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Resveratrol prevents nicotine-induced teratogenesis in cultured mouse embryos

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ABSTRACT

Nicotine, a major toxic component in tobacco smoke, leads to severe embryonic damage during organogenesis in embryos. We investigated whether resveratrol would positively influence nicotine-induced teratogenesis in mouse embryos (embryonic day 8.5) cultured for 48 h using a whole embryo culture system. Embryos exposed to nicotine (1 mM) revealed significantly severe morphological anomalies, increased levels of caspase-3 mRNA and lipid peroxidation, and decreased levels of cytoplasmic superoxide dismutase (SOD), mitochondrial manganese SOD, cytosolic glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, hypoxia-inducible factor 1α, Bcl-x_L, and sirtuin1 (SIRT1) mRNAs and SOD activity compared to those in the normal control group. However, when resveratrol (1×10^{-8} μM or 1×10^{-7} μM) was added concurrently to the embryos exposed to nicotine, all the parameters in above improved conspicuously. These findings indicate that resveratrol has a noted protective effect against nicotine-induced teratogenesis in mouse embryos through its antioxidative and anti-apoptotic effects.

1. Introduction

The uterine environment is relatively hypoxic during the early postimplantation period, which is important for early organogenesis, as it is the most vulnerable stage of embryogenesis especially to environmental oxidative stress [1]. Smoking prevalence among women in reproductive age has increased in the world over the last decades and reached 28% in the USA in 2006 [2]. Maternal cigarette smoking can result in fetal growth restriction, increased rates of spontaneous abortion, premature placental abruption, perinatal lethality, decreased birth weight, and sudden infant death syndrome [3]. Nicotine, a major toxic component of cigarette smoke, crosses the placental barrier and acts directly and indirectly on fetal systems [4]. Reactive oxygen species (ROS) damage mitochondrial and nuclear DNA, proteins, and lipids, and these have been recognized as one of the most important toxic mechanisms of nicotine [5–7]. Administration of nicotine to rats leads to higher lipid peroxidation and a subsequent decrease in antioxidant enzymes [8]. Human and animal studies have demonstrated that cigarette smoke induces a high load of ROS that perturbs the oxidant/antioxidant balance and leads to embryonic and fetal oxidative stress [9].

Endogenous tissue antioxidants counteract the damaging effects of cigarette smoke [10]. Dietary antioxidants reduce hepatotoxicity and elevations in lipid peroxidation and pro-inflammatory interleukin-6 due to smoking [11]. Therefore, high antioxidant capacity has been proposed as a promising strategy to prevent cigarette smoke-induced diseases. Resveratrol is a member of the phytoalexin family found in a wide diversity of plants including grapes, raspberries, mulberries, pistachios, and peanuts [12]. Fresh grape skin contains about 50–100 mg of resveratrol per gram [13]. Resveratrol has a number of beneficial health effects such as anti-cancer, anti-viral, neuroprotective, anti-aging, anti-inflammatory, and lifespan-prolonging effects [14,15]. Resveratrol (gavage feeding 100 mg/kg body weight) not only prevented oxidative stress and apoptosis in whole embryos associated with diabetic embryopathy, but also improved glucose homeostasis and lipid metabolism of diabetic dams [16]. Resveratrol crosses the blood–brain barrier and protects brain in traumatic injured animals [17]. In particular, recent studies indicate that resveratrol activates the protein deacetylase enzyme silent information regulator 2/sirtuin 1 (SIRT1) and protects pulmonary endothelial cells against the adverse effects of cigarette smoking-induced oxidative stress and apoptosis [18].

Many animal studies have demonstrated that antioxidants effectively counteract the deleterious effects of nicotine and attenuate oxidative damage [19,20,8]. As non-toxic antioxidants might be an efficient and inexpensive way to reduce the rate of some serious fetal anomalies, some additional appropriate clinical pregnancy studies are needed. In the present study, we investigated whether resveratrol has a protective effect against nicotine-induced teratogenesis in cultured mouse embryos using a whole embryo culture system.

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2. Materials and methods

2.1. Experimental animals

Male and female ICR mice (8–10 weeks old) were purchased from a commercial breeder (Biogenomics Co., Seoul, Korea). One male and three female mice were housed in a cage for mating. The environmental conditions were controlled with an ambient temperature of 21 ± 2 °C, 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 standard mouse chow (Samyang Ltd., Incheon, Korea) and tap water *ad libitum* throughout the experimental period. Pregnancy was confirmed the following morning (08:00) by the presence of vaginal plugs or spermatozoa detected in a vaginal smear after mating the previous evening (20:00); this was considered embryonic day (E) 0.5. Under cervical dislocation euthanasia, pregnant mice were sacrificed and embryos were obtained at E8.5. 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).

2.2. Rat serum preparation

Serum of Sprague-Dawley male rats (10–12 weeks old) was prepared as embryo culture fluid as follows. After collection, blood samples were immediately centrifuged for 10 min at 3000 rpm and 4 °C to clear the plasma fraction of cells. Then, the supernatant was transferred to new tubes, which were recentrifuged for 10 min at 3000 rpm and 4 °C to separate the blood cells. The clear serum supernatant was decanted and pooled, and the pooled serum was heat-inactivated for 30 min at 56 °C in a water bath. It was then either used immediately or stored at −70 °C. Serum was incubated at 37 °C and filtered through a 0.2 μm filter prior to use in the whole embryo culture.

2.3. Whole embryo culture and nicotine and/or resveratrol treatments

The whole embryo culture system was based on a previously described model [21]. Animals were sacrificed *via* cervical dislocation at E8.5 between 09:00 and 10:00, and only embryos with somite numbers of 4–8 were utilized. After removing 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 different concentrations (1×10^{-8} μM or 1×10^{-7} μM) of resveratrol (Sigma, St. Louis, MO, USA) dissolved in dimethyl sulfoxide (Sigma) to a concentration of less than 0.001% and/or 1 mM of nicotine (163.8 μg/mL serum; Sigma), which were determined by our preliminary experiments based on the previous studies [22,23]. Embryos were randomized into four groups: (1) control group, (2) nicotine group, (3) nicotine plus resveratrol (1×10^{-8} μM) group, and (4) nicotine plus resveratrol (1×10^{-7} μM) group. The embryos were incubated at 37 ± 0.5 °C in sealed culture bottles (three embryos/bottle) 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 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). All embryos were cultured for 48 h using a whole embryo culture system (Ikemoto Rika Kogyo, Japan).

2.4. Morphological scoring

At the end of the 48 h culture period, the embryos were evaluated according to the morphological scoring system developed by Van Maele-Fabry et al. [24]. Only viable embryos with yolk sac circulation and a heartbeat were utilized for morphological scoring. Measurements of each viable embryo were obtained with 17 standard scoring items, plus the yolk sac diameter, crown-rump length, and head length. The morphological features that were assessed included embryonic flexion, heart, caudal neural tube, brain (forebrain, midbrain, and hindbrain), otic and optic systems, olfactory organs, branchial arch, maxilla, mandible, limb buds (forelimb and hindlimb buds), yolk sac circulation, allantois, and somites.

2.5. Lipid peroxidation measurement

Lipid peroxidation was determined by thiobarbituric-acid-reactive species (TBARS) levels as described by Ohkawa [25] with minor modifications. The malondialdehyde (MDA) level in the embryos was measured spectrophotometrically through the TBARS concentration. The TBARS results are expressed as nmol/mg protein. Embryos in each group were homogenized in chilled 10 mM phosphate buffer and were then mixed thoroughly with 8.1% sodium dodecyl sulfate, 20% acetic acid, and 0.75% 2-thiobarbituric-acid solution. The solution was heated for 30 min in a 95 °C oven. After cooling, the flocculent was removed by centrifugation at 3500 rpm for 15 min. The absorbance of the upper layer was measured at 532 nm with a spectrophotometer and compared to the value from the prepared 1,1,3,3,-tetramethoxypropane standard curve. The protein content of the embryos was determined according to the method of Lowry [26] using bovine serum albumin as the standard.

Table 1
Primer sequences used in the study.

Primer	Sequence (5′–3′)	Accession no.
β-Actin	Forward: TTT CCA GCC TTC CTT CTT GGG TAT G Reverse: CAC TGT GTT GGC ATA GAG GTC TTA C	NM_007393
SOD1	Forward: TGC GTG CTG AAG GGC GAC Reverse: GTC CTG ACA ACA CAA CCT GGT TC	NM_011434
SOD2	Forward: GGA GCA AGG TCG CTT ACA GA Reverse: GTG CTC CCA CAC GTC AAT C	NM_013671
GPx-1	Forward: TGT TTG AGA AGT GCG AAG TG Reverse: GTG TTG GCA AGG CAT TCC	NM_008160
GPx-4	Forward: TAA GAA CGG CTG CGT GGT Reverse: GTA GGG GCA CAC ACT TGT AGG	NM_008162
HIF-1α	Forward: CAC CAG ACA GAG CAG GAA Reverse: TCA GGA ACA GTA TTT CTT TGA TTC A	NM_010431
Bcl-x_L	Forward: TGACCACCTAGAGCCTTGGA Reverse: TGTTCCCGTAGAGATCCACAA	NM_009743
Caspase-3	Forward: AAA GCC GAA ACT CTT CA TCA T Reverse: GTC CCA CTG TCT GTC TCA	NM_009810
SIRT1	Forward: TCG TGG AGA CAT TTT TAT CAG G Reverse: GCT TCA TGA TGG CAA GTG G	NM_019812

2.6. Quantitative real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from cultured mouse embryos using a Trizol Reagent kit (Invitrogen, Carlsbad, CA, USA). RNA samples were purified with a column using an RNA Clean-up kit (Macherey-Nagel). Total RNA (2 μg) was used in a cDNA Synthesis kit (Invitrogen). Real-time PCR was carried out in a 20 μg reaction volume using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and mouse embryonic cDNA (1.6 μg) as the template. Reactions were performed using a 7500 Real-Time PCR System (Applied Biosystems), according to the manufacturer's instruction. Gene-specific primers were designed by TIB Mol-Bio Synthesis (Berlin, Germany). Primers to mouse cytoplasmic superoxide dismutase (SOD1), manganese SOD (SOD2), cytoplasmic glutathione peroxidase (GPx-1), phospholipid hydroperoxide GPx (GPx-4), hypoxia inducible factor 1α (HIF-1α), Bcl-x_L, caspase-3, and SIRT1 were used (Table 1). Beta-actin primers were used as an internal standard to normalize target transcript expression. Data were analyzed from nine independent runs using a comparative Ct method, as previously described by Livak and Schmittgen [27].

2.7. SOD activity assay

Total SOD activity was assayed with a SOD Assay kit-WST (Dojindo Laboratories, Kumamoto, Japan). Briefly, the mouse embryos were homogenized, and the protein concentrations of the supernatants were analyzed by the Bradford method [28]. The supernatants were incubated with an assay reagent containing xanthine, xanthine oxidase, and a water-soluble tetrazolium salt, WST-1. The superoxide free radicals generated from the xanthine substrate by xanthine oxidase reduced WST-1 to WST-1 diformazan, which absorbs maximally at 450 nm. SOD in the embryos inhibited the WST-1 reduction as it catalyzed the dismutation of superoxide ions to molecular oxygen and hydrogen peroxide. The reduction of WST-1 was measured spectrophotometrically at 450 nm. SOD activity was calculated as an inhibition rate in which 1 U was defined as a 50% decrease from the control value over a period of 30 min at 37 °C. The results were presented as specific activity, which was determined as the total activity per embryo divided by the total amount of protein per embryo.

2.8. Statistical evaluation

Group differences in each gene expression, lipid peroxidation, and SOD activity data were assessed *via* one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Morphological score data were compared using the Kruskal–Wallis non-parametric ANOVA and Dunn's multiple comparison *post hoc* test. A $p < 0.05$ was considered significant. All data are expressed as mean ± SEM. All analyses were conducted using the SPSS for Windows software, version 10.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Protective effect of resveratrol on nicotine-induced developmental defects in mouse embryos

Embryos exposed to nicotine alone (1 mM) exhibited severe growth retardation and developmental abnormalities (Fig. 1 and Table 2). In particular, nicotine-treated embryos showed significantly lower morphological scores for yolk sac diameter and