Effects of Genistein on the Gene Expressions of Glutathione Peroxidases and Superoxide Dismutases in Ethanol-Treated Mouse Fetuses

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ABSTRACT

Genistein is a product of naturally occurring isoflavones at relatively high levels in soybeans. The harmful effects of ethanol are attributed to the induction of biological processes which lead to an increase in the generation of reactive oxygen species in fetuses. In this study, we investigated the effects of genistein (1×10^{-8} and 1×10^{-7} μg/ml) on gene expressions of the representative cellular antioxidative enzymes in ethanol ($1\ \mu$l/ml)-treated mouse fetuses during the critical period (embryonic days 8.5~10.5) of organogenesis using a semi-quantitative RT-PCR analysis. The mRNA levels of *cytosolic glutathione peroxidase* (*GPx*), *phospholipid hydroperoxide GPx*, *cytosolic Cu,Zn-superoxide dismutase* (*SOD*), and *mitochondrial SOD* were significantly decreased in ethanol-treated fetuses. However, the mRNA levels of ethanol plus genistein-treated fetuses were significantly higher than those of ethanol alone fetuses. These results indicate that genistein can up-regulate the expressions of GPx and SOD mRNAs reduced by the ethanol treatment in fetuses.

(Key words : ethanol, genistein, GPx, SOD, mouse fetuses)

INTRODUCTION

Fetal alcohol syndrome of congenital multiple malformation is a major cause for ethanol consumption by maternal (Abel and Sokol, 1991; Abel and Hannigan, 1995; Livy *et al.*, 2003; Martinez-Frias *et al.*, 2004). The mechanism by which ethanol induces perturbation of embryonic development is not clear. However, the evidence of the enhanced reactive oxygen species (ROS) as well as the decreased level of antioxidant enzymes in ethanol- exposed offspring has been recognized as one of the predominant causes of ethanol toxicity (Kotch *et al.*, 1995; Green *et al.*, 2006).

Mammals have endogenous antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) as a protector from ROS. SODs are the first line of antioxidant enzyme defense systems against ROS and particularly superoxide anion radicals and GPxs catabolize or neutralize H_2O_2 by using glutathione. At present, three valid isoforms of SOD have been identified in mammals, and their genomic structure, cDNA, and proteins have been reported. Two isoforms of SOD have Cu and Zn in their catalytic center and are placed to either intracellular cytoplasmic compartments (SOD1) or to extracellular elements (SOD3). SOD1 has been detected in the cytoplasm, nuclear compartments, and lysosomes in mammalian cells. SOD3 was first discovered in extracellular fluids including human plasma, lymph, and synovial fluid. SOD2 is a manganese-containing enzyme exclusively to the mitochondrial spaces. (Zelko *et al.*, 2002; Miao and St Clair, 2009). GPx isoenzymes share catalytic triad in their active center, consisting of selenocysteine, glutamine and tryptophan residues. The GPx isoenzymes such as the ubiquitously expressed cytosolic GPx (GPx1) and phospholipid hydroperoxide GPx (PHGPx), and the epithelium-specific gastrointestinal GPx (GPx2) are located intracellular except for the secreted plasma GPx (Papp *et al.*, 2007; Steinbrenner and Sies, 2009).

Genistein, a soybean isoflavone, is a polyphenolic compound and prevents hypertension, cancer, cardiovascular and liver diseases, inflammation, and apoptosis (Mahn *et al.*, 2005; Mo-

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hamed *et al.*, 2009; Pavese *et al.*, 2010; Bonacasa *et al.*, 2011).

In this study, the gene expression patterns of the representative antioxidative enzymes *GPxs* and *SODs* were investigated in the developing mouse embryos exposed to ethanol and/or genistein during the critical period (embryonic days 8.5～10.5) of organogenesis.

MATERIALS AND METHODS

1. Chemicals and Animals

Genistein (Sigma, St. Louis, MO, USA) was diluted with dimethylsulfoxide (Amresco, St. Louis, USA) to a concentration of less than 0.001%. Ethanol was purchased from Calbiochem (Darmstadt, Germany) and was diluted with phosphate-buffered saline. Male and female ICR mice (8～10 weeks old) were purchased from a commercial breeder, Biogenomics Co. (Seoul, South Korea). One male and three female mice were housed in a cage for mating. The environmental conditions were controlled throughout, with an ambient temperature of 21 ± 2℃, relative humidity of 55 ± 10%, air ventilation rate of 10 cycles per hour, and a 12:12 h light:dark cycle. The animals were fed a standard mouse chow (Samyang Ltd., Incheon, South Korea) and tap water *ad libitum* throughout the experimental period. Pregnancy was confirmed in the morning (08:00 am) by the presence of vaginal plugs or spermatozoa detected in a vaginal smear after mating during the previous evening (20:00 pm); this was considered to be ED 0.5. The pregnant mice were sacrificed by cervical dislocation and embryos were obtained at ED 8.5. All experiments were approved and carried out according to the "Guide for Care and Use of Animals" (Chungbuk National University Animal Care Committee, according to NIH #86-23).

2. Rat Serum Preparation

The serum of Sprague-Dawley male rats (10～12 weeks old) was prepared for embryo culturing as follows: after collection, the blood samples were immediately centrifuged at 3,000 rpm for 10 min at 4℃ to clear the cells from the plasma fractions. Next, the supernatant was transferred to new tubes and centrifuged again at 3,000 rpm for 10 min at 4℃ to remove any remaining blood cells. The cleared serum was decanted and pooled, heat-inactivated for 30 min in a 56℃ water bath, and then either used immediately or stored at −70℃. The serum was incubated at 37℃ and filtered through a 0.2 μm filter prior to use in culture.

3. Whole Embryo Culture

The whole embryo culture technique used in our study was based on a previously described model (New, 1978). Pregnant ICR mice were sacrificed by cervical dislocation at ED 8.5 between 09:00 and 10:00 am, and only embryos with somites number of 4～8 were used for this experiment. After removal of the decidua and Reichert's membranes, embryos with intact visceral yolk sacs and ectoplacental cones were placed randomly into sealed culture bottles (three embryos/bottle) containing 3 ml of culture medium, and either ethanol alone (1 μl/ml), or ethanol plus genistein (1×10^{-8} and 1×10^{-7} μg/ml). The cultures were incubated at 37 ± 0.5℃ and rotated at 25 rpm. The culture bottles were initially gassed with a mixture of 5% O_2, 5% CO_2, and 90% N_2 over a 17 h period at a flow rate of 150 ml/min. Subsequent gassing was performed at the same rate over periods of 7 h (20% O_2, 5% CO_2, and 75% N_2) and 24 h (40% O_2, 5% CO_2, and 55% N_2). Embryos were cultured using the whole embryo culture system (Ikemoto Rika Kogyo, Japan).

4. RT-PCR Analysis

Total RNA of each sample was extracted using TRIzol (Takara, Tokyo, Japan) according to the company's explanation. RNA sample was measured with a spectrophotometer under 260 nm and normalized before reverse transcription. Two μg of total RNA was reverse transcribed to first strand cDNA. To synthesis cDNA oldigo dT and M-MLV reverse transcriptase (Promega, MO, USA) were used. Taq polymerase (Takara) was used for PCR. The primer sets were used to amplify *cytosolic GPx* (*GPx1*), *phospholipid hydroperoxide GPx* (*PHGPx*), *cytoplasmic Cu,Zn SOD* (*SOD1*), and *Mn SOD* (*SOD2*) and *glyceraldehyde phosphate dehydrogenase* (*GAPDH*) as an internal control (Table 1). The PCR products were separated on a 2%-agarose gel in Tris-borate-EDTA buffer. The spot densities of DNA bands were measured with Alpha Easy 5.0 program (Alpha Innotech Co, U.S.A).

5. Statistical Analysis

Group differences were assessed via one-way ANOVA followed by Tukey's multiple comparison tests. All analyses were conducted using the Statistical Package for Social Sciences for Windows software, version 10.0 (SPSS Inc., Illinois, USA).

Table 1. Primer list

Gene name	Primer	Annealing temperature	Gene accession No.
GPx1	Forward : 5'-tacattgttttgagaagtgcg-3' Reverse : 5'-gacagcagggtttctatgtc-3'	57	NM_008160
PHGPx	Forward : 5'-atgcacgaattctcagccaag-3' Reverse : 5'-ggcaggtccttctctat-3'	57	NM_008162
SOD1	Forward : 5'-caatacacaaggctgtacca-3' Reverse : 5'-tgctctcctgagagtgagat-3'	57	NM_011434
SOD2	Forward : 5'-acaccattttctggacaaac-3' Reverse : 5'-actgaaggtagtaagcgtgc-3'	57	NM_013671
GAPDH	Forward : 5'-aacggatttggycgtattgg-3' Reverse : 5'-agccttctccatggtggtgaagac-3'	57	NM_008084

Statistical significance was assessed at $p<0.05$. All data are expressed as the mean ± SD.

RESULTS

1. Gene Expression Patterns of Antioxidative Enzymes in the Embryos Treated with Ethanol under the Presence or Absence of Genistein

1) Expression Pattern of GPx1 mRNA (Fig. 1)

The levels of *GPx1* mRNA in ethanol-treated embryos (78%) were significantly lower than those of normal control embryos (100%; $p<0.05$). However, co-treatments with genistein at two different concentrations resulted in significant increases in *GPx1* mRNA levels (115% and 109% for 1×10^{-8} and 1×10^{-7} μg/ml, respectively) as compared to ethanol alone embryos ($p<0.05$).

2) Expression Pattern of *PHGPx* mRNA (Fig. 2)

The levels of *PHGPx* mRNA in ethanol-treated embryos (65%) were significantly lower than those of control embryos ($p<0.05$). However, co-treatments with genistein resulted in a significant increase in the expression levels (129% and 125% for 1×10^{-8} and 1×10^{-7} μg/ml, respectively) as compared to ethanol alone embryos ($p<0.05$).

3) Expression Pattern of *SOD1* mRNA (Fig. 3)

The levels of *SOD1* mRNA in ethanol-treated embryos (67%) were significantly lower than those of control embryos ($p<0.05$). However, co-treatments with genistein resulted in a significant increase in the expression levels (137% and 119% for 1×10^{-8} and 1×10^{-7} μg/ml, respectively) as compared to ethanol alone embryos ($p<0.05$).

4) Expression Pattern of *SOD2* mRNA (Fig. 4)

The levels of *SOD2* mRNA in ethanol-treated embryos (82%) were significantly lower than those of control embryos ($p<0.05$). However, the level of *SOD2* mRNA (105%) in the

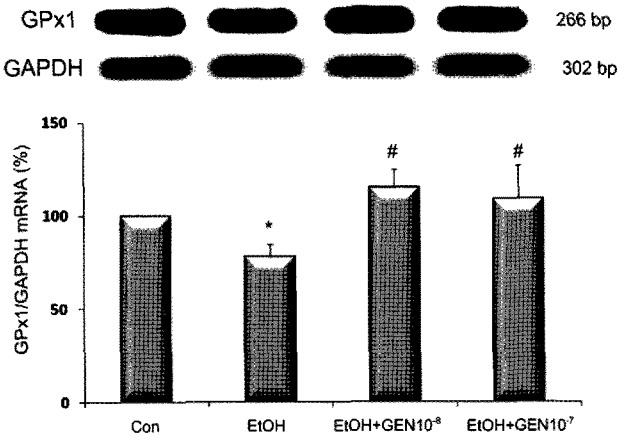

Fig. 1. Semi-quantitative RT-PCR analysis of *GPx1* mRNA levels in mouse embryos exposed *in vitro* at ED 8.5 to ethanol with the presence or absence of genistein for 2 days. The level of *GPx1* mRNA was significantly decreased by ethanol treatment (EtOH; 1 μ l/ml) as compared to control embryos (Con; *), but was restored by co-treatment with genisteins (GEN; 1×10^{-8} and 1×10^{-7} μ g/ml) as compared to ethanol group (#). Data represent five independent assays (mean ± SD) performed in triplicate at $p<0.05$.

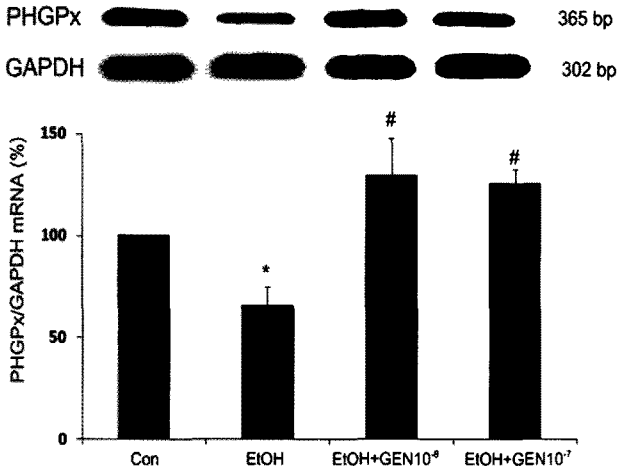

Fig. 2. Semi-quantitative RT-PCR analysis of *PHGPx* mRNA levels in mouse embryos exposed *in vitro* at ED 8.5 to ethanol with the presence or absence of genistein for 2 days. The level of *PHGPx* mRNA was significantly decreased by ethanol treatment (EtOH; 1 μ l/ml) as compared to control embryos (Con; *), but was restored by co-treatment with genisteins (GEN; 1×10^{-8} and 1×10^{-7} μ g/ml) as compared to ethanol group (#). Data represent five independent assays (mean ± SD) performed in triplicate at $p<0.05$.

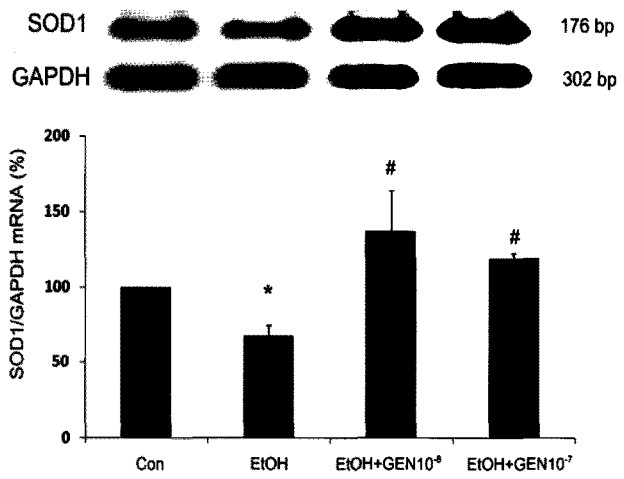

Fig. 3. Semi-quantitative RT-PCR analysis of *SOD1* mRNA levels in mouse embryos exposed *in vitro* at ED 8.5 to ethanol with the presence or absence of genistein for 2 days. The level of *SOD1* mRNA was significantly decreased by ethanol treatment (EtOH; 1 μ l/ml) as compared to control embryos (Con; *), but was restored by co- treatment with genisteins (GEN; 1×10^{-8} and 1×10^{-7} μ g/ml) as compared to ethanol group (#). Data represent five independent assays (mean ± SD) performed in triplicate at $p<0.05$.

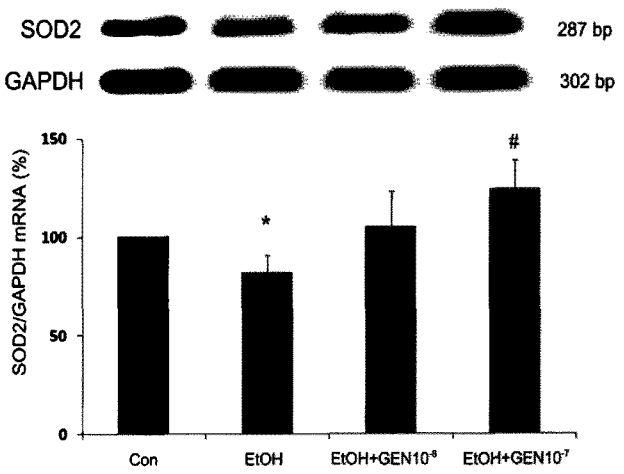

Fig. 4. Semi-quantitative RT-PCR analysis of *SOD2* mRNA levels in mouse embryos exposed *in vitro* at ED 8.5 to ethanol with the presence or absence of genistein for 2 days. The level of *SOD2* mRNA was significantly decreased by ethanol treatment (EtOH; 1 μ l/ml) as compared to control embryos (Con; *), but was restored by co-treatment with genisteins (GEN; 1×10^{-8} and 1×10^{-7} μ g/ml) as compared to ethanol group (#). Data represent five independent assays (mean ± SD) performed in triplicate at $p<0.05$.

embryos treated with ethanol plus 1×10^{-8} μ g/ml of genistein was increased similarly to that of the control embryos and further SOD2 mRNA level in the embryos co-treated with 1×10^{-7} μ g/ml of genistein resulted in a significant increase (124%) as compared to ethanol alone embryos ($p<0.05$).

DISCUSSION

Prenatal exposure to ethanol can lead to dysmorphogenesis and the generation of ROS (Smith, 1997; Ornoy, 2007). During particular periods in embryo development, the embryo is more susceptible to teratogens-induced oxidative stress, which can modify endogenous antioxidant status. Therefore, antioxidnats such as ascorbic acid, capsaicin, and black ginseng can obviate these effects (Dennery, 2007; Kim *et al.*, 2008; Lee *et al.*, 2009).

Genistein is a major element of soybean isoflavone. The cardioprotective and anti-osteoporosis activities of genistein have demonstrated in conjunction with its antioxidant effect (Pesce *et al.*, 2000; Guo *et al.*, 2002; Migliaccio and Anderson, 2003; Zielonka *et al.*, 2003; Bonacasa *et al.*, 2011).

The antioxidant enzymes (GPxs and SODs) are a vital element to embryonic development. *cGPx* and *SOD1* mRNAs were

specifically expressed in various cells and tissues in developing embryo (Baek *et al.*, 2005; Yon *et al.*, 2007). Because deletion of the *PHGPx* gene leads to early embryonic lethality, it is essential for a normal mouse development. Also, *PHGPx* mRNA was strongly observed in tissues of proliferation and differentiation during embryogenesis (Imai *et al.*, 2003; Schneider *et al.*, 2006). The mutant mice of SOD2 that is required for normal biological function of tissues exhibit slow growth, fatty liver, dilated cardiomyopathy, and premature death (Li *et al.*, 1995; Melov *et al.*, 1999). In current study, the mRNA levels of *GPx1, PHGPx, SOD1,* and *SOD2* in cultured embryos were significantly decreased by ethanol treatment, but were restored to those of control (vehicle-treated) embryos or significantly increased by co-treatment with genistein as compared to ethanol alone embryos. *GPx1* mRNA level revealed significant elevation in both LNCaP and PC-3 cells for the preventive role of genistein (Suzuki *et al.*, 2002). Borras *et al.* (2006) have suggested that 0.5 μM genistein prevented MCF-7 cells via ERK1/2 and nuclear factor κB and increased SOD2 mRNA levels.

Taken together, these results indicate that genistein up-regulates the expressions of GPx and SOD mRNAs reduced by the ethanol treatment in fetuses, suggesting that genistein may have a protective effect against ethanol-induced teratogenicity via its antioxidative activity.

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