ARTICLE
Journal of Cellular Biochemistry 112:1311–1317 (2011)

Journal of Cellular Biochemistry

Effects of Phenytoin and *Echinacea purpurea* Extract on Proliferation and Apoptosis of Mouse Embryonic Palatal Mesenchymal Cells

Xiao Hu,[1] Zhenguo Chen,[2] Xiaoyan Mao,[1] and Shijie Tang[1*]

[1] *Cleft Lip and Palate Treatment Center, Second Affiliated Hospital, Shantou University Medical College, Shantou 515041, People's Republic of China*

[2] *Laboratory of Cell Senescence, Shantou University Medical College, Shantou 515041, People's Republic of China*

ABSTRACT

Cleft palate is one of the most common birth defects. Several environment factors are involved in the disorder, such as smoking, vitamin deficiency and teratogens. We investigated the teratogenic agent phenytoin and extract of the immunostimulant *Echinacea purpurea* in the etiology of cleft palate associated with the proliferation and apoptosis of mouse embryonic palatal mesenchymal (MEPM) cells. We measured the effects of phenytoin, *E. purpurea* extract, and the mixture of phenytoin and *E. purpurea* extract on the cell viability of MEPM cells by CCK-8 assay and on the proliferation and apoptosis of MEPM cells by BrdU labeling assay, flow cytometry, and TUNEL assay. Exposure to phenytoin for 24 h inhibited cell proliferation and increased cell apoptosis of MEPM cells, and *E. purpurea* extract had the reverse effect. Importantly, treatment with the mixture of phenytoin and *E. purpurea* extract increased the proliferation and decreased the apoptosis of MEPM cells as compared with treatment with phenytoin alone. The teratogenic effect of phenytoin on cleft palate is associated with the proliferation and apoptosis of MEPM cells, and *E. purpurea* extract may have a protective effect. J. Cell. Biochem. 112: 1311–1317, 2011.

KEY WORDS: CLEFT PALATE; ECHINACEA PURPUREA EXTRACT; MOUSE EMBRYONIC PALATAL MESENCHYMAL CELLS; PHENYTOIN

Cleft palate is one of the most common congenital disorders. International data from 57 registries for 1993–1998 suggest a prevalence of 1.3–25.3 per 10,000 births [Mossey and Castillia, 2003]. Palatal shelves being too small to contact one another is believed to be the most common reason for cleft palate. Mesenchymal cell number, determined by a balance between cell proliferation and apoptosis, is a major contributor to the shelf size. Insufficient mesenchyme delays or inhibits the growth of palatal processes in embryos [Wilkie and Morriss-Kay, 2001; Hanumegowda et al., 2002; Dhulipala et al., 2004; He et al., 2008; Takechi et al., 2008; Risley et al., 2009; Vanhoutteghem et al., 2009].

At least three major classes of environmental triggers have been studied in facial embryogenesis. One of these classes is teratogens. For example, maternal smoking has been recognized as an important covariate in clefting [Little et al., 2004]. Other teratogens that increase the risk of clefting through maternal ingestion include pharmaceuticals, such as the anticonvulsant phenytoin and benzodiazepines, or pesticides, such as dioxin. The effect of a second class of environmental trigger, infection, is less clear. However, some evidence demonstrates that immune modulation factors are essential for palate development [Suzuki et al., 2000; Kondo et al., 2002]. Finally, nutrients (e.g., vitamins or trace elements) and cholesterol metabolism are increasingly being seen as being influencing embryonic development [CDC, 2004; Edison and Muenke, 2004].

Phenytoin, an important first-line anti-epileptic drug, is widely prescribed throughout the world. It is also the best-known teratogenic drug used in human and laboratory study. Phenytoin was first reported in 1964 to cause cleft palate with or without cleft lip in fetuses [Janz and Fuchs, 1964]. Animal experiments showed

Abbreviations used: CK, cytokeratin; DMEM, Dulbecco's modifed Eagle's medium; E, Embryonic day; FBS, fetal bovine serum; GSK-3β, glycogen synthase kinase-3β; MEPM, mouse embryonic palatal mesenchymal; PBS, phosphate buffered saline; PI, propidium iodide.

Grant sponsor: Guangdong Science and Technology Department 2008B080701034.

*Correspondence to: Shijie Tang, PhD, Cleft Lip and Palate Treatment Center, Second Affiliated Hospital, Shantou University Medical College, North Dongxia Road, Shantou 515041, Guangdong, People's Republic of China.
E-mail: tang2302@163.com

Received 5 December 2010; Accepted 19 January 2011 • DOI 10.1002/jcb.23044 • © 2011 Wiley-Liss, Inc.
Published online 10 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

that it induced 10–20% of palate and lip malformations in mice [Karolyi et al., 1990]. Recently, treatment with phenytoin during pregnancy for women with epilepsy was found to result in serious adverse outcomes, including cleft palate in 10.7% of cases [Meador et al., 2006]. The possible mechanism of phenytoin-induced cleft palate is interference with palatal growth by inhibiting DNA, RNA, and/or protein synthesis [Sonawane and Goldman, 1981] or preventing medial-edge epithelial breakdown [Goldman et al., 1983].

Echinacea, or purple coneflower, has become a top-selling medicinal herb and food supplement in Europe and the United States [Ernst, 2002]. Three major species, *E. purpurea*, *E. angustifolia*, and *E. pallida*, have been studied for their possible pharmacological and immunological effects [Rininger et al., 2000; Nieri et al., 2003]. *E. purpurea* is the most commonly consumed species in the United States [Borchers et al., 2000] and is used widely in preventing or treating the common cold, coughs, bronchitis, influenza, and inflammation of the mouth and pharynx [Barrett, 2003]. *E. purpurea* was reported to increase neutrophil chemotoxicity and bactericidal activity against Staphylococcus infection [Roesler et al., 1991], upregulate production of TNFα, IL-10, IL-6, and IL-1β in macrophages [Burger et al., 1997] and simulate digestion on human peripheral blood mononuclear cells [Rininger et al., 2000]. Also, *E. purpurea* is an effective radioprotective agent with low cytotoxicity and few side effects [Mishima et al., 2004]. Stimulation of the maternal immune system can decrease or prevent drug-induced embryonic abnormalities [Holladay et al., 2000, 2002; Prater et al., 2004]. Mahabady et al. [2006] reported that *E. purpurea* extract can decrease phenytoin-induced cleft palate incidence in fetuses of mice, and mean weight and length of fetuses of mice that received phenytoin and *E. purpurea* extract were significantly greater than those receiving only phenytoin.

We hypothesized that the possible mechanism of phenytoin-induced cleft palate may be to modulate the number of palatal mesenchymal cells, and we determined whether *E. purpurea* extract could rescue cell number after treatment with phenytoin.

MATERIALS AND METHODS

MEPM CELLS

C57BL/6J mice were purchased from the Medical Animal Center of Sun-Yat-sen University (Guangzhou, China). C57BL/6J mice at embryonic day (E) 15 were sacrificed by cervical dislocation, and palatal shelves were dissected from fetuses with use of microsurgical instruments (Medical Needle Co., Ningbo, China). Animal experiments were approved by the Laboratory Animal Ethical Committee of Medical College of Shantou University.

Excised palatal shelves were washed with Ca^{2+}–Mg^{2+} free phosphate buffered saline (PBS; Sigma, St. Louis, MO) and cut to 1 mm^3. Palatal shelf tissues were seeded uniformly at the bottom of 25-cm^2 cell culture flasks (Nunc, Kamstrup, Roskilde, Denmark) at 16 pieces per flask, which were pre-moistened with Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G, 0.1 mg/ml streptomycin). After being kept upside down at 37°C for 4 h in a 95% air/5% CO_2 incubator (Thermo Scientific, Waltham, MA), culture flasks were turned over and tissues were submerged in DMEM/F12 containing 10% FBS and antibiotics, with the medium replaced every other day. Seven days later, the tissues were discarded, and remaining cells were incubated with trypsin (2.5 g/L)/EDTA (0.38 g/L) for 1–2 min. The action of trypsin was inhibited with DMEM/F12 containing 10% FBS, and the mixture was centrifuged at 500*g* for 5 min to pellet the cells. The cell pellet was resuspended in DMEM/F12 containing 10% FBS and antibiotics. The number of cells was determined by use of a hemocytometer, and cells were seeded at 3×10^5 cells/25 cm^2 cell culture flask. Cells at 70% confluence were subcultured at a ratio of 1:3. Cells at passage 2 were trypsinized, resuspended, and seeded in DMEM/F12 without FBS. After culture for 30–50 min, the medium without FBS was replaced by fresh DMEM/F12 including 10% FBS and antibiotics to acquire highly pure cells, because under the condition of free FBS epithelial cells adhered to the bottom of flask more slowly than did MEPM cells. Cells at passage 3 were used for further study. DMEM/F12, FBS, antibiotics, and trypsin/EDTA were purchased from Gibco (Grand Island, NY).

IMMUNOFLUORESCENCE STAINING

Cells at passage 3 were trypsinized, resuspended, and seeded at 3×10^5 cells/well in six-well plates (Nunc) for immunofluorescence staining of MEPM cells with a standard protocol. Briefly, cells were divided into 3 groups (3 wells/group) and incubated at 4°C overnight with the antibodies mouse monoclonal anti-vimentin IgG1 (1:100; Abcam, Cambridge, UK), mouse monoclonal anti-cytokeratin (CK) IgG1 (1:300; Abcam) or PBS vehicle alone (negative control), then the secondary antibody goat anti-mouse Alexa Fluor® 488 IgG (1:300; Invitrogen, Carlsbad, CA). Then, DAPI (Beyotime, Haimen, Jiangsu, China) was added, and cells were kept at room temperature for 5 min. Vimentin-positive and CK-positive cells were assessed by use of a Leica DM 2500 microscope (Wetzlar, Germany).

FLOW CYTOMETRY

Cells were collected for flow cytometry to assess the purity of MEPM cells by a standard protocol. Cells were divided into two groups (10^6 cells/group), one treated with the antibodies mouse monoclonal anti-vimentin IgG1 (1:100) and goat anti-mouse Alexa Fluor® 488 IgG (1:300), and in the other group, the monoclonal anti-vimentin IgG1 antibody was replaced by PBS as a negative control. The purity of MEPM cells was calculated by FACSCalibur™ flow cytometry (Becton-Dickinson, Franklin Lakes, NJ).

DETECTION OF MEPM CELLS VIABILITY

MEPM cells were transferred to 96-well plates (Corning, NY) and seeded from 10^4 cells/well with 100 μl DMEM/F12, 10% FBS and antibiotics, with 6 repeats for each density. At 37°C for 24 h, phenytoin (Sigma), *E. purpurea* extract (Layn Natural Ingredients, Guilin, China) or a mixture of phenytoin and *E. purpurea* extract, 10 μl, was added to each well, and cells were incubated at 37°C. Then, CCK-8 solution (Dojindo, Japan), 10 μl, was added to each well, and cells were incubated at 37°C for an additional 3 h. The absorbance was read at 450 nm on an automatic microwell plate reader (Thermo Scientific). Cells without any treatment were the control.

PROLIFERATION ASSAY OF MEPM CELLS

Cell proliferation was analyzed on an automatic microwell plate reader (Thermo Scientific) by use of the BrdU Cell Proliferation Assay kit (Calbiochem, Merk, Darmstadt, Germany). MEPM cells were transferred to 96-well plates and seeded from 10^4 cells/well with 90 μl DMEM/F12, 10% FBS and antibiotics, with 6 repeats for each density. At 37°C for 24 h, phenytoin, *E. purpurea* extract or a mixture of phenytoin and *E. purpurea* extract, 10 μl, was added to each well, and cells were incubated at 37°C for 24 h. Then, BrdU label solution (Calbiochem), 20 μl, was added to each well, and cells were incubated at 37°C for an additional 18 h. We added 200 μl fixative/denaturing solution to each well for 30 min at room temperature after removing the contents of wells. Then, we incubated anti-BrdU antibody for 1 h and peroxidase goat anti-mouse IgG HRP conjugate for 30 min at room temperature. The absorbance was read at 450–540 nm on an automatic microwell plate reader (Thermo Scientific). Cells without any treatment were the control.

QUANTIFICATION OF MEPM CELLS APOPTOSIS

MEPM cells were treated with phenytoin, *E. purpurea* extract or a mixture of phenytoin and *E. purpurea* extract to detect cell apoptosis; cells without any treatment were the control.

Cell apoptosis was analyzed on a flow cytometer by annexin V-FITC and propidium iodide (PI) staining by use of the Annexin V-FITC Apoptosis Detection kit (Calbiochem) according to the manufacturer's instructions. Data for 10,000 cells were collected in the list mode.

Cell apoptosis was also analyzed by TUNEL assay by use of the TdT-FragEL™ DNA Fragmentation Detection Kit (Calbiochem) according to the manufacturer's instructions.

STATISTICAL ANALYSIS

All experiments were carried out in duplicate, and all results are expressed as mean ± SEM of at least three independent experiments. Data analysis involved use of Windows SPSS v13.0 (SPSS, Chicago, IL). Differences between groups were analyzed by independent sample *t* tests. $P < 0.05$ was considered statistically significant.

RESULTS

CHARACTERIZATION OF MEPM CELLS

After adhering to the bottom of the culture flask, cultured MEPM cells were similar to fibroblasts morphologically, with a fusiform shape and 2–4 enations (Fig. 1A). Most cells were positive for vimentin (mesenchymal cells) on immunofluorescence, and a few were positive for CK [epithelia; Fig. 1B; Yano et al., 1996]. On flow cytometry, the proportion of vimentin-positive cells was nearly 90% that of controls (Fig. 1C).

VIABILITY OF MEPM CELLS

After culture for 24 h, MEPM cells were treated with phenytoin and *E. purpurea* extract at seven concentrations for five times (Fig. 2A,B). Treatment with phenytoin at 200 μg/ml for 24 h significantly inhibited the cell viability to 51.42% that of control. Treatment with *E. purpurea* extract at 150 μg/ml for 24 h promoted the cell viability to 128.81% that of control. Thus, we used 200 μg/ml phenytoin, 150 mg/ml *E. purpurea* extract, and this mixture of phenytoin and *E. purpurea* extract to treat MEPM cells for 24 h (Fig. 2C). The mixture of phenytoin and *E. purpurea* extract promoted cell viability as compared with phenytoin alone ($P < 0.001$). As compared with controls, treatment with phenytoin inhibited MEPM cell viability ($P < 0.001$) and treatment with *E. purpurea* extract stimulated cell viability ($P = 0.0003$).

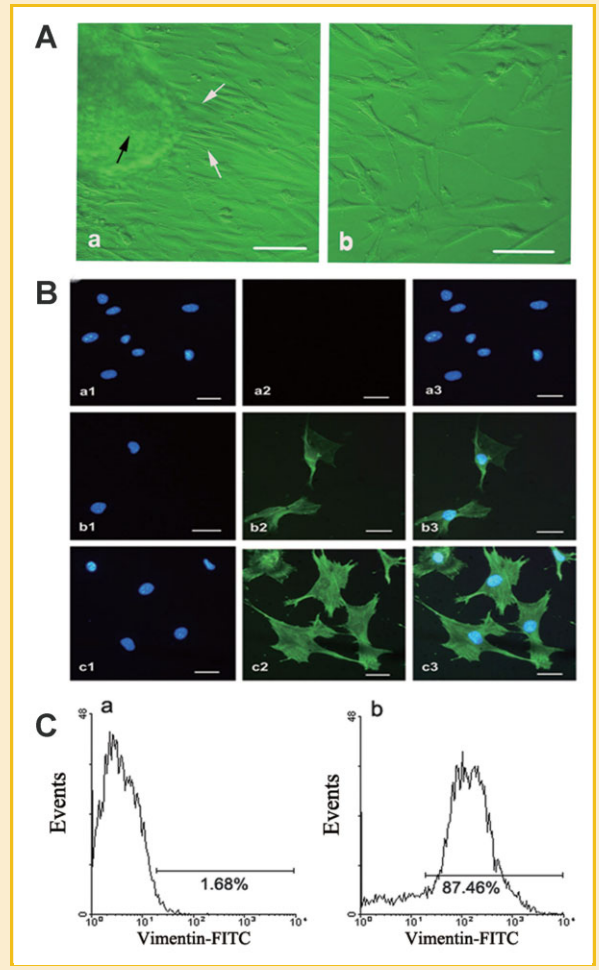

Fig. 1. Identity and purity of mouse embryonic palatal mesenchymal (MEPM) cells. A: Morphology of MEPM cells. a: MEPM cells (white arrowhead) shown emerging from palatal shelf tissues (black arrowhead); (b) Morphology of MEPM cells after purification. B: MEPM cells by immunofluorescence staining. a: Negative control, (b) Cytokeratin (CK)-positive cells, (c) Vimentin-positive cells; (1) Nucleus stained with DAPI, (2) Cytoplasm stained with vehicle alone (negative control), mouse monoclonal to CK, and mouse monoclonal to vimentin, (3) Merged images. C: The purity of MEPM. a: Control group; (b) Experimental (vimentin) group. Scale bars: 100 μm in (A), 20 μm in (B).

PROLIFERATION OF MEPM CELLS

As compared with controls, treatment with phenytoin significantly inhibited the cell proliferation ($P < 0.001$). *E. purpurea* extract promoted the cell proliferation ($P = 0.022$), and the mixture of phenytoin and *E. purpurea* extract significantly promoted the proliferation of MEPM cells ($P < 0.001$; Fig. 3).

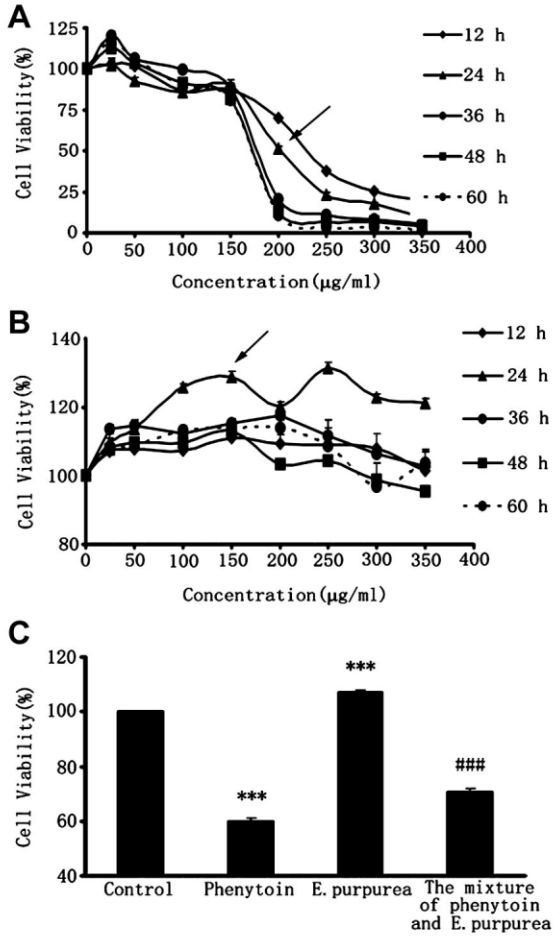

Fig. 2. Effect of treatment with phenytoin, *E. purpurea* extract and the mixture of phenytoin and *E. purpurea* extract on viability of MEPM cells. A: Effect of phenytoin for five times at seven concentrations. Exposure to phenytoin (200 μg/ml for 24 h; arrowhead) was considered optimal treatment for further study. B: Treatment with *E. purpurea* extract for five times at seven concentrations. Exposure to *E. purpurea* extract (150 μg/ml for 24 h; arrowhead) was considered the optimal treatment for further study. C: Treatment with 200 μg/ml phenytoin, 150 μg/ml *E. purpurea* extract, and the mixture of phenytoin and *E. purpurea* extract for 24 h. Data are mean ± SEM. $^{***}P < 0.001$, compared to control groups. $^{###}P < 0.001$, compared to the phenytoin only groups.

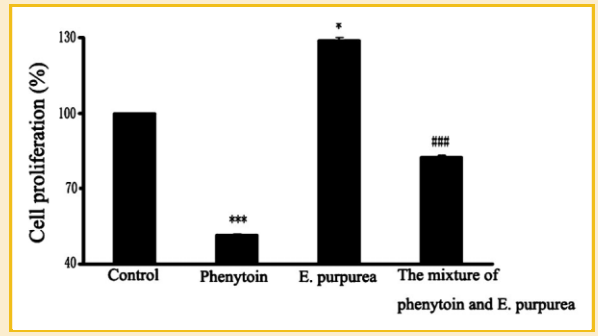

Fig. 3. Effect of treatment with phenytoin, *E. purpurea* extract and the mixture of phenytoin and *E. purpurea* extract on proliferation of MEPM cells. Treatment with 200 μg/ml phenytoin, 150 μg/ml *E. purpurea* extract, and the mixture of phenytoin and *E. purpurea* extract for 24 h. Data are mean ± SEM. $^{***}P < 0.001$, $^{*}P < 0.05$, compared to control groups. $^{###}P < 0.001$, compared to the phenytoin only groups.

APOPTOSIS OF MEPM CELLS

We analyzed the number of apoptotic cells after treatment by flow cytometry with annexin V-FITC and PI staining (Fig. 4A) and by TUNEL assay (Fig. 4B,C). The percentage of early apoptotic cells in the control group was 1.62%, whereas phenytoin treatment produced apoptosis in about one-third of cells (33.05%), which was 20-fold that of controls. *E. purpurea* extract produced 0.33% early apoptotic cells, less than that of controls. As well, the mixture of phenytoin and *E. purpurea* extract produced 16.48% early apoptotic cells, significantly less that with phenytoin only. TUNEL assay revealed that as compared with controls, phenytoin treatment induced a significantly increased rate of apoptotic cells ($P < 0.001$), *E. purpurea* extract treatment no change ($P = 0.243$), and the mixture of phenytoin and *E. purpurea* extract a decreased rate ($P < 0.001$).

DISCUSSION

In this study, we focused on the possible functions of phenytoin, *E. purpurea* extract and the mixture of phenytoin and *E. purpurea* extract in the proliferation and apoptosis of MEPM cells to investigate the involvement of environmental factors in cleft palate. Exposure to phenytoin for 24 h inhibited cell proliferation and increased cell apoptosis, and *E. purpurea* extract had the reverse effect. Treatment with the mixture of phenytoin and *E. purpurea* extract increased the proliferation and decreased the apoptosis of MEPM cells as compared with phenytoin alone. Considering extant results about combining phenytoin and *E. purpurea* extract in vivo [Mahabady et al., 2006], we concluded that the teratogenic effect of phenytoin on cleft palate is associated with the proliferation and apoptosis of MEPM cells, and *E. purpurea* extract may have a protective effect.

Nonsyndromic orofacial clefts, which include cleft lip, cleft lip and palate, and cleft palate alone, comprise a range of disorders affecting the lips and oral cavity, the causes of which remain largely unknown. Effects on speech, hearing, appearance, and cognition can lead to long-lasting adverse out comes for health and social integration. Prevention is the ultimate objective for clefting, and a prerequisite of this aim is to elucidate causes of the disorders [Mossey et al., 2009]. Several studies confirmed that the genetic basis of nonsyndromic clefting is complex; variations in numerous genes, together with environmental factors, are known to play a role in its etiology [Murray, 2002; Mossey et al., 2009]. In terms of cytomorphology, at least three processes are involved in the development of the secondary palate. First, the palate shelf fusion requires epithelial apoptosis, which is the major fate of the midline cells during physiological palatal fusion [Cuervo et al., 2002; Hay, 2005]. Mechanisms of normal disintegration of the midline epithelial seam have been reviewed, together with pathologic

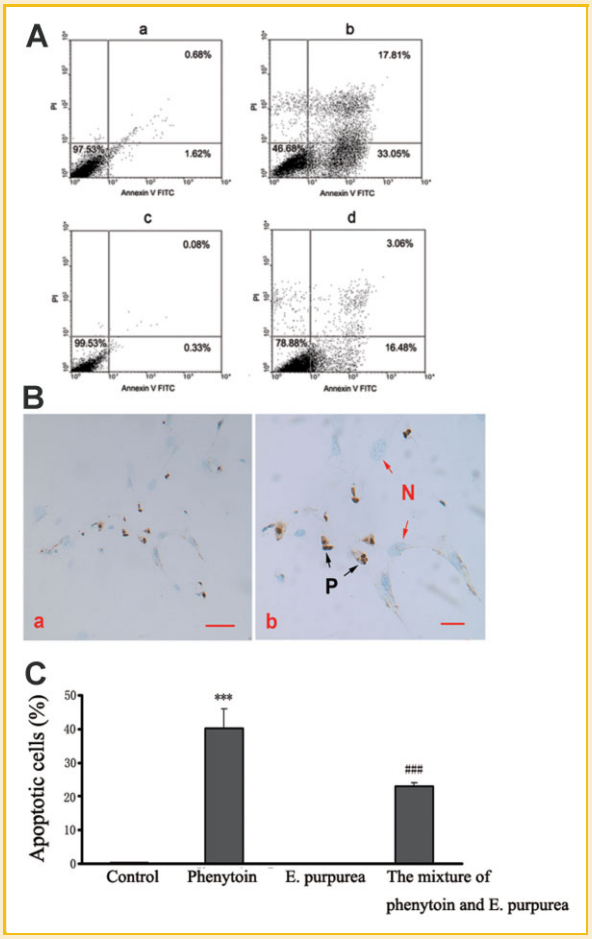

Fig. 4. Effect of treatment with phenytoin, *E. purpurea* extract and the mixture of phenytoin and *E. purpurea* extract on apoptosis of MEPM cells. A: Treatment with phenytoin (b; 200 μg/ml for 24 h), *E. purpurea* extract (c; 150 μg/ml for 24 h) and the mixture of phenytoin and *E. purpurea* extract (d). a: Control group. Cells were stained with annexin V-FITC and propidium iodide (PI), and data for 10,000 cells were collected. Lower left quadrant: viable cells (FITC and PI negative); upper right quadrant: necrotic or late apoptotic cells (PI positive and FITC negative); lower right quadrant: early apoptotic cells (FITC positive and PI negative). B: Dark brown DAB signal indicates apoptotic cell (positive, P) and blue-green signifies normal cell (N). Scale bars: 50 μm in a, 20 μm in (b). C: The rate of apoptotic cells (%). Treatment with phenytoin at 200 μg/ml for 24 h and *E. purpurea* extract at 150 μg/ml for 24 h. $^{***}P < 0.001$, compared to control group. Combination of phenytoin and *E. purpurea* extract for 24 h. Data are mean ± SEM. $^{\#\#\#}P < 0.001$, compared to phenytoin treatment.

processes that prevent this disintegration and causes cleft palate [Dudas et al., 2007]. Second, growth of the palate shelves depends on the survival and continued proliferation of mesenchymal cells that originate from neural crest and mesodermal cells of the first pharyngeal arch [Murray and Schutte, 2004]. Finally, epithelial–mesenchymal transition processes are important during normal development of the secondary palate; failure of these processes causes cleft palate [Yang and Weinberg, 2008; Thiery et al., 2009]. We consider the three processes as an entirety and each may contribute equally. Signaling between the palatal epithelium and mesenchyme is known to have a key role in regulation of palatal growth; factors involved include FGF10 signals from the palatal mesenchyme to its receptor FGF2b, which is expressed in the palatal epithelium. Loss of function of FGF10 or FGFR2b causes reduced mesenchymal proliferation and increased apoptosis, thus leading to truncation of the palatal shelves [Rice et al., 2004]. So, some environmental factors, through regulating gene expression to control mesenchymal proliferation and apoptosis, cause cleft palate.

Phenytoin at a low concentration, 25 μg/ml, for a moderate time, 36 h, could slightly increase cell viability as compared with control treatment. However, 150 μg/ml phenytoin for12–60 h decreased cell viability. We chose 200 μg/ml phenytoin for 24 h as the optimal concentration and time, because it decreased cell viability approximately 50%. Compared to phenytoin, *E. purpurea* extract showed no regular changes on cell viability over time (Fig. 2B). The active phytochemicals in this plant may vary with the age of plant, the portion of plant used, growth conditions, geographical location, and extraction method [Perry et al., 2001]. The most common compounds in *Echinacea* include alkamides, cichoric acid, glycoproteins/polysaccharides and polyacetylenes; cichoric acid, alkamides, and glycoproteins/polysaccharides possess immunomodulatory activity. Our *E. purpurea* extracts were extracted by ethanol, and its ingredients are mainly polysaccharides (4%) and cichoric acid (2%). So, which ingredient was effective or both is unknown. Several studies reported that *E. purpurea* extract stimulated various immune cells, including macrophages, natural killer cells, and dendritic cells [Pugh et al., 2008; Benson et al., 2010], but *E. purpurea* extract may not be effective for embryonic palatal mesenchymal cells. However, with increased concentrations, we found that *E. purpurea* extract could decrease cell viability slightly. Burger et al. [1997] also showed that at low concentrations *E. purpurea* induced the secretion of IL-1, IL-6, IL-10, and TNF-α. We chose the same time as for phenytoin, 24 h, 150 μg/ml *E. purpurea* extract for further study. For cell viability detection, *E. purpurea* extract and the mixture of phenytoin and *E. purpurea* extract for the same time revealed no effect of *E. purpurea* extract. However, the mixture of phenytoin and *E. purpurea* extract could increase the cell viability of MEPM cells as compared with phenytoin only.

Because a change in cell viability maybe due to changes in proliferation or apoptosis, we examined cell proliferation by BrdU labeling (Fig. 3) and cell apoptosis by annexin V FITC-PI double staining and TUNEL assay (Fig. 4). Phenytoin prominently inhibited the proliferation of MEPM cells and promoted apoptosis as compared with control treatment. Importantly, the mixture of phenytoin and *E. purpurea* extract could promote the proliferation of MEPM cells and decrease apoptosis as compared with phenytoin only. These is a discrepancy between cell viability (Fig. 2C) and proliferation (Fig. 3) assay for the effect of the mixture of phenytoin and *E. purpurea* extract compared with phenytoin only on MEPM cells, which maybe due to the limitations of a cell viability assay used.

It was reported that MEPM cells from C57BL/6J mouse fetuses involved at E13 [Han et al., 2006], but we found dissecting fetuses at this stage difficult, and we could not confirm whether these tissues were palatal shelves. Our MEPM cells were cultured from palatal shelves dissected from C57BL/6J mouse fetuses at E15 for

comparability of data. The secondary palate develops as an outgrowth of the maxillary prominences at about E11.5 in the mouse. The palate shelves initially grow vertically down the side of the tongue (E12.5) and then elevates above the tongue as it drops in the oral cavity (E13.5); with continued growth, the shelves appose in the midline (E14.5) and fuse [E15.5; Murray and Schutte, 2004]. So, our MEPM cells from between the shelves appose in the midline and fuse.

Liu et al. [2007] discovered a genetic requirement for glycogen synthase kinase-3β (GSK-3β) in midline development; homozygous null mice display cleft palate. However, chemical (rapamycin) rescue of cleft palate and midline defects occur in conditional GSK-3β mice. Therefore, we can change the environmental factors, including teratogens, infection, nutrients, and cholesterol metabolism to rescue the most common human birth defects-cleft palate. Factors include enhancing maternal immune system, anti-infection, and increasing maternal nutrients. Findings of these interactions can inform decisions about public health strategies.

In conclusion, phenytoin plays an important role in the proliferation and apoptosis of MEPM cells, and *E. purpurea* extract has an effect opposite to that of phenytoin. Phenytoin depressed cellular proliferation and increased cell apoptosis, and *E. purpurea* extract had the reverse effect and rescued the effects on MEPM cells induced by phenytoin. Investigation of phenytoin may help in understanding one of the factors involved in the development of cleft palate.

ACKNOWLEDGMENTS

We thank Professors. Tianhua Huang, Yucai Fu, Dr. Pingnan Sun, Dr. Xiaoling Zhou, Dr. Mingyan Xu and Dr. Xiaoling Deng for thoughtful discussion and advice.

REFERENCES

Barrett B. 2003. Medicinal properties of *Echinacea*: A critical review. Phytomedicine 10:66–86.

Benson JM, Pokorny AJ, Rhule A, Wenner CA, Kandhi V, Cech NB, Shepherd DM. 2010. *Echinacea purpurea* extracts modulate murine dendritic cell fate and function. Food Chem Toxicol 48:1170–1177.

Borchers AT, Keen CL, Stern JS, Gershwin ME. 2000. Inf iammation and native American medicine: The role of botanicals. Am J Clin Nutr 72:339–347.

Burger RA, Torres AR, Warren RP, Caldwell VD, Hughes BG. 1997. *Echinacea*-induced cytokine production by human macrophages. Int J Immunopharmacol 19:371–379.

Centers for Disease Control and Prevention (CDC). 2004. Spina bifida and anencephaly before and after folic acid mandate—United States, 1995–1996 and 1999–2000. Morb Mortal Wkly Rep 53:362–365.

Cuervo R, Valencia C, Chandraratna RA, Covarrubias L. 2002. Programmed cell death is required for palate shelf fusion and is regulated by retinoic acid. Dev Biol 245:145–156.

Dhulipala VC, Hanumegowda UM, Balasubramanian G, Reddy CS. 2004. Relevance of the palatal protein kinase A pathway to the pathogenesis of cleft palate by secalonic acid D in mice. Toxicol Appl Pharmacol 194:270–279.

Dudas M, Li WY, Kim J, Yang A, Kaartinen V. 2007. Palatal fusion-where do the midline cells go? A review on cleft palate, a major human birth defect. Acta Histochem 109:1–14.

Edison RJ, Muenke M. 2004. Central nervous system and limb anomalies in case reports of first-trimester statin exposure. N Engl J Med 350:1579–1582.

Ernst E. 2002. The risk-benefit profile of commonly used herbal therapies: Ginkgo, St. John's Wort, Ginseng, Echinacea, Saw Palmetto, and Kava. Ann Intern Med 136:42–53.

Goldman AS, Baker MK, Piddington R, Herold R. 1983. Inhibition of programmed cell death in mouse embryonic palate in vitro by cortisol and phenytoin: Receptor involvement and requirement of protein synthesis. Proc Soc Exp Biol Med 174:239–243.

Han J, Xiao Y, Lin J, Li Y. 2006. PDGF-C controls proliferation and is down-regulated by retinoic acid in mouse embryonic palatal mesenchymal cells. Birth Defects Res B Dev Reprod Toxicol 77:438–444.

Hanumegowda UM, Judy BM, Welshons WV, Reddy CS. 2002. Selective inhibition of murine palatal mesenchymal cell proliferation in vitro by secalonic acid D. Toxicol Sci 66:159–165.

Hay ED. 2005. The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev Dyn 233:706–720.

He F, Xiong W, Yu X, Espinoza-Lewis R, Liu C, Gu S, Nishita M, Suzuki K, Yamada G, Minami Y, Chen Y. 2008. Wnt5a regulates directional cell migration and cell proliferation via Ror2-mediated noncanonical pathway in mammalian palate development. Development 135:3871–3879.

Holladay SD, Sharova L, Smith BJ, Gogal RM, Jr., Ward DL, Blaylock BL. 2000. Nonspecific stimulation of the maternal immune system. I. Effects on teratogen-induced fetal malformations. Teratology 62:413–419.

Holladay SD, Sharova LV, Punareewattana K, Hrubec TC, Gogal RM, Jr., Prater MR, Sharov AA. 2002. Maternal immune stimulation in mice decreases fetal malformations caused by teratogens. Int Immunopharmacol 2:325–332.

Janz D, Fuchs U. 1964. Are anti-epileptic drugs harmful during pregnancy? Dtsch Med Wochenschr 89:241–248.

Karolyi J, Erickson RP, Liu S, Killewald L. 1990. Major effects on teratogen-induced facial clefting in mice determined by a single genetic region. Genetics 126:201–205.

Kondo S, Schutte BC, Richardson RJ, Bjork BC, Knight AS, Watanabe Y, Howard E, Lima RLD, Daack-Hirsch S, Sander A, McDonald-McGinn DM, Zackai EH, Lammer EJ, Aylsworth AS, Ardinger HH, Lidral AC, Pober BR, Moreno L, Arcos-Burgos M, Valencia C, Houdayer C, Bahuau M, Moretti-Ferreira D, Richieri-Costa A, Dixon MJ, Murray JC. 2002. Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. Nat Genet 32: 285–289.

Little J, Cardy A, Munger RG. 2004. Tobacco smoking and oral clefts: A meta-analysis. Bull World Health Organ 82:213–218.

Liu KJ, Arron JR, Stankunas K, Crabtree GR, Longaker MT. 2007. Chemical rescue of cleft palate and midline defects in conditional GSK-3beta mice. Nature 446:79–82.

Mahabady MK, Ranjbar R, Arzi A, Papahn AA, Najafzadeh H. 2006. A comparison study of effects of *Echinacea* extract and levamisole on phenytoin-induced cleft palate in mice. Regul Toxicol Pharmacol 46:163–166.

Meador KJ, Baker GA, Finnell RH, Kalayjian LA, Liporace JD, Loring DW, Mawer G, Pennell PB, Smith JC, Wolff MC, NEAD Study Group. 2006. In utero antiepileptic drug exposure: Fetal death and malformations. Neurology 67: 407–412.

Mishima S, Saito K, Maruyama H, Inoue M, Yamashita T, Ishida T, Gu Y. 2004. Antioxidant and immuno-enhancing effects of *Echinacea purpurea*. Biol Pharm Bull 27:1004–1009.

Mossey P, Castillia E. 2003. Global registry and database on craniofacial anomalies. Geneva: World Health Organization.

Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. 2009. Cleft lip and palate. Lancet 374:1773–1785.

Murray JC. 2002. Gene/environment causes of cleft lip and/or palate. Clin Genet 61:248–256.

Murray JC, Schutte BC. 2004. Cleft palate: Players, pathways, and pursuits. J Clin Invest 113:1676–1678.

Nieri P, Adinolfi B, Morelli I, Breschi MC, Simoni G, Martinotti E. 2003. Genetic characterization of the three medicinal *Echinacea* species using RAPD analysis. Planta Med 69:685–686.

Perry NB, Burgess EJ, Glennie VL. 2001. *Echinacea* standardization: Analytical methods for phenolic compounds and typical levels in medicinal species. J Agric Food Chem 49:1702–1706.

Prater MR, Zimmerman KL, Ward DL, Holladay SD. 2004. Reduced birth defects caused by maternal immune stimulation in methylnitrosourea-exposed mice: Association with placental improvement. Birth Defects Res A Clin Mol Teratol 70:862–869.

Pugh ND, Tamta H, Balachandran P, Wu X, Howell J, Dayan FE, Pasco DS. 2008. The majority of in vitro macrophage activation exhibited by extracts of some immune enhancing botanicals is due to bacterial lipoproteins and lipopolysaccharides. Int Immunopharmacol 8:1023–1032.

Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Thesleff I, Rice DP. 2004. Disruption of Fgf10/Fgfr2b-coordinated epithelial–mesenchymal interactions causes cleft palate. J Clin Invest 113:1692–1700.

Rininger JA, Kickner S, Chigurupati P, McLean A, Franck Z. 2000. Immunopharmacological activity of *Echinacea* preparations following simulated digestion on murine macrophages and human peripheral blood mononuclear cells. J Leukoc Biol 68:503–510.

Risley M, Garrod D, Henkemeyer M, McLean W. 2009. EphB2 and EphB3 forward signalling are required for palate development. Mech Dev 126:230–239.

Roesler J, Emmendörffer A, Steinmüller C, Luettig B, Wagner H, Lohmann-Matthes ML. 1991. Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to test subjects mediates activation of the phagocyte system. Int J Immunopharmacol 13:931–941.

Sonawane BR, Goldman AS. 1981. Susceptibility of mice to phenytoin-induced cleft palate correlated with inhibition of fetal palatal RNA and protein synthesis. Proc Soc Exp Biol Med 168:175–179.

Suzuki K, Hu D, Bustos T, Zlotogora J, Richieri-Costa A, Helms JA, Spritz RA. 2000. Mutations of PVRL1, encoding a cell–cell adhesion molecule/herpesvirus receptor, in cleft lip/palate-ectodermal dysplasia. Nat Genet 25:427–430.

Takechi R, Taniguchi A, Ebara S, Fukui T, Watanabe T. 2008. Biotin deficiency affects the proliferation of human embryonic palate mesenchymal cells in culture. J Nutr 138:680–684.

Thiery JP, Acloque H, Huang RY, Nieto MA. 2009. Epithelial–mesenchymal transitions in development and disease. Cell 139:871–890.

Vanhoutteghem A, Maciejewski-Duval A, Bouche C, Delhomme B, Hervé F, Daubigney F, Soubigou G, Araki M, Araki K, Yamamura K, Djian P. 2009. Basonuclin 2 has a function in the multiplication of embryonic craniofacial mesenchymal cells and is orthologous to disco proteins. Proc Natl Acad Sci USA 106:14432–14437.

Wilkie AO, Morriss-Kay GM. 2001. Genetics of craniofacial development and malformation. Nat Rev Genet 2:458–468.

Yang J, Weinberg RA. 2008. Epithelial–mesenchymal transition: At the crossroads of development and tumor metastasis. Dev Cell 14:818–829.

Yano H, Yoshimoto H, Ohtsuru A, Ito M, Yamashita S, Fujii T. 1996. Characterization of cultured rat embryonic palatal mesenchymal cells. Cleft Palate Craniofac J 33:379–384.