Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
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A preliminary study on the teratogenesis of dexamethasone and the preventive effect of vitamin B_{12} on murine embryonic palatal shelf fusion in vitro*

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Received Dec. 24, 2007; revision accepted Feb. 14, 2008

Abstract: Excessive dexamethasone (Dex) administrated into pregnant mice during critical periods of palatal development can produce a high incidence of cleft palate. Its mechanisms remain unknown. Vitamin B_{12} has been shown to antagonize the teratogenic effects of Dex, which, however, remains controversial. In this study, we investigated the effects of Dex and vitamin B_{12} on murine embryonic palatal shelf fusion using organ culture of murine embryonic shelves. The explanted palatal shelves on embryonic day 14 (E14) were cultured for 24, 48, 72 or 96 h in different concentrations of Dex and/or vitamin B_{12}. The palatal shelves were examined histologically for the morphological alterations on the medial edge epithelium (MEE) and fusion rates among different groups. It was found that the palatal shelves were not fused at 72 h or less of culture in Dex group, while they were completely fused in the control and vitamin B_{12}-treated groups at 72 and 96 h, respectively. The MEE still existed and proliferated. In Dex+vitamin B_{12} group the palatal shelves were fused at each time point in a similar rate to controls. These results may suggest that Dex causes teratogenesis of murine embryonic palatal shelves and vitamin B_{12} prevents the teratogenic effect of Dex on palatogenesis on murine embryos in vitro.

Key words: Dexamethasone, Vitamin B_{12}, Organ culture, Cleft palate, Medial edge epithelial cells
doi:10.1631/jzus.B0710625 **Document code:** A **CLC number:** R78

INTRODUCTION

Cleft lip and palate is one of the most frequent congenital malformations in humans. Its etiology is complex and multifactorial. Both genetic and environmental factors are involved and regulated spatially and temporally by complicated molecular mechanisms (Gritli-Linde, 2007; Rice, 2005). The formation of the mammalian secondary palate requires several critical steps, including growth, elevation, contact, medial edge epithelium (MEE) disappearance, and, finally, bilateral palatal shelves fusion. Interruption of any of these steps may cause cleft palate.

B-vitamins are a group of water-soluble vitamins, including B_6, B_{12}, folic acid, etc. Deficiency of vitamins is associated with many illness and birth defects (Botto *et al*., 2004; Herrmann *et al*., 2007; Krapels *et al*., 2004). Epidemiological studies of cleft lip and palate showed that a pregnant female taking certain doses of vitamin B_{12} may decrease the incidence of cleft and palate in her offspring (van Rooij *et al*., 2003). However, others also reported that children with congenital malformations did not show any serum vitamin deficiencies compared with a normal population (Stoll *et al*., 1999).

It was reported that palatal development in vitro was similar to in vivo (Koch and Smiley, 1981; Shimizu *et al*., 2001). In this study we have investigated the teratogenic effect of dexamethasone (Dex) on the palate and employed vitamin B_{12} to antagonize the teratogenic effects of Dex on palate fusion in mouse

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* Project (No. 30530730) supported by the National Natural Science Foundation of China

embryos in vitro, using the organ culture method (Hassell, 1975).

MATERIALS AND METHODS

Animals

Adult C57BL/6J mice, weighting 15~20 g, one male and two females per cage, were mated overnight in a temperature-controlled (22 °C) SPF (specified-pathogens free) room, water and food ad libitum. Vaginal plugs appeared in the following morning, which was designated as the embryonic day 0 (E0).

Dissection and organ culture

On E14, mouse embryos were quickly immersed in GMF-PBS (Ca^{2+} and Mg^{2+} free-phosphate buffed solution, pH 7.2) and flushed lightly several times. Then the palatal shelves were removed using microsurgical shears and forceps under a dissecting microscope. Isolated palatal shelves were cultured according to the previous methods (Hassell, 1975). Briefly, the palatal shelves were placed in pairs on 0.4 μm porosity millipore filters (Millipore Corporation, USA), nasal epithelium down, media edges in contact, on 35 mm-tissue culture dishes (FALCON, France). The culture medium was composed of DMEM/F12 (Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham's) (Hyclone Corporation, USA), 10% fetal bovine serum (FBS, Hyclone Corporation), and 100 U/ml Penicillin-Streptomycin, with or without 20 ng/ml Dex (Sigma, USA) and/or 10 ng/ml vitamin B_{12} (Sigma, USA).

Transplants were divided into the control, the Dex-treated, the vitamin B_{12}-treated, and the Dex+ vitamin B_{12}-treated groups, with 8 samples in each group. Samples were cultured for 24, 48, 72 or 96 h at 37 °C in an incubator with 5% CO_2 (Leica, Germany). All the medium and treatments were replaced every 24 h.

Histology

At the end of incubation, transplants were immediately immersed in 4% neutral paraformaldehyde and embedded in paraffin. Then, 5 μm-thick serial sections were cut and stained with eosin and haematoxylin. These sections were then photographed using a Leica photographic system (Germany).

Scanning electron microscopy (SEM) preparation

Transplants were immersed in 2.5% glutaraldehyde solution for 2 h at room temperature, and then followed were ethanol gradient dehydration, isoamylacetate replacement, critical point drying, and gold plating. At last, the samples were scanned to observe surface changes of the palatal shelves.

Statistical analysis

Statistical analysis was performed with the chi-square test and variance analysis (SPSS 13.0). Different groups in the same designated time and in the same group across different incubation times were compared. Values of $P<0.05$ were considered significant.

RESULTS

The numbers of palatal shelf fusion in various experimental groups are presented in Table 1. The palatal shelves were not fused at the 72 h or less of culture in Dex group, compared with controls ($P<0.0025$), indicating the teratogenesis of Dex. In Dex+vitamin B_{12} group the palatal shelves were fused at each time point in a similar rate to the control and vitamin B_{12} groups, significantly different from the Dex group ($P<0.05$). These results indicate the antagonistic effect of vitamin B_{12} against Dex.

Table 1 The numbers of palatal shelf fusion in various experimental groups

Group	Number of palatal shelf fusion			
	24 h	48 h	72 h	96 h
Control	3	7	8	8
Dex	0	1	2	2
Vitamin B_{12}	4	6	6	8
Dex+vitamin B_{12}	5	6	7	7

Light microscope

Transplants were classified in three groups according to Vargas (1967) method: no fusion, partial fusion, and complete fusion. Complete fusion or mesenchymal coalescence means no MEE is observed, and the epithelial laminae have been broken down and resorbed. All of the palates in the control group completely fused after 48 h (Fig.1b), and no

MEE was observed. All of the palates in the vitamin B_{12}-treated group also completely fused at 96 h. There was no significant difference between control and vitamin B_{12}-treated groups (P>0.5) after 48, 72 and 96 h, which suggests that vitamin B_{12} had no effect on normal palatal fusion (Figs.1e and 1f). In the Dex-treated group at 24 h no fusion was observed (Fig.1c) and the MEE existed; at 48 h only 1 out of 8 (Fig.1d) and at 72 h 2 out of 8 fused (Fig.1g), and MEE did not disappear but thickened. In Dex+vitamin B_{12} group at 24 (Fig.1g), 48 (Fig.1h), 72 and 96 h, 5, 6, 7 and 7 out of 8 samples fused, respectively, indicating the antagonism of vitamin B_{12} against Dex.

Scanning electron microscopy

Figs.2a and 2b showed situation of the palatal shelves in the control group, and incubated after 24 h, bilateral palatal shelves have achieved completely fusion. The spheroidal structures in MES (medial epithelial seam) seemed likely deciduous perithelial cells from MEE and migrated to surface. The observations of palatal shelves of Dex-treated group showed in Figs.2c and 2d. Opposing palatal shelves seemed fused incompletely and retained seam between bilateral palates. The spheroidal structures in MES and palatal surface were less than the control group, and the spheroidal structures trapped, which suggested perithelial cells that underwent slough and migration were less than the control group. The perithelial cells did not slough and migrate, which resulted in basal cell of MEE and could not contact and induced cleft palate. Some fibroblast-like cells were observed on the surface of palatal shelves after vitamin B_{12} treatment (Figs.2e and 2f), but fusion of shelves was normal (Fig.2g). Possibly, function of vitamin B_{12} involved in cell morphous was needed further study. In vitamin B_{12}+Dex-treated group, surface morphous of palatal shelves was similar to that of the control group, and bilateral shelves fused but retained some feature like vitamin B_{12}-treated group (Figs.2h, 2i and 2j).

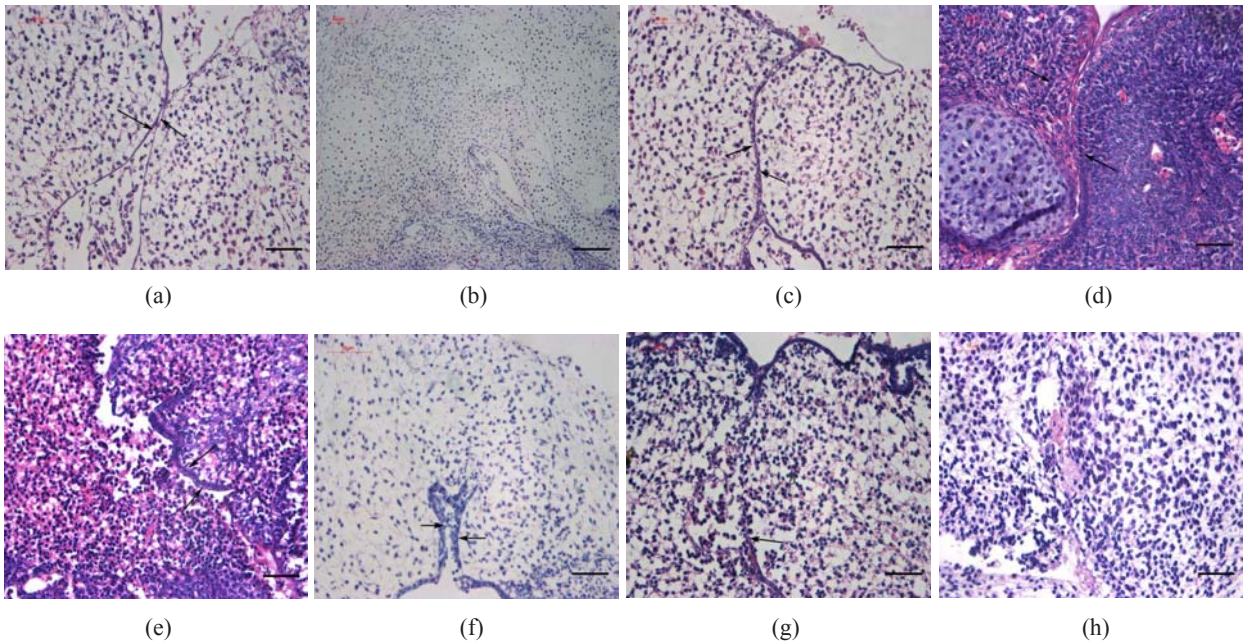

Fig.1 Hematoxylin and eosin (H & E)-stained sections of the palatal shelves. In the control group at 24 h of culture (a), the MEE was observed, and at 48 h (b), the MEE disappeared and the bilateral palatal shelves fused. In Dex group at 24 h (c) and 48 h (d), the MEE was still seen and after 48 h the MEE thickened. In vitamin B_{12}-treated group at 24 h (e) and 48 h (f), the MEE also disappeared. In Dex+vitamin B_{12} group at 24 h (g) and 48 h (h) the bilateral palatal shelves fused

Black arrows show the MEE; Scale bars=50 μm

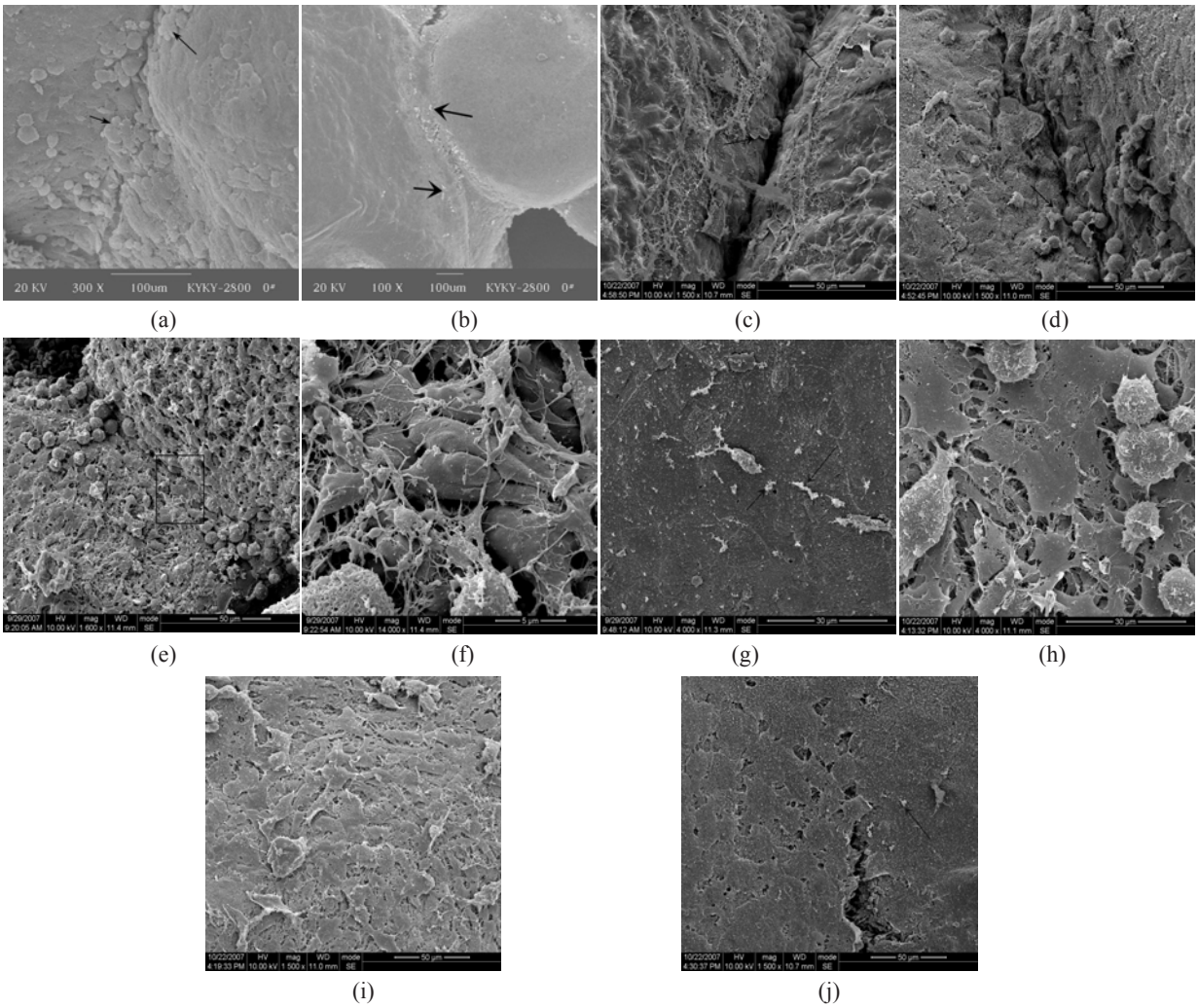

Fig.2 SEM observation of palates fusion. In the control group (a), the black arrows show some spheroidal structures in the medial edge area at 24 h and fusion at 48 h (b). In the Dex-treated group at 24 h the bilateral palatal shelves contacted and few filopodia were observed at 24 h (c). In Dex-treated group at 48 h, the spheroidal structures did not disappear and seemed trapped in the medial edge seam (d). In the vitamin B_{12}-treated group at 24 h (e), compared with Dex-treated group (c), more spheroidal structures and some fibroblast-like cells were seen as the black arrows indicate. The square (f) in (e) is shown at a higher magnification. Few spheroidal structures were observed in the medial area at 48 h in vitamin B_{12} group (g). Vitamin B_{12}+Dex group had some spheroidal structures and some fibroblast-like cells were seen at 24 h (h), and at 48 h, many fibroblast-like cells were observed, with a few spheroidal structures (i); and at 72 h no spheroidal structures were observed and in some areas a few apoptosis cells were seen, with few filopodia (j)

(a), (b) Scale bars=100 μm; (c), (d), (e), (i), (j) Scale bars=50 μm; (g), (h) Scale bars=30 μm; (f) Scale bars=5 μm

DISCUSSION

Bilateral palatal fusion includes two phases: the first phase in which palates move from a vertical position to a horizontal position, and the second phase in which the two palates contact and fuse to form the secondary palate. The potential of the palatal shelves to fuse in vitro depends on the age of embryo, which varies among species. Pourtois (1966) found that in the rat the potential to fuse was acquired 24 h before the actual time of the secondary palatal fusion, whereas Vargas (1967) found that in the mouse it was at least 40 h before the actual time of fusion in vivo. Teratogens that disturb any of these developmental

events may induce a failure of the palates to fuse.

Studies both in vivo and in vitro demonstrated that environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a cleft palate teratogen, not only disrupts the differentiation and proliferation of the bilateral MEE in mice, but also has effects on the expression of some growth factors (Abbott and Birnbaum, 1990a; 1990b; Abbott *et al*., 1989a; 1992; 2003; 2005b; Bock and Köhle, 2006; Hassoun and Dencker, 1982; Miettinen *et al*., 2004; Ryan *et al*., 1989). Other teratogens also showed effects on the fusion of the palatal shelves, such as retinoic acid (RA), which induces cleft palate when administered in excess to the maternal mouse, depending on the stage of embryonic development exposed (Abbott *et al*., 1989b; Newall and Edwards, 1981). Both in vivo and in vitro studies showed that RA changed the regulation of cell proliferation, which was often associated with the altered expression of epidermal growth factor receptor (EGFR) (Abbott and Birnbaum, 1990c; Abbott *et al*., 2005a) and transforming growth factor beta (TGF-β) (Degitz *et al*., 1998; Nugent *et al*., 1998). Dex, one of the glucocorticoids (GC) that are well-known teratogens, is a fat-soluble hormone that penetrates the cell membrane and binds to GC receptor (GR) in the cytoplasm, inhibiting palatal mesenchymal proliferation, and resulting in smaller palates that fail to contact and fuse (Greene and Kochhar, 1975; Hackney, 1980; Pratt *et al*., 1984; Shah *et al*., 1989). Sensitivity to Dex varies by species and stages of pregnancy, and the species differences in susceptibility are related to H-2 histocompatility (Kusanagi, 1984; Montenegro and Palomino, 1989). A population-based case-control study of cleft lip and palate showed that maternal corticosteroid use during pregnancy is associated with moderately increased risk of delivering an infant with an orofacial cleft (Carmichael *et al*., 2007).

Various in vitro methods have been employed in the investigation on the effects of Dex on the palate, including mesenchymal culture, or palatal epithelial and mesenchymal co-incubation, and organ culture. The methods vary in their ability to stimulate palate development and fusion, and the organ culture has significant advantages in this regard (Chou *et al*., 2004). In the current study, we observed that Dex appeared to stimulate the thickening of the MEE. When exposed to 20 ng/ml Dex for 24 h, the palatal shelves from E14 pregnant mice retained the MEE, and even after 96 h the MEE still existed. SEM images show that the spheroidal structures did not disappear and seemed trapped in the medial edge seam. Compared with the control group, few filopodia were observed.

Vitamin B_{12} is one of the most important coenzymes and participates in many biochemical reactions in vivo (Selhub, 2002). Population-based epidemiological investigations showed that pregnant women taking certain doses of vitamin B_{12} could reduce the risk of non-syndromic craniofacial clefts (van Rooij *et al*., 2003; Vujkovic *et al*., 2007). It was demonstrated that the vitamin B_{12} concentration of 185 pmol/L or less and the pyridoxal-5′-phosphate (PLP) concentration of 44 nmol/L or less in mothers increased the risk of having a child with cleft lip with or without cleft palate (van Rooij *et al*., 2003). Animal experiments showed that when vitamin B_{12} level decreased, the level of amniotic homocysteine, a risk factor for the malformation of the palate, increased (Weingärtner *et al*., 2007). Experiments on mice from E10~E13 demonstrated that vitamin B_{12} could antagonize the Dex-induced cleft palate (Natsume *et al*., 1986). However, the main mechanism of how vitamin B_{12} antagonized the Dex-induced craniofacial clefts is still unknown.

In the current studies, in the vitamin B_{12}-treated group 10 ng/ml vitamin B_{12} was added in the medium, and the palate fuse rate and SEM images were similar to the control, suggesting that vitamin B_{12} had no effect on palatal MEE. With exposure to 20 ng/ml Dex+10 ng/ml vitamin B_{12}, at 48 h incubation, the fuse rate significantly increased to near the one in controls and SEM images became similar to the control as well. These results demonstrate that vitamin B_{12} could antagonize the Dex-induced failure of the palatal fusion in mouse embryos.

CONCLUSION

In summary, our results may suggest that Dex causes teratogenesis of murine embryonic palatal shelves and vitamin B_{12} prevents the teratogenic effect of Dex on palatogenesis on murine embryos in vitro. We, therefore, propose that a proper concentration of vitamin B_{12} may effectively antagonize the

effects of Dex that leads to MEE thickening, which is possibly a consequence of excessive MEE proliferation or reduced cell death. Investigations into the molecular mechanisms of how Dex interacted with vitamin B_{12} were not examined and further studies are needed to clarify the preventive effects of vitamin B_{12} on cleft lip and palate.

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