Genistein Prevents Ethanol-Induced Teratogenesis in Mouse Embryos

Jung-Min Yon[1], Chunmei Lin[1], A Young Jung[1], Jong Geol Lee[1], Ki Youn Jung[1], In-Jeoung Baek[2], Beom Jun Lee[1], Young Won Yun[1] and Sang-Yoon Nam[1,*]

[1]*Department of Veterinary Medicine, College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea*

[2]*Laboratory of Mammalian Molecular Genetics, Department of Biochemistry, College of Science, Yonsei University, Seoul 120-749, Korea*

ABSTRACT

Drinking of excessive ethanol during pregnancy induces a fetal alcohol syndrome. Genistein is one of naturally occurring isoflavones at relatively high levels in soybeans. In this study, we investigated the effects of genistein (1×10^{-8} and 1×10^{-7} μg/ml) on the ethanol (1 μl/ml)-induced teratogenesis of developing mouse embryos during the critical period (embryonic days 8.5~10.5) of organogenesis using a whole embryo culture system and then morphological scoring analysis. Ethanol-treated embryos exhibited a variety of developmental abnormalities. However, the total morphological scores for ethanol plus genistein groups were significantly higher than those of ethanol alone group ($p<0.05$). In particular, there were significant increases in the ethanol plus 1×10^{-8} μg/ml of genistein group on the scores for heart, optic system, branchial bar, mandibular process, and caudal neural tube and further in the ethanol plus 1×10^{-7} μg/ml of genistein group on the scores for heart, hind-, mid-, and forebrains, optic system, branchial bars, maxillary and mandibular processes, caudal neural tube, forelimb, hindlimb, and somites as compared with those of ethanol alone group ($p<0.05$). These results indicate that genistein has a preventive effect against ethanol-induced teratogenesis.

(Key words : ethanol, genistein, teratogenesis, whole embryo culture)

INTRODUCTION

Experimental evidence has demonstrated that 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 (ROS) (Cederbaum *et al.*, 2009; Brocardo *et al.*, 2011). ROS are produced following single electron reductions of molecular oxygen. While physiological concentrations of ROS in aerobic organisms are beneficial and include cell signaling pathways, an unbalanced and increased concentration of ROS may cause the occurrence of various diseases, such as cancer, hypertension, diabetes, atherosclerosis, inflammation, and premature aging (Fantel, 1996; Valko *et al.*, 2007).

Maternal ethanol consumption during conception can cause developmental anomalies, such as prenatal and postnatal growth retardation, central nervous system dysfunction, behavioral abnormalities, cardiac defects, and facial dysmorphology in newborns, which is known as fetal alcohol syndrome in human (Abel and Sokol, 1991; Abel and Hannigan, 1995; Livy *et al.*, 2003; Martinez-Frias *et al.*, 2004).

Isoflavonoids are plant polyphenolic antioxidants that are extracted in legumes, especailly in soybean and various soybean-based food products (Liggins *et al.*, 2000; Zhu *et al.*, 2005). Genistein is a product naturally occurring isoflavones at relatively high (3 mg/g) levels in soybeans. Several studies have reported that genistein exerts a protective effect against lipid peroxidation of low density lipoproteins and inhibits the expression of tyrosine kinases and proliferation of human cancer cell lines (Xu and Loo, 2001; Lai and Yen, 2002; Sarkar and Li, 2003; Pavese *et al.*, 2010).

In this study, the effect of ethanol and/or genistein administration in the developing mouse embryos during the critical period (embryonic days 8.5~10.5) of organogenesis was investigated using a whole embryo culture system.

† This work was supported by the research grant of the Chungbuk National University in 2009.

* Correspondence: E-mail: synam@cbu.ac.kr

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. Morphologic Scoring

After the 48-hr culture period, cultures were ended and embryos were placed into a petridish with pre-warmed sterile Tyrode's solution (pH 7.2) and assessed based on the morphologic scoring system of Van Maele-Fabry *et al.* (1990). Only viable embryos that have presence of yolk sac circulation and heart circulation were selected for assessement. Diameters of the yolk sacs were estimated and vascularization and circulation were examined. The evaluated morphological items included yolk sac diameter (mm), yolk sac circulation, allantois, flexion, crown-rump length (mm), head length (mm), heart, forebrain, midbrain, hindbrain, otic system, optic system, branchial bars, maxillary process, mandibular process, olfactory system, caudal neural tube, fore limb, hind limb, and somites.

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). Score data were compared by Kruskal-Wallis non-parametric ANOVA and Dunn's multiple comparison *post hoc* test. Statistical significance was assessed at $p<0.05$. All data are expressed as the mean ± SD.

RESULTS

As shown in Fig. 1, Fig. 2 and Table 1, abnormalities and a significant decrease of the total morphological scores were observed in the embryos exposed to ethanol during a critical organogenic stage compared to control embryos ($p<0.05$).

Ethanol-treated embryos exhibited severe developmental abnormalities in most of the organs examined. In particular, the ethanol- treated embryos had significantly lower morphological scores for their heart, hindbrain, midbrain, forebrain, otic and optic systems, olfactory systems, branchial bars, maxillary process, caudal neural tube, forelimb, hindlimb, and somites than those of control embryos ($p<0.05$). However, the total morphological scores for ethanol plus genistein groups were signifi-

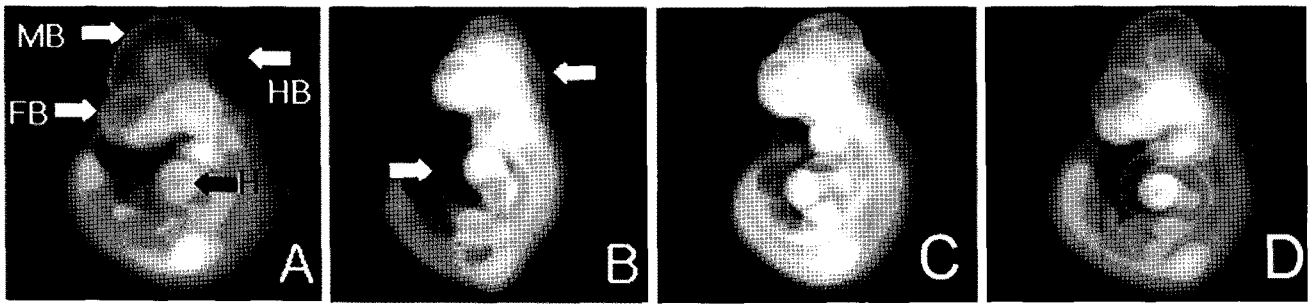

Fig. 1. Morphological features of cultured mouse embryos. A: Control embryo treated with vehicle only. B: Embryo exposed to ethanol (1 μl/ml) exhibits an abnormal head shape, open hindbrain, and heart anomalies (arrows). C & D: Embryos that were exposed to ethanol plus genisteins (1×10^{-8} and 1×10^{-7} μg/ml). FB; forebrain, MB; midbrain, HB; hindbrain, H; heart.

Table 1. Morphological scores of the mouse embryos exposed to ethanol (EtOH) and/or 1×10^{-8} μg/ml of genistein ($Gen10^{-8}$) and 1×10^{-7} μg/ml of genistein ($Gen10^{-7}$) *in vitro* at embryonic day 8.5 for 2 days

Group	Con	EtOH	EtOH+$Gen10^{-8}$	EtOH+$Gen10^{-7}$
No. of embryo	n = 20	n = 20	n = 25	n = 17
Yolk sac circulation	4.23 ± 0.44	3.73 ± 0.50	3.82 ± 0.58	3.90 ± 0.40
Allantois	2.65 ± 0.43	2.28 ± 0.38	2.52 ± 0.36	2.51 ± 0.40
Flexion	4.63 ± 0.36	4.10 ± 0.38	4.52 ± 0.41	4.41 ± 0.51
Heart	3.98 ± 0.11	3.23 ± 0.47*	3.78 ± 0.29#	3.74 ± 0.31#
Hindbrain	4.60 ± 0.50	3.35 ± 0.65*	3.70 ± 0.51	4.12 ± 0.52#
Midbrain	4.73 ± 0.44	3.40 ± 0.70*	3.78 ± 0.47	4.09 ± 0.51#
Forebrain	4.78 ± 0.53	3.78 ± 0.68*	4.04 ± 0.49	4.20 ± 0.60#
Otic system	4.60 ± 0.45	3.45 ± 0.60*	3.80 ± 0.35	3.91 ± 0.40
Optic system	4.75 ± 0.34	3.35 ± 0.65*	3.96 ± 0.37#	3.82 ± 0.61#
Branchial bars	2.60 ± 0.60	1.33 ± 0.54*	2.02 ± 0.17#	1.96 ± 0.40#
Maxillary process	2.13 ± 0.58	1.63 ± 0.43*	1.88 ± 0.32	2.19 ± 2.92#
Mandibular process	1.90 ± 0.50	1.50 ± 0.16	1.90 ± 0.35#	1.91 ± 0.44#
Olfactory system	2.18 ± 0.54	1.63 ± 0.36*	1.90 ± 0.28	1.88 ± 0.33
Caudal neural tube	4.30 ± 0.66	3.93 ± 0.47*	4.24 ± 0.60#	4.35 ± 0.68#
Forelimb	2.90 ± 0.21	1.95 ± 0.60*	2.08 ± 0.46	2.32 ± 0.50#
Hindlimb	1.60 ± 0.50	0.93 ± 0.44*	1.26 ± 0.35	1.50 ± 0.43#
Somites	4.80 ± 0.41	4.35 ± 0.49*	4.64 ± 0.37	4.65 ± 0.49#
Total score	61.36 ± 7.60	47.92 ± 8.50*	53.84 ± 6.73#	59.06 ± 9.94#

Each value represents mean ± SD. * Versus control group at $p<0.05$. # Versus ethanol group at $p<0.05$. Control; Con.

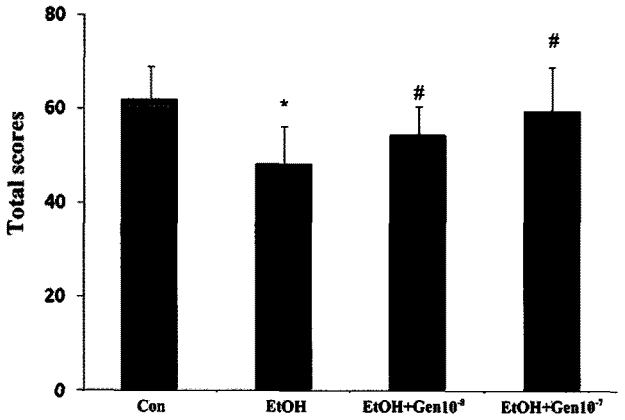

Fig. 2. Total morphological scores in ethanol (1 μl/ml) and/ or genisteins (GEN: 1×10^{-8} and 1×10^{-7} μg/ml)-treated embryos. Data are expressed as the mean ± SD. Significant differences between treatment groups versus control (Con; #) or ethanol (EtOH; *) group were evaluated by one-way ANOVA at $p<0.05$.

cantly higher than those of ethanol alone group ($p<0.05$). There were significant increases in the ethanol plus 1×10^{-8} μg/ml of genistein group in the scores for heart, optic system, branchial bar, mandibular process, and caudal neural tube compared with those of ethanol alone group ($p<0.05$). Furthermore, the morphological scores of ethanol plus 1×10^{-7} μg/ml of genistein group were significantly alleviated in heart, hind-, mid-, and forebrains, optic system, branchial bars, maxillary and mandibular processes, caudal neural tube, forelimb, hindlimb, and somites as compared with those of ethanol alone group ($p<0.05$).

DISCUSSION

Prenatal exposure to ethanol, the most prevalent teratogen, induces mental retardation, neurobehavioral deficits, and facial defects via embryonic oxidative stress linked to the metabolism of ethanol (Smith, 1997; Ornoy, 2007). Genistein is a major element of soybean isoflavone and has a variety of functions against oxidative stress, acetaldehyde, cancer and inflammatory cytokines, and cardiovascular diseases (Mahn *et al.*, 2005; Pavese *et al.*, 2010; Bonacasa *et al.*, 2011; Suzuki and Hara, 2011).

In this study, we used a whole embryo culture system to explore the effect of genistein on ethanol-induced toxicity in the mouse embryos during critical ontogenetic periods. The embryo culture system allows the *in vitro* development and growth of embryos presented from the early stage of somite to late period of organogenesis when most primary organ have taken a form, and has been used in many toxicological studies(New, 1978; Klug *et al.*, 1985). It has been reported that the embryonic crown-rump length, head length and somite number were all decreased and the total morphologic score that represented the general development and growth of the major organs of embryo such as midbrain, forebrain, and heart decreased in ethanol exposure groups. Also, it showed that unclosed neural tube and abnormal heart were the most frequent occurrence detected in the embryos treated with ethanol (Xu *et al.*, 2005). In the present study, significant embryonic morphological improvements were detected in the groups treated with genistein and ethanol concurrently compared to the ethanol alone group. Especially, there were a significant increase in ethanol plus 1×10^{-8} μg/ml of genistein group on the scores for heart, optic system, branchial bar, mandibular process, and caudal neural tube and in ethanol plus 1×10^{-7} μg/ ml of genistein group on the scores for heart, hind-, mid-, and forebrains, optic system, branchial bars, maxillary and mandibular processes, caudal neural tube, forelimb, hindlimb, and somites as compared with those of ethanol alone group. These findings suggest that genistein can effectively protect against ethanol-induced morphological abnormalities during embryonic organogenesis.

REFERENCES

Abel EL and Sokol R. 1991. A revised conservative estimate the incidence of FAS and its economic impact. Alcohol Clin. Exp. Res. 15:514-524.

Abel EL and Hannigan JH. 1995. Maternal risk factors in fetal alcohol syndrome: provocative and permissive influences. Neurotoxicol. Teratol. 17:445-462.

Bonacasa B, Siow RC and Mann GE. 2011. Impact of dietary soy isoflavones in pregnancy on fetal programming of endothelial function in offspring. Microcirculation 18:270-285.

Brocardo PS, Gil-Mohapel J and Christie BR. 2011. The role of oxidative stress in fetal alcohol spectrum disorders. Brain Res. Rev. 67:209-225.

Cederbaum AI, Lu Y and Wu D. 2009. Role of oxidative stress in alcohol-induced liver injury. Arch. Toxicol. 83:519-548.

Fantel AG. 1996. Reactive oxygen species in developmental toxicity: review and hypothesis. Teratology 53:196-217.

Klug S, Lewandowski C and Neubert D. 1985. Modification and standardization of the culture of early postimplantation

embryos for toxicological studies. Arch. Toxicol. 58:84-88.

Lai HH and Yen GC. 2002. Inhibitory effect of isoflavones on peroxynitrite-mediated low-density lipoprotein oxidation. Biosci. Biotechnol. Biochem. 66:22-28.

Liggins J, Bluck LJ, Runswick S, Atkinson C, Coward WA and Bingham SA. 2000. Daidzein and genistein content of fruits and nuts. J. Nutr. Biochem. 11:326-331.

Livy DJ, Miller EK, Maier SE and West JR. 2003. Fetal alcohol exposure and temporal vulnerability: Effects of binge-like alcohol exposure on the developing rat hippocampus. Neurotoxicol. Teratol. 25:447-458.

Mahn K, Borrás C, Knock GA, Taylor P, Khan IY, Sugden D, Poston L, Ward JP, Sharpe RM, Viña J, Aaronson PI and Mann GE. 2005. Dietary soy isoflavone induced increases in antioxidant and eNOS gene expression lead to improved endothelial function and reduced blood pressure *in vivo*. FASEB J. 19:1755-1757.

Martinez-Frias ML, Bermejo E, Rodriguez-Pinilla E and Frias J. 2004. Risk for congenital anomalies associated with different sporadic and daily doses of alcohol consumption during pregnancy: a case-control study. Birth Defects Res. A Clin. Mol. Teratol. 70:194-200.

New DA. 1978. Whole-embryo culture and the study of mammalian embryos during organogenesis. Biol. Rev. Camb. Philos. Soc. 53:81-122.

Ornoy A. 2007. Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. Reprod. Toxicol. 24:31-41.

Pavese JM, Farmer RL and Bergan RC. 2010. Inhibition of cancer cell invasion and metastasis by genistein. Cancer Metastasis Rev. 29:465-482.

Sarkar FH and Li Y. 2003. Soy isoflavones and cancer prevention. Cancer Invest. 21:744-757.

Smith SM. 1997. Alcohol-induced cell death in the embryo. Alcohol Health Res. World 21:287-297.

Suzuki T and Hara H. 2011. Role of flavonoids in intestinal tight junction regulation. J. Nutr. Biochem. 22(5):401-408.

Xu J and Loo G. 2001. Different effects of genistein on molecular markers related to apoptosis in two phenotypically dissimilar breast cancer cell lines. J. Cell Biochem. 82:78-88.

Xu Y, Xiao R and Li Y. 2005. Effect of ethanol on the development of visceral yolk sac. Hum. Reprod. 20:2509-2516.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M and Telser J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39:44-84.

Van Maele-Fabry G, Delhaise F and Picard JJ. 1990. Morphogenesis and quantification of the development of post-implantation mouse embryos. Toxicol. *In Vitro* 4:149-156.

Zhu D, Hettiarachchy NS, Horax R and Chen P. 2005. Isoflavone contents in germinated soybean seeds. Plant Foods Hum. Nutr. 60:147-151.

(접수: 2011. 4. 18 / 심사: 2011. 4. 19 / 채택: 2011. 4. 26)