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World J Gastroenterol 2002;8(2):363-366
World Journal of Gastroenterology
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• BASIC RESEARCH •

Protective effect of estradiol on hepatocytic oxidative damage

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Received 2001-08-09 **Accepted** 2001-08-23

Abstract

AIM: **To examine the protective effect of estradiol on the cultured hepatocytes under oxidative stress.**

METHODS: **Hepatocytes of rat were isolated by using perfusion method, and oxidative stress was induced by a serum-free medium and FeNTA. MDA level was determined with TBA method. Cell damage was assessed by LDH assay. Apoptosis of hepatocytes was assessed with cytoflowmetric analysis. Expression of Bcl-xl in cultured hepatocytes was detected by Western blot. The radical-scavenging activity of estradiol was valued by its ability to scavenge the stable free radical of DDPH.**

RESULTS: **Oxidative stress increased LDH (from $168\pm25\times10^{-6}$IU.cell^{-1} to $780\pm62\times10^{-6}$IU.cell^{-1}) and MDA (from $0.28\pm0.07\times10^{-6}$nmol.cell^{-1} to $1.35\pm0.12\times10^{-6}$ nmol.cell^{-1}) levels in cultured hepatocyte, and estradiol inhibited both LDH and MDA production in a dose dependent manner. In the presence of estradiol 10^{-6} mol.L^{-1},10^{-7}mol.L^{-1} and 10^{-8}mol.L^{-1},the LDH levels are $410\pm53\times10^{-6}$IU.cell^{-1}($P<0.01$ *vs* oxidative group), $530\pm37\times10^{-6}$IU.cell^{-1} ($P<0.01$ *vs* oxidative group), $687\pm42\times10^{-6}$IU.cell^{-1}($P<0.05$ *vs* oxidative group) respectively, and the MDA level are $0.71\pm0.12\times10^{-6}$ nmol.cell^{-1}($P<0.01$ *vs* oxidative group),$0.97\pm0.11\times10^{-6}$ nmol.cell^{-1}($P<0.01$ *vs* oxidative group) and $1.27\pm0.19\times10^{-6}$nmol.cell^{-1} respectively. Estradiol suppressed apoptosis of hepatocytes induced by oxidative stress, administration of estradiol(10^{-6}mol/L)decreased the apoptotic rate of hepatocytes under oxidative stress from 18.6±1.2% to 6.5±2.5%, $P<0.01$. Bcl-xl expression was related to the degree of liver cell damage due to oxidative stress, and estradiol showed a protective action.**

CONCLUSION: **Estradiol protects hepatocytes from oxidative damage by means of its antioxidant activity.**

Liu Y,Shimizu I, Omoya T, Ito S, Gu XS, Zuo J.Protective effect of estradiol on hepatocytic oxidative damage. *World J Gastroenterol* 2002;8(2):363-366

INTRODUCTION

Hepatic fibrosis is a common consequence of chronic liver injury from many causes[1-7], and the critical event in hepatic fibrosis is the activation of lipocyte (also known as the stellate, fat-storing or Ito cell) which is the main source of extracelluar matrix in fibrosis formation [8-20]. The putative impetus to lipocyte activation came from cytokines released from Kupffer cells or leukocytes in liver injury[21]. An alternative way is that parenchymal-cell necrosis itself may activate lipocyte; such activation could be mediated by the lipid peroxides formed after the membrane of parenchymal cells is injured[22-28]. Therefore, preventing hepatocyte from injury is a matter of primary importance in blocking the fibrogenic pathway. We have reported the inhibitory effect of estradiol on activation of rat lipocytes[29,30] and the suppressive effect on fibrogenesis in rat model[31]. The present study is initiated to investigate the role of estradiol on the hepatocyte under oxidative stress, and to elucidate the mechanism of its inhibitory effect on hepatic fibrogenesis.

MATERIALS AND METHODS

Hepatocyte isolation and induction of lipid peroxidation

Hepatocytes were isolated from the liver of male Wistar rats(500-600g) with *in situ* perfusion method as previously described. Inocula of 2×10^5 cells per well were introduced into 12-well plate, (Nunc). The cells were cultured in 1ml Williams medium E supplemented with 50ml·L^{-1} FBS, 105U·L^{-1} penicillin,100mg·L^{-1} strptomycin, and 10g·L^{-1} L-glutamine at 37℃ in 50ml·L^{-1} CO_2 atmosphere and 100% humidity. After 4h, the cell medium was removed and lipid peroxidation was induced by incubating hepatocytes in serum-free Williams medium E with 100μmol·L^{-1} FeNTA(ferric nitrilotriacetate). Three groups of hepatocyte were analysed in parallel in each experiment including the hepatocytes cultured under normal condition and the hepatocyte cultured under oxidative stress in the presence or absence of 17-β-estradiol(Sigma).

Detection of (MDA) and lactate dehydrogenase (LDH) level in culture medium

Lipid peroxidation in cultured hepatocyte was determined by detecting the level of malondialdehyde (MDA), end product of lipid peroxidation in culture medium. The cell medium was collected, centrifuged at 450g to remove cell debris, and the MDA contents was determined by using a colorimetic reaction with thiobarbituric acid. A calibration curve was constructed from the conversion of tetraethoxypropane to MDA. The degree of cell damage was assessed by detecting the lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. The LDH activity was determined by using LDH detection Kit(Sigma). The time course of the MDA and LDH levels in culture medium was constructed to examine the lipid peroxidation and cell damage of hepatocyte during cultivation.

Flowcytometric analysis for apoptosis in hepatocytes

Rat hepatocytes were isolated and inoculated in 24-well plate as described, After oxidative stress was induced for 24h, apoptotic hepatocytes were detected by flowcytometry. Apoptotic cells expose their phosphatidylserine (PS) in the outer leaflet of cell membrane.

The exposed PS can be revealed by FITC-conjugated annexin V. Annexin V Kits(BD pharmingen) was used ,and the samples were treated according to the instruction enclosed in the kit as mannul described and analysed on the flowcytometer(Coulter Epics).

Western blot analysis of Bcl-xl

Rat hepatocytes were cultured in 35mm diameter dishes(Nunc) which were divided into 3 groups: the normal group, the oxygen stress and the oxygen stress plus estradiol group. In the presence or absence of 10^{-6}mol·L^{-1} estradiol for the indicated time period,the dishes were then washed twice with ice cold PBS and lysed directly in 1ml SDS loading buffer(50mmol·L^{-1} Tris, pH6.7, 20g·L^{-1}SDS, 100g·L^{-1} glycerol, 0.6g·L^{-1}bromophenol blue, 100mmol·L^{-1} dithiothreitol). The samples were boiled for 5 min and applied to a standard 120g·L^{-1} SDS polyacrylamide protein gel. After electrophoresis, protein transfer was performed onto Hybond-ECL(Amersham Pharmacia Biotech) using a semi-dry blotting apparatus. The membrane was treated first with 100ml·L^{-1} non-fat milk in PBS at room temperature for 2h and next with the Bcl-xl or Bcl-2 monoclonal antibody (Tanslab; diluted 1:500)for two hours at room temperature. After washing, the membrane was then incubated with HRP conjugated goat antimouse IgG(Amersham Pharmacia Biotech; diluted 1:1000)for one hour at room temperature. Immunoreactive bands were visualized using the ECL western blotting detection system kit (Amersham Pharmacia Biotech) according to the manufacturer's recommended protocol. The membranes used for Bcl-xl or Bcl-2 detection were reprobed with actin polyantibody and the corresponding secondary antibody to normalize the signal strength of Bcl-xl and Bcl-2.

Radical-scavenging activity of estradiol

The radical-scavenging activity of estradiol was determined from its ability to scavenge the stable free radical of 1,1-diphenyl-2-picrylhydrazyl (DPPH,Wako) and was compared with that of the well-known antioxidant, α-tocopherol. 5μl of estradiol or α-tocopherol (2,4,6,8, 10μmol·L^{-1}) was added to 2.5ml 100μmo·L^{-1}l DPPH, in 20mmol·L^{-1} 2-(N-morpholino) ethanesulfonic acid (MES) (pH 5.5) and the change in optical absorbance at 517nm was measured 30min thereafter.

Statistical analysis

Experimental results were analyzed by Student's *t* test for multiple comparisons. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Effect of estradiol on MDA and LDH level in hepatocytes under oxidative stress

The MDA and LDH levels in the medium of hepatocytes cultured under normal condition and oxidative stress were shown respectively in Figure 1 and Figure 2. The MDA level increased rapidly 5h after the oxidative stress and was maintained at a considerably high concentration during the period of continued cultivation. In normal control, MDA was kept at a low concentration in the incubating time, indicating a low level of lipid peroxidation in the normal condition. LDH level increased steadily after oxidative stress, whereas the LDH level in normal control increased slightly during culture. These data revealed that oxidative stress initiate lipid peroxidation and cause cell membrane damage in hepatocytes under stress. Twenty-four' oxidative stress was selected as the time point to examine the effect of estradiol administration on the MDA and LDH level in hepatocytes under oxidative stress. As the data showed in Table 1,estradiol decreased the MDA and LDH level in the culture medium in a dose dependent manner.That means estradiol could inhibit the lipid peroxidation and subsequent hepatocytic membrane damage under oxidative stress.

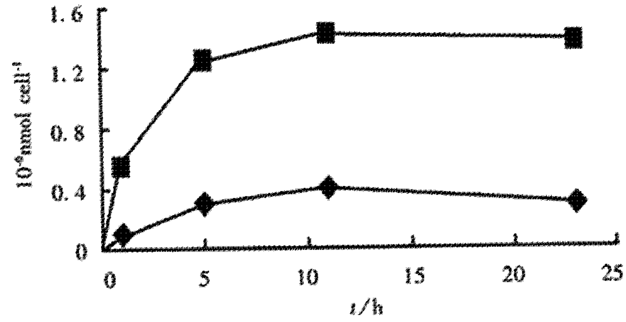

Figure 1 Effect of estradiol on MDA level in hepatocytes cultured under normal condition (◆) and oxidative stress (■)

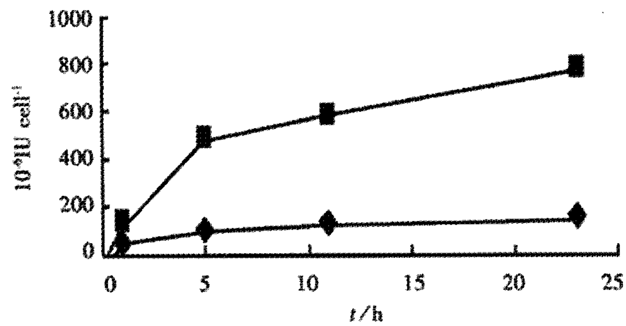

Figure 2 Effect of estradiol on LDH level in hepatocytes cultured under normal contion (◆) and oxidative stress (■)

Table 1 Effect of estradiol on MDA and LDH level in culture medium of hepatocytes under oxidative stress

Stress	MDA(10^{-6}nmol·cell^{-1})	LDH (10^{-6}IU·cell^{-1})
780±62		1.35±0.12
10^{-8}mol·L^{-1}estradiol	1.27±0.19	687±42^a
10^{-7}mol·L^{-1}estradiol	0.97±0.11^b	530±37^b
10^{-6}mol·L^{-1}estradiol	0.71±0.12^b	410±53^b
Normal control	0.28±0.07^b	168±25^b

^{a}*P*<0.05, ^{b}*P*<0.01 *vs* stress group.

Flowcyto-metric analysis for apoptotic hepatocytes

The aim of this investigation was to ascertain whether oxidative stress could induce apoptosis of hepatocytes, and whether estradiol could protect hepatocytes from such damage. The apoptotic rate of hepatocyte under normal culture condition was 5.9±1.7%, but in hepatocytes under oxidative stress it was 18.6±1.2%. With the administration of 10^{-6}mol·L^{-1} estradiol,the apoptotic rate of hepatocytes under oxidative stress decreased to 6.5±2.5%. These data suggest that estradiol inhibit hepatocytic apoptosis induced by oxidative stress (results obtained from three distinct experiments).

Western blot analysis of Bcl-xl

Bcl-2 family has been investigated extensively for its proapoptotic or antiapoptotic property; Bcl-xl and Bcl-2 are well-known negative regulator of apoptosis[32-36]. Western blot was used to examine the relationship between Bcl-xl and Bcl-2 level and estradiol administration. Estradiol applied in this experiment was 10^{-6}mol·L^{-1}. The result was depicted in Figure 3 (showing one representative result from three independent experiments). Bcl-xl level was upregulated in hepatocytes under oxidative stress for 24h as compared with the normal control, while estradiol administration attenuated the increased expression of Bcl-xl induced by oxidative stress. After culturing for 48h, Bcl-xl expression was increased in hepatocytes both in the absence or presence of estradiol under oxidative stress. Bcl-xl level in hepatocytes was increased under oxidative stress.Bcl-2 expression was not detectable even though two antibodies from two different companies were used, indicating its low level in hepatocyte.

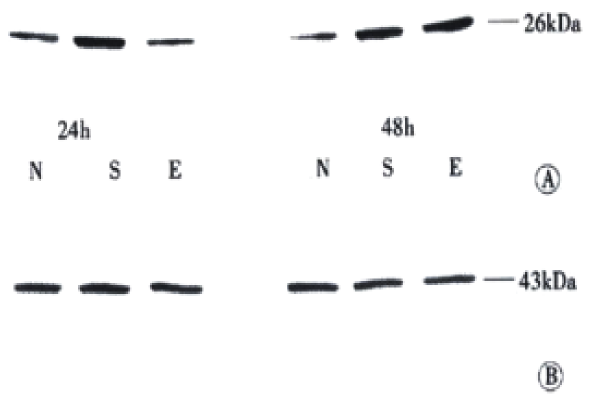

Figure 3 (A) Effect of estradiol on Bcl-xl expression in cultured hepatocytes for 24h and 48h.
(B) Actin expression in cultured hepatocytes for 24h and 48h. N: Normal condition. S: Oxidative stress. E: Oxidative stress supplemented with 10^{-6}mol $\cdot L^{-1}$ estradiol.

Radical-scavenging activity of estradiol

Radical-scavenging activity of estradiol was determined by monitoring the decrease in absorbance at 517nm. Estradiol caused an immediate decrease in DPPH absorbance in a dose dependent manner(Figure 4). Its radical scavenging activity potency was approximately half that of α-tocopherol.

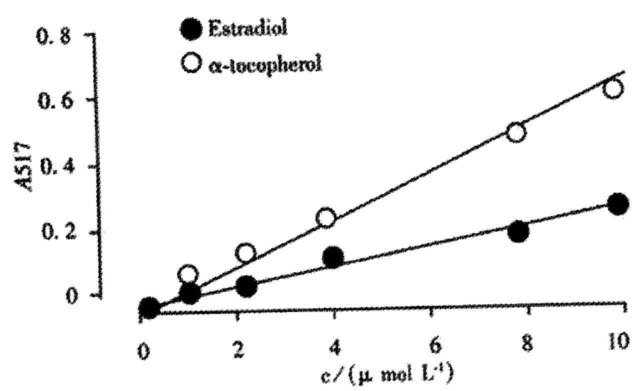

Figure 4 DPPH radical-scavenging activity of estradiol

DISCUSSION

Hepatic fibrosis and cirrhosis occur more frequently in men than in women. The ratio of male:female has been reported to range from 2.3:1 to 2.6:1 which indicates that sex hormones may play a role in the development of hepatic fibrosis and subsequent cirrhosis. We have found that estradiol treatment resulted in reduced hepatic fibrosis in male rat in which hepatic fibrosis had been induced by dimethylnitrosamine(DMN), and this phenomenon had been proven to be associated with the inhibitory effect of estradiol on the activation of lipocyte which is essential for hepatic fibrogenesis. Lipocyte can be activated by inflammatory factors realeased from Kupffer cells or free radicals and lipid peroxides formed in injuried liver. Therefore, we examined the effect of estradiol on the cultured hepatocytes to evaluate its protective activity against oxidative damage of the liver. Estradiol is a steroidal compound that binds to specific intracellular receptors which act as transcription factors. Although the liver is not the classical sex hormone target ,but livers in both men and women contain high affinity, low capacity estadiol receptors, and they are believed to respond to estradiol regulation. Another transcription independent activity of the molecule estradiol is its intrinsic antioxidant activity which makes it a potential chemical shield for cells[37,38]. The neuroprotective effect of estradiol from oxidative damage has been extensively investigated in recent years[39,40]. It is also noteworthy that oxygen-derived free radicals and lipid peroxidation have been implicated in hepatic injury[41]. Our data also demonstrated that estradiol could inhibit the lipid peroxidation in hepatocyte and the free radicals induced-hepatocytic injury,and thus exerted its suppressive effect on liver fibrosis.

Hepatocytic damages induced by oxidative stress include two forms: cell necrosis and apoptosis. We also found that estradiol could inhibit the apoptosis of hepatocytes in the face of oxidative challenges. To elucidate the mechanism of its antiapoptotic effect, we examined the relationship between estradiol administration and the Bcl-2 and Bcl-x expression level by Western blotting. Figure 3 showed that Bcl-xl level in hepatocytes was increased under oxidative stress, while administration of estradiol attenuated the increased expression of Bcl-x induced by oxidative stress.These data suggested that Bcl-x expression responsed directly to oxidative stress as a protective reaction, and estradiol could counteract the oxidative stress and stabilized the Bcl-xl expression before its antioxidant activity was depleted. In this study, Bcl-2 expression was not detectable even though two antibodies from different companies were used because of its low level in hepatocytes. But in another investigation we found that estradiol administration dramatically increased the Bcl-2 level in fibrotic liver tissue of rat induced by DMN injection(data not presented). Therefore, the mechanism of the antiapoptotic effect of estradiol is still elusive. The regulators, which participate in this pathway need further investigation. In conclusion, our findings suggested that estradiol could protect hepatocytes from oxidative stress , and the transcription-independent antioxidant activity of estradiol molecule may play a major role in this pathway.

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Edited by Lu HM