Original Article

Estrogen and/or Progesterone Effects on HepG2 Human Cell Lines; Oxidant or Antioxidant?

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Abstract

Objective: Although much is known about estrogen and progesterone, their toxicity or protective effects on hepatocytes are yet to be fully understood. This study investigated the probable oxidant or antioxidant effects of estrogen and progesterone on HepG2 cell line in the presence or absence of H2O2. **Methods:** HepG2 cells were cultured with two concentrations of estrogen and progesterone (10PM and 1μM) and H2O2 (50 and 200 μM) separately, and in combination, for 24 hours. The effects of selected doses after MTT assay on: 1) cellular integrity, 2) GR and GPx activity, 3) cellular levels of GSH and 4) ALT and AST activities were assessed. **Results:** MTT assay showed toxic effect of 200 μM H2O2 on the cells. According to MTT results, 10nM and 1 μM doses of estrogen and progesterone and 1μM of each in combination, in the presence of 50 μM H2O2 were selected for the rest of the experiments. Incubation of the cells with H2O2 caused a remarkable decrease in GPx and GR activities as well as GSH level, and an increase in ALT and AST levels. However, treatment with estrogen attenuated further changes and estrogen in combination with progesterone led to a more pronounced amelioration of H2O2-induced toxicity. **Conclusion:** Our results demonstrated that while high level of oxidative stress is severely cytotoxic, estrogen and progesterone might significantly improve the antioxidant defense within hepatocytes which undergo a low-intensity oxidative exposure.

Introduction

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive mediators or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals which potentially damage all components of the cell, including proteins, lipids, and DNA. Furthermore, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. Our body possesses enzymatic

and non-enzymatic defense mechanisms against oxidative stress (1).

The enzymatic antioxidants including superoxide dismutase (SOD), catalase and gluthation peroxidase (GPx), act as the first defense against reactive oxygen species (ROS) through catalyzing their conversion to less reactive or insert species. Some low molecular-weight molecules inside the cell such as glutathione (GSH) provide secondary defense against free radicals (2,3).

Estrogens own a phenolic hydroxyl group and a variety of in vivo beneficial effects, like protection against coronary heart disease, osteoporosis, Alzheimer's disease and stroke(4) and hormone therapy demonstrates many beneficial effects in the treatment of these diseases (5).On the other hand, quinoids, quinoid radicals and phenoxy radicals are formed from estrogens and may be responsible for adverse effects such as carcinogenesis (6). The biological effects of estrogens involve their dual role as pro-oxidants and antioxidants. GSH, glutathione peroxidase (GPX), have shown an antioxidant role which increases detoxification of free radicals formation and thus prevents many pathophysiologic processes (3). Massafra et al. suggested that the physiological production of ovarian estradiol during menstrual cycle may have an important role in regulating erythrocyte glutathione peroxidase activity (7). Dantas et al. showed that estrogen at physiological concentrations act as an antioxidant in vivo. They have proposed that NADPH oxidase inhibition attenuates superoxide production in the absence of estrogen indicating that estrogen has a modulatory role on NADPH oxidase activity (8).

Similarly, progesterone is reported to have neuroprotective properties following cerebral ischemia and traumatic brain injury and may ameliorate the oxidative stress by its membrane stabilizing effect. However, some studies found that progesterone could result in toxic effects (9, 10).

Aspartate aminotransferase (11) and alanine aminotransferase (ALT) are raised in acute liver damage, and the AST to ALT ratio is often useful in differentiating the causes of liver damage. In the present study, we tried to evaluate the antioxidation or oxidant effects of sex hormones estrogen and progesterone in HepG2 cell line in the presence or absence of H2O2. We measured AST and ALT to evaluate cellular damage against H2O2 and probable antioxidant effects of sex hormones on the rate of cell damage in the presence of H2O2. We also assessed the effect of these hormones on the enzymatic activity of GPx and GR and GSH level on this hepatocyte cell.

Materials and methods

Materials

Glutathione reductase (GR), tert-butyl hydroperoxide (t-BuOOH), bovine serum albumin (BSA), Triton X-100, di-methyl sulfoxide (DMSO) were purchased from Sigma Chemical Co (Poole, Dorset, UK). RPMI, FBS (fetal bovine serum), Penicillin and streptomycin were obtained from Gibco-BRL (Paisley, UK) and estrogen and progesterone was provided by Mehr Darou (Tehran, Iran). Tripure Isolation Reagent was from Roche Applied Sciences (Indianapolis, IN). cDNA Synthesis Kit was purchased from Fermentas, EU and SYBR green DNA PCR Master Mix was from AB Company (Foster City, CA USA).

Cell Culture and treatments

The human hepatocellular cell line (HepG2) was obtained from NCBI (National Cell Bank of Iran, Pasteur Institute, Tehran). HepG2 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37 °C in 5% CO2. The cells were seeded at a density of 5×10^5 cell in a flask (25 cm2) and then incubated with different treatments in 12 conditions for 24 hours (Table 1).

MTT assay

A cell viability assay was carried out using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described by Mosmann (12), with minor modifications. Briefly, HepG2 cells (1×10^4 cells/well in 96-well plate) were first incubated with applied treatments for 24 h at 37°C. The cells were then incubated in serum-free medium to which MTT (0.5 mg/mL, 10 µl) was added. Following 3.5 h incubation, 100 µl of DMSO was added to dissolve the formazan crystals and the absorbance was determined in an ELISA reader at 570/650 nm. The number of metabolically competent cells was determined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells which served as control. The experiment was repeated five times.

Determination of glutathione peroxidase (GPx) activity

The procedures of Fecondo and Augustey (13), was used for the determination of GPx activity, with minor modifications (14).

The enzyme activity in clear supernatant of HepG2 cell lysate was expressed as µMol of NADPH oxidized/min/mg cell protein using a molar extinction coefficient of $6.22×10^6$ M-1cm-1 for NADPH. One unit of GPx was defined as mU/mg cell protein.

Table 1. The twelve different conditions applied for HEPG2 cells treatment.

Control	Control+ Ethanol	Progesterone (P1) (10nM)	Progesterone (P2) (1µM)	Estrogen (E1) (10nM)	Estrogen (E2) (1µM)
E2+P2	H2O2 (50µM)	H2O2 (200µM)	H2O2+ P2	H2O2 +E2	H2O2+E2+P2

Determination of glutathione reductase (GR) activity

The activity of GR was assessed using the method described by Racker, with minor modifications (11). The decrease in absorbance, which reflects the oxidation of NADPH during reduction of GSSG by GR present in the sample, was monitored spectrophotometrically at 340 nm for 3 min. Results were based on a molar extinction coefficient for NADPH of $6.22×10^6$M-1cm-1. One unit of GR was defined as mU/mg cell protein.

Determination of glutathione

The assay of GSH with DTNB was performed followed by a standard Ellman's method (15). Clear supernatant of cell lysate was analyzed for GSH level. Potassium phosphate buffer (0.2 M, pH 7.6) was added to 0.2 ml of cell lysate supernatant and then DTNB (0.001 M) was added to the solution. An absorbance of the products was observed after 5 min at 412 nm.

Determination of ALT, AST

Following 24 h treatment, cell culture media from each of the 25 cm2 culture flasks were collected, centrifuged at 3000×g for 10 min and stored at -70°C until assessments. ALT and AST activities were measured using a kit (Zist-Chimie, Tehran, Iran) based on the colorimetric method of Reitman and Frankel (16).

Statistical analysis

The effect of the varying treatments was assessed on the various study parameters using a repeated measures statistical design. All data were analyzed by Kruskal-Wallis and Mann-Whitney tests for group comparison and expressed as mean ± standard error of the mean. $P<0.05$ was taken as significant. The SPSS.16 software package was used for data analysis.

Results

Effect of treatments on cell survival in HepG2 cells

MTT test was used as an indicator of cytotoxicity induced by treatments in cultured HepG2 cells. The cells were exposed to all concentrations of treatments for 24 h and the maximal ethanol concentration was used to solubilize and dilute estrogen and progesterone as vehicle. According to our findings, treatments with 200µM H2O2 induced 51% cell damage while others had no significant effects on cell viability as shown in Figure 1. Treatments with 200µM H2O2 were not continued in other experiments (Figure 1).
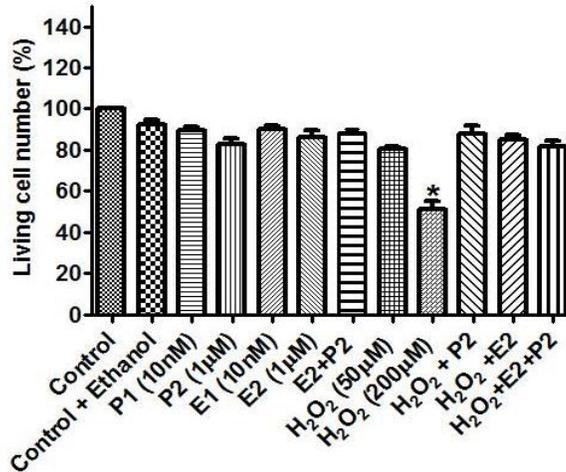
Figure 1. The effect of treatments on cell viability in HepG2 cells. Following 24 h of treatment, the viability of HepG2 cells was evaluated using the MTT assay. Values are reported as mean ± standard error of the mean (SEM). Sample size (n) = 5. *P < 0.05 indicates significant change as compared to the control and Φ as compared to the 50 μM H2O2.

Effects of treatments on GPx activity

As demonstrated in Figure 2, 50μM H2O2 induced 33% decrease in GPx activity, however treatment with P2 (1μM progesterone) did not ameliorate this reduction while E2 (1μM estrogen) and P2+E2 increased GPx activity. Compare to control, it failed to show any significant difference. On the other hand, E1, E2 and E2+P2 without H2O2 had an increasing effect on GPx activity however this induction was insignificant (Figure 2).
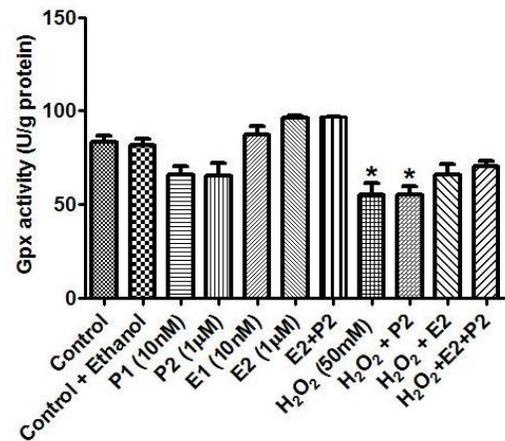
Figure 2. The effect of treatments on the activity of glutathione peroxidase activity in HepG2 cultured cells. Data are presented as mean ± standard error of the mean (SEM). Sample size (n) = 4. *P < 0.05 indicates significant change as compared to the control and Φ as compared to the 50 μM H2O2.

Effects of treatments on GR activity

Treatment of HepG2 cultured cells with different treatments, resulted in a 40.5% decrease in GR activity at 50μM of H2O2. As shown in Figure 3, E2 had a mild increasing effect on GR activity while E2+P2 induced a significant increase. However, P2 treatment could not ameliorate the H2O2 reduction effect on GR activity (Figure 3).
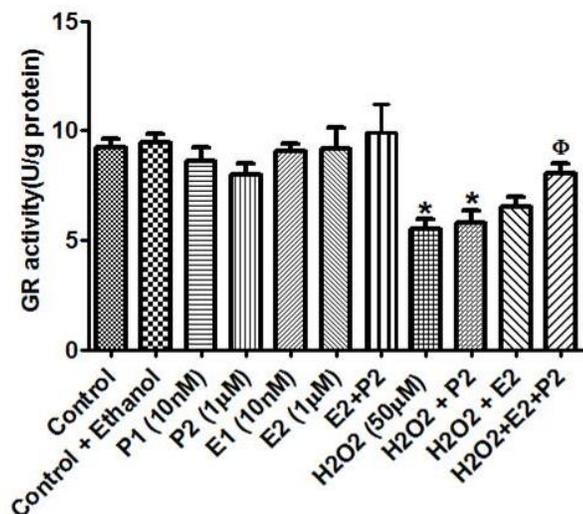
Figure 3. The effect of treatments on the activity of glutathione reductase activity in HepG2 cultured cells. Data are presented as mean ± standard error of the mean (SEM). Sample size (n) = 4.*P < 0.05 indicates a significant change as compared to the control and Φ as compared to the 50 µM H2O2.

Effects of treatments on GSH level

As shown in Figure 4, the GSH level in HepG2 cell lysate after 24h treatment 50µM of H2O2 was significantly reduced by 25.5% compared to the control group (P< 0.05) and interestingly, only P2+E2 could compensate this effect (Figure 4).
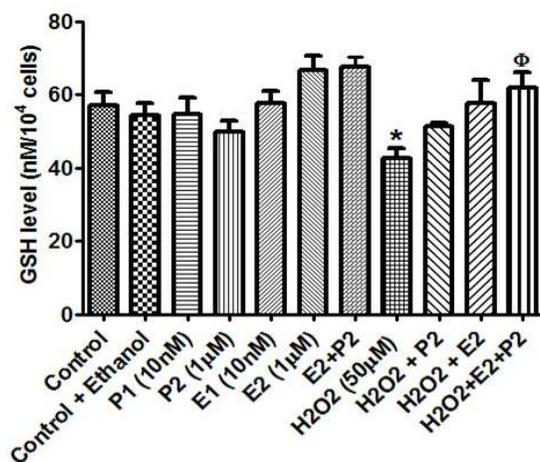
Figure 4. The effect of treatments on glutathione level in HepG2 cultured cells. Data are presented as mean ± standard error of the mean (SEM). Sample size (n) = 4.*P < 0.05 indicates significant change as compared to the control (no treatment) and Φ as compared to 50 µM H2O2.

Effects of treatments on ALT and AST

The cell damage associated with estrogen and progesterone was assessed by the measurement of ALT (Figure 5A), AST (Figure 5B). According to our results, ALT and AST levels were significantly increased in the presence of H_2O_2 (up to 77.1% and 55.7%, respectively) whereas such an increase was changed to 26.7% and 9.8% after P2 treatment and to 38.5% and 15.55% following E2 exposure. Interestingly, P2+E2 alone could ameliorate H_2O_2 toxicity effects in a significant way.

Discussion

Gluthation hemostasis is an important antioxidant defective system in the cells (17). In this study we evaluate the effect of sex hormones, estrogen and progesterone on oxidative stress in HepG2 cell line in the presence and absence of H_2O_2. Based on our findings, specific doses of estrogen and progesterone retained antioxidant effects and increased GR and GSH activity in the presence of H_2O_2 in HepG2 human cell line. We also observed that when estrogen and progesterone are used in combination, they can potentiate the anti-oxidative defense system in the cells, hence protect them from oxidative damage.

Guan et al. showed that Andrographolide, a bioactive molecule isolated from Andrographis plant, causes an increasing enzymatic activity of GR, GPx and total GSH in the lung cells of the mice exposed to cigarette smoking (18). Wang et al. observed APO A1 has an antioxidant effect on fatty liver in mice and increases the GPx activity (19). Ozacmak et al. offered evidence that estrogens and progesterone or the combination of both exert a remarkable neuroprotective effect reducing oxidative stress (20). Estrogen provides protection against vascular disease suggesting the clinical importance of the above mechanisms. Despite the reaction to angiotensin system and calcium channel blockers and lipid lowering agents, Estrogen has a protective effect on vessels. This might in part be due to increasing nitric oxide synthesis (21). Tang et al. suggested that 4-hydroxy estrone has an antioxidant role in plasma upon lipid peroxidation processes (22). Arteaga et al. showed that in contrast with the potent antioxidant effect of estradiol, progesterone and testosterone did not have any pro- or antioxidant effects on low-density lipoprotein in vitro. Likewise, progestins did not -
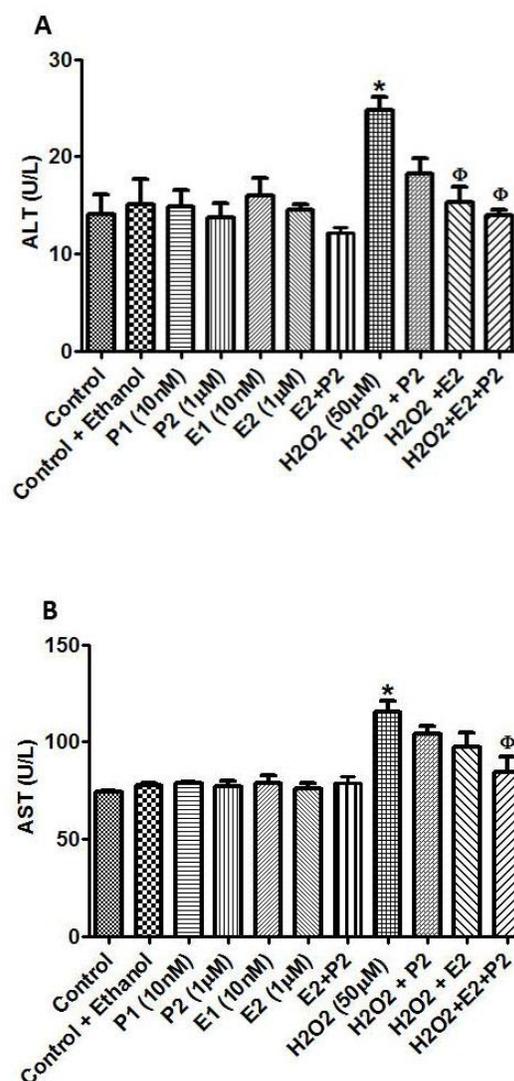
Figure 5. (A) ALT, (B) AST were measured based on the colorimetric method. Data are expressed as mean ± SEM. Sample size (n) = 3. *$P < 0.05$ for significant change as compared to control (no treatment) and Φ as compared to the 50 µM H_2O_2.

counteract the antioxidant effect of estradiol in vitro (23). Tate et al. suggested that H_2O_2 can act as extracellular signal in epithelial cells of the retina and increase antioxidant enzymes and other proteins enabling protection against oxidative damages (21). It is shown that H_2O_2 plays a role in aging process and apoptosis. Bladier et al. tried different doses of H_2O_2 on fibroblast cells and observed that at the dose of 50 µM H_2O_2, the aging process is promoted and cell proliferation is notably

decreased. The dose of 300 to 400 µM H2O2, was shown sufficient to trigger apoptosis (24).

Taken together, our results demonstrated that the specific doses of estrogen and progesterone retain antioxidant effects and increase GSH level in the presence of H2O2 in HepG2 human cell line. While both hormones revealed antioxidant effects, the effect of estrogen was more pronounced. Moreover, despite some other studies, our data showed that the combination of both hormones leads to a synergistic effect and potentiates the antioxidant capacity (9). Given the fact that oral contraceptive pills contain both mentioned hormones, they may not only hold detrimental effects on the liver cells, but also in some instances, such pills might serve possible protective effects against harmful substances.

Acknowledgment

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