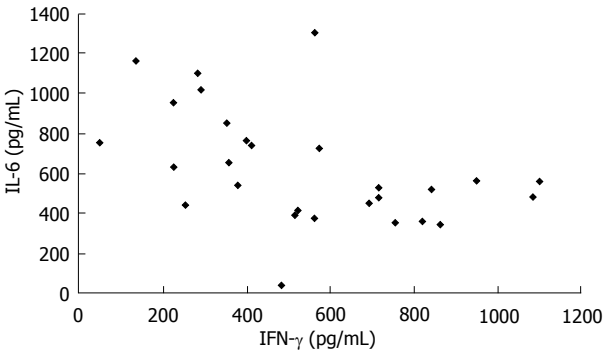


Figure 2 Correlation between liver IFN-γ and IL-6. *r* = -0.36, *P* < 0.001.

of estrogen on various types of liver injury other than obstruc-tive 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[18,32]. In general, benign and malignant bile duct obstructions are mostly seen in the 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 maintained 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 d 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 less significant in extent in group 2 with the lowest estrogen level than in the other groups, considering the seven parameters yielding statistical difference among groups (Table 4). This finding has shown that estrogen has an 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 that both physiological and supraphysiological levels of estrogen potentiated, not only liver injury, but also reactive responses (Table 4). These data correlate well with the results of studies reporting that estrogen accelerates liver injury caused by alcohol and carbon tetrachloride[13,15,17].

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[33,34].

Another striking result of this study was the statistically significant difference in cytokine levels among groups. There has been an inverse correlation between IFN-γ and IL-6 ($r < -0.5$). In group 2, with the lowest estrogen level, IL-6 was significantly lower ($P < 0.001$) while IFN-γ was significantly higher ($P < 0.001$) than in the other groups in which IL-6 was higher and IFN-γ lower (Table 4). 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 hepatocyte growth factor, transforming growth factor-α, and epidermal growth factor; however, to be responsive to these growth factors, it must be sensitized with tumor necrosis factor-α and IL-6[35]. Kupffer cells have important effects on liver regeneration and in one study 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[36]. It has been accepted that IL-6 accelerates regeneration[35,37,38] and the most important synthesizers of IL-6 are Kupffer cells and macrophages infiltrating liver[39]. Besides, it has been suggested that IL-6 increases fibrosis because transforming growth factor-β, that enhances fibrosis as a response to liver injury, seems to decrease in rats deprived of IL-6[40]. In this study, group 2 with low serum estrogen and liver IL-6 levels showed decreased regeneration and fibrotic activity unlike the other groups with higher IL-6 levels, and this supported the data about IL-6 in the literature.

IFN-γ increases fibrosis in liver and in the absence of lymphocytes, responsible for the production of this cytokine, more intense fibrosis results[41,42]. In our study, IFN-γ was found to be high in group 2 with the lowest estrogen level and in group 2 regeneration and fibroblastic activity were decreased unlike the other groups with low IFN-γ levels. This suggested that IFN-γ had a role opposite to that of IL-6.

It has been reported that, estrogen suppresses[43-45], stimulates[46] 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[47] and serum[48] IL-6 levels are increased transiently after obstructive jaundice, the effects of estrogen on liver tissue cytokine environment is not well understood. Influence of estrogen on IFN-γ secretion is also contradictory; some reports have suggested suppression[49,50], while others have claimed inhibition[51,52].

In this study, we showed for the first time, even after 14 d of bile duct ligation, secretion of IL-6 was found to be increased while IFN-γ was decreased in a reverse correlation under the influence of estrogen. Increased IL-6 and decreased IFN-γ, 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 Kupffer cell changes in groups 1 and 3 with high level of estrogen, reminded us that changing of liver cytokine levels 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 effects 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. This study has also suggested that, in liver disease occurring before menopausal period, detrimental effects of physiological levels of estrogen(s) on the liver can perhaps be prevented by using specific estrogen blockers.

COMMENTS

Background

Different responses depend on differences in hormones between the sexes and have been the subject of several studies for several years. The main target hormone, estrogen, plays a critical role in several mechanisms in the body. Among these, estrogen receptors found in liver tissue have a key role for the estrogen effects on liver and related mechanisms. As women are more prone to bile stones and elderly postmenoposal patients have a higher incidence of bile obstruction, a role for estrogen must be considered in these pathologies.

Research frontiers

It was shown that estrogen has a proliferative effect in partial hepatectomy; however, it increases the susceptibility of the liver to injury. On the other hand, bile duct obstruction, bile stasis and related cytokine changes by Kupffer cells have a distinct entity that must be investigated. Histopathological changes including Kupffer cells and cytokine release related to estrogen is still an unclear mechanism.

Innovations and breakthroughs

As several studies mentioned the regenerative effect and increased injury due to hepatectomy, our study is performed in bile duct obstruction model. Not only the cholangiocyte proliferation, but also the cytokine levels, liver regeneration and liver function tests were also evaluated. Women are more prone to bile stones and the rate of bile duct obstruction increases with age. Bile duct obstruction related to bile stones is more likely to be seen in postmenopausal women. In this point of view, estrogen status and expected situations must be investigated.

Applications

Although some points have been mentioned here, there are still unknown mechanisms for the actions of estrogen. Cytokine release, thromboemboli and coagulation are thought to be potential mechanisms. However, no clear definition has been made. We feel that this study will help in further clinical studies and will be an investigative source for other estrogen and bile duct obstruction related studies.

Peer review

This is an interesting investigation of effects of estrogens on liver injury and regeneration using the bile duct ligation model. The presentation and readability of the manuscript is good. It 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.

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