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Inhibition of Programmed Cell Death in the Fetal Palate by Cortisol (41084)

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Abstract. In an *in vitro* model, cortisol inhibits a precisely timed process of palatal development, the lysosomally mediated cell death of the medial edge palatal epithelium. These steroid effects occur only in palatal shelves from mouse strains with high incidences of corticosteroid-induced cleft palate. In studies *in vivo* using steroid-sensitive mice, corticosteroid delays shelf elevation but does not prevent shelf contact and alters lysosomal enzyme distribution in the medial edge palatal epithelia. Thus, susceptibility to corticosteroid-promoted palatal clefting is correlated with the inhibition of programmed cell death in the medial edge epithelium *in vitro* and *in vivo*.

Fetal development of the secondary palate in mammals concludes with the contact and adhesion of apposing palatal shelves in the midline. The medial edge epithelia of these shelves form a seam which then breaks down permitting the fusion of palatal mesenchyme (1). These normal developmental changes have been experimentally reproduced in culture by explanting paired palatal shelves (2–4). In addition, the process of epithelial breakdown has been demonstrated in single palatal shelves *in vitro* and, interestingly, appears in culture at a time corresponding to the same event *in vivo* (5–7). This programmed cell death of the palatal epithelium is mediated by the intracellular release of hydrolytic enzymes from lysosomes (8–10). We report here that cortisol prevents medial edge autolysis in single shelves *in vitro* and that this steroid inhibition of programmed cell death is restricted to fetal shelves from mouse strains with high incidences of steroid-induced cleft palate. We also present *in vivo* evidence indicating that corticosteroid permits shelf contact but alters the synthesis and/or release of lysosomal enzymes in the medial edge epithelia of palatal shelves in steroid-sensitive mice.

Materials and Methods. Timed-pregnant Dublin ICR and CD-1 mice were obtained from Flow Laboratories and Charles River Breeding Laboratories, respectively. A/J, C57BL/6J(B6), and C57BL/10ScSn-(B10) breeding stocks were obtained from the Jackson Laboratory. A/J, B6, and B10 females were mated with syngenic males either overnight or for a 4 hr period. Palatal shelves from 13-day, 16-hr ICR, 13-day, 10-hr CD-1, 13-day, 21-hr A/J, 13-day, 18-hr B6, and 13-day, 21-hr BIO fetuses were cultured as single shelves in normal medium with cortisol (cortisol-21-phosphate, Sigma Chemical Company; 0.0001 to 10 μg/ml); controls were without steroid. Homotypic pairs of shelves were frequently used to compare responses in controls and steroid-treated cultures. In any case, controls were included in each experimental protocol. The culture medium consisted of 10% fetal bovine serum (Microbiological Associates) plus 2% penicillin–streptomycin mixture (5000 units-μg of each/ml; Microbiological Associates) in either Eagle's basal medium or Eagle's minimal essential medium with Earle's salts (Microbiological Associates or Flow Laboratories). Each shelf was placed oral side up on a Millipore filter (0.45 μm; Millipore Corporation) supported by a glass ring in the well of a depression dish. The cultures were incubated at 37–38° in a humidified, 5% CO_2–air atmosphere. These culture techniques are similar to *in vitro* conditions which have been shown to permit normal disruption of the medial edge epithelium in single shelves and typical epithelial breakdown and mesenchymal fusion in paired shelves (5). For lysosomal

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enzyme histochemistry, shelves were collected from culture, fixed in a formaldehyde–calcium solution, infiltrated with gum arabic–sucrose, and then frozen. Cryostat sections were cut at 6 μm, stained for trimetaphosphatase by the method of Doty *et al.* (11) and counterstained with 0.1% toluidine blue. For general histology, the shelves were collected, fixed in a paraformaldehyde–glutaraldehyde solution (Karnovsky's fixative), dehydrated, and embedded in Epon 812. Sections were cut with glass knives at 0.5 μm and stained with 0.1% toluidine blue. Serial sections of single palatal shelves have been examined. Development of the medial edge epithelium was observed to be the same near anterior and posterior borders of these shelves but slightly more advanced in the middle third. As a consequence, the figures in this paper are representative sections through the middle third of both controls and steroid-treated palatal shelves.

For *in vivo* studies, cortisone (100 mg/kg) in dimethyl sulfoxide (DMSO) was injected subcutaneously into pregnant A/J and B10 females on Days 11 through 14 of gestation; controls received only DMSO. At appro-

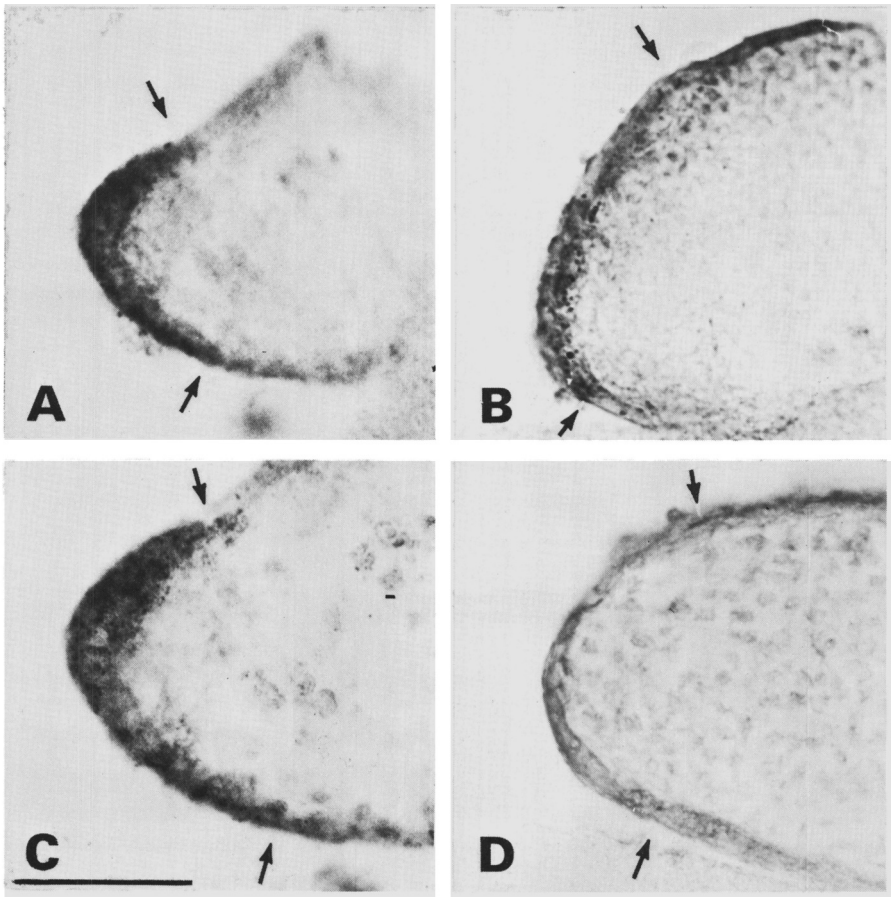

FIG. 1. Lysosomal enzyme histochemistry of palatal shelves cultured with or without steroid for 18 to 24 hr. (A) and (B) are the B6 and A/J controls, respectively, showing, in both cases, the medial edge epithelium (between arrows) darkly stained for trimetaphosphatase. (C) and (D) are the corresponding B6 and A/J shelves treated with cortisol (0.1 μg/ml) showing clearly higher levels of enzyme staining in the medial edge epithelium (between arrows) of the steroid-resistant (B6) strain. The magnification for all figures is the same; the bar in (C) represents 50 μm.

priate times thereafter, the fetuses were collected and their heads were removed and frozen in hexane at −70° (12). Cryostat sections were cut at 10 μm and then air dried on glass slides at room temperature for 1 to 3 hr. The sections were fixed in diacetone alcohol as per Burstone (13) and stored desiccated at 4° for periods up to 1 week. Sections were then stained with hematoxylin and eosin for general histological examination. Alternatively, sections were stained with the Barka and Anderson method (14) for acid phosphatase and counterstained with 1% methyl green.

Results. The effects of cortisol were compared in single palatal shelves from steroid-sensitive (A/J or CD-1) and steroid-resistant (B6) mouse strains, i.e., strains with high (100 or 90%) and low (19%) incidences of fetal clefting in response to steroid injected into pregnant mice (15, 16). Shelves from 13-day, 10-hr CD-1, 13-day, 21-hr A/J, and 13-day, 18-hr B6 mice were cultured in medium containing cortisol (0.1 μg/ml) for 18 to 24 hr, that is, for a period shortly prior to normal epithelial breakdown. Appropriate steroid-free controls were cultured simultaneously. All shelves were collected and examined histochemically for the marker lysosomal enzyme trimetaphosphatase. Figures 1A and B show that the medial edge palatal epithelium in both B6 and A/J controls is darkly stained for the enzyme. Similarly, the cortisol-treated B6 shelf has considerable enzyme staining at the medial edge (Fig. 1C). In contrast, the steroid-treated A/J palate shows little or no trimetaphosphatase activity (Fig. 1D); this characteristic response to cortisol by A/J is also exhibited by shelves from steroid-sensitive CD-1 mice. Furthermore, results are identical when acid phosphatase is substituted as the marker lysosomal enzyme. Palatal shelves were also cultured for longer periods and prepared for general histological examination. By 48 to 60 hr *in vitro* the medial edge epithelium has broken down completely in shelves from B6 and A/J controls as well as in the B6 shelf cultured with cortisol (Figs. 2A, B and C). The corresponding epithelium of the steroid-treated A/J palatal shelf persists despite the sloughing of some cells (Fig. 2D). The medial edge epithelia of A/J (and CD-1) palatal shelves remain intact even after 9 days of culture with steroid (17). Shah and Travill (18) show that the medial edge palatal epithelia in fetal hamsters *in utero* also persist in response to maternally injected cortisol.

Medial edge breakdown in steroid-sensitive (A/J and CD-1) palates is, in large measure, inhibited by cortisol at levels of 0.01 μg/ml and higher but is not affected by steroid at 0.0001 μg/ml (Table I). This low concentration is below normal physiological levels for corticosteroids in maternal plasma and probably in fetal plasma as well during the midgestation period (19). Intermediate results with 0.001 μg cortisol/ml in A/J and CD-1 shelves apparently reflect the limits of palatal sensitivity to steroid under these culture conditions. A similar pattern of cortisol responses has been demonstrated for the steroid-sensitive ICR strain; these results and their significance are discussed below. Epithelial cell loss in B6 (steroid-resistant) shelves is refractory to cortisol in concentrations ranging from 0.0001 to 10 μg/ml. Epithelial breakdown is also largely unaffected by cortisol in shelves from steroid-resistant B10 shelves (22% incidence of steroid-induced fetal cleft palate (20)).

Corticosteroids cause a delay in the elevation of A/J and CD-1 palatal shelves from a vertical to a horizontal position (21, 22). Shelf elevation is a normal developmental event and its delay has been suggested to account for the subsequent failure of contact between apposed shelves (23, 24). However, an *in vivo* study by Greene and Kochhar (12) using frozen, cryostat-sectioned fetal heads from steroid-sensitive ICR mice (95% incidence of steroid-induced fetal cleft palate) show that, despite the steroid-promoted delay, palatal shelves make contact but then fail to undergo epithelial breakdown and fusion. In view of these results, we have tested in culture the effects of steroid on medial edge breakdown in single palatal shelves from ICR mice. The data (Table I) indicate that ICR palates largely mimic the *in vitro* re-

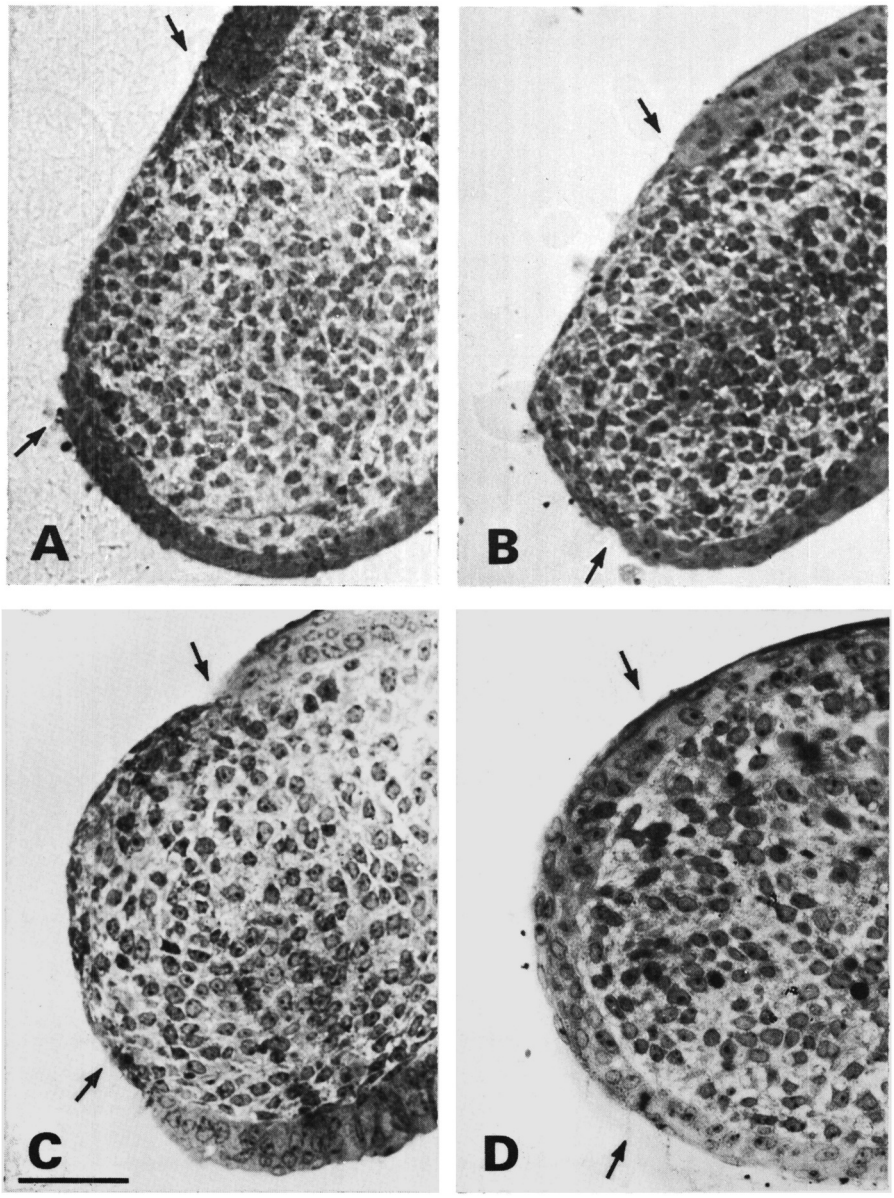

FIG. 2. General histology of palatal shelves cultured with or without steroid for 48 hr. (A) and (B) are the B6 and A/J controls, respectively, in which the epithelium at the medial edge (between arrows) has completely broken down. (C) and (D) are the corresponding B6 and A/J shelves treated with cortisol (0.1 μg/ml) showing loss of the medial edge epithelium (area between arrows) in the palatal shelf from the steroid-resistant (B6) animal and retention of this epithelial population (between arrows) in the shelf from the steroid-sensitive (A/J) mouse. In all figures, the upper surface is oral and the lower surface is nasal. In these studies, epithelial breakdown is defined as the complete loss of at least some epithelial lining at the medial edge. The palatal medial edge is not always maintained precisely in its normal anatomical position in single shelves in culture. For this reason, some of the palatal sections are angled slightly to provide easily compared views of the medial edge areas. The magnification for all figures is the same; the bar in (C) represents 50 μm.

TABLE I. EFFECTS OF CORTISOL ON MEDIAL EDGE EPITHELIAL BREAKDOWN IN SINGLE PALATAL SHELVES *IN VITRO*

	Mouse Strain				
	Dub (ICR)	CD-1	A/J	C57BL/6J(B6)	C57BL/10ScSn(B10)
Control	20/20	20/20	19/19	11/11	21/21
Cortisol (0.01 to 10 μg/ml)	3/16[a]	3/26[a]	2/19[a]	19/19	13/15
Cortisol (0.001 μg/ml)	1/3	2/4	2/4	—	2/2
Cortisol (0.0001 μg/ml)	0/1	5/5	3/3	7/7	3/4

Note. Column entries represent number of cultured shelves with epithelial breakdown per total number of shelves.

[a] These cortisol data are significantly different than the control values ($P < 0.001$, χ^2) the few cases of epithelial breakdown exhibited by the steroid-sensitive strains in this category occurred randomly at cortisol concentrations of 0.01, 0.1, 1, and 10 μg/ml.

sponse to cortisol exhibited by shelves from A/J and CD-1 mice. The ICR findings also prompted us to examine the effects of steroid on normal palatal development in one of the other steroid-sensitive strains (A/J). Using the techniques of the ICR *in vivo* study, we now have evidence that shelf contact occurs in A/J subsequent to a lengthy steroid-promoted delay in shelf elevation (Figs. 3A and B); the percentage of cortisone-treated fetal heads with shelves in contact peaks at about 30% during this period. The lysosomal activity in the steroid-treated A/J shelves in contact is clearly limited to superficial epithelial cells along the medial edge and apparently reduced in amount as well (Figs. 3C and D). However, acid phosphatase may be localized in the Golgi or primary or secondary lysosomes or may be generally distributed in the cytoplasm; its precise intracellular localization could influence the intensity of staining. In preliminary studies with steroid-resistant B10 mice, shelf contact occurs virtually simultaneously in 60 to 70% of both control and cortisone-treated fetal heads. In this instance, the distribution of lysosomal activity in shelves exposed to steroid (Fig. 4B) is similar to that of controls (Fig. 4A).

Discussion. These investigations examine the actions of cortisol *in vitro* on a process of palatal development. In this culture model, cortisol prevents the programmed breakdown of the medial edge palatal epithelium and alters in this epithelial population the synthesis and/or release of lysosomal enzymes. Significantly, these changes in response to cortisol are restricted to palatal shelves from mouse strains with high incidences of steroid-induced cleft palate. It is also of interest that only mouse strains which are sensitive to corticosteroids have high palatal concentrations of cytoplasmic receptors for these steroids (25, 26). It suggests that cortisol-receptor interactions are likely implicated in the steroid inhibition of programmed cell death in this palatal epithelium.

A recent report by Walker and Patterson (22) using living CD-1 fetuses exteriorized from the uterus shows that the fetal membranes and tongue are major obstacles to shelf elevation in corticosteroid-treated mice. Removal of these tissues permits shelves from steroid-treated fetuses as well as controls to move without delay to the horizontal and fuse. This indicates that corticosteroid induces cleft palate by interfering with shelf elevation. However, even under these conditions, shelves exposed to steroid appear to fuse less readily than controls. This result as well as the major conclusions of the Greene and Kochhar ICR *in vivo* study (12) and our A/J and B10 *in vivo* studies suggest that postelevation events may also be important in

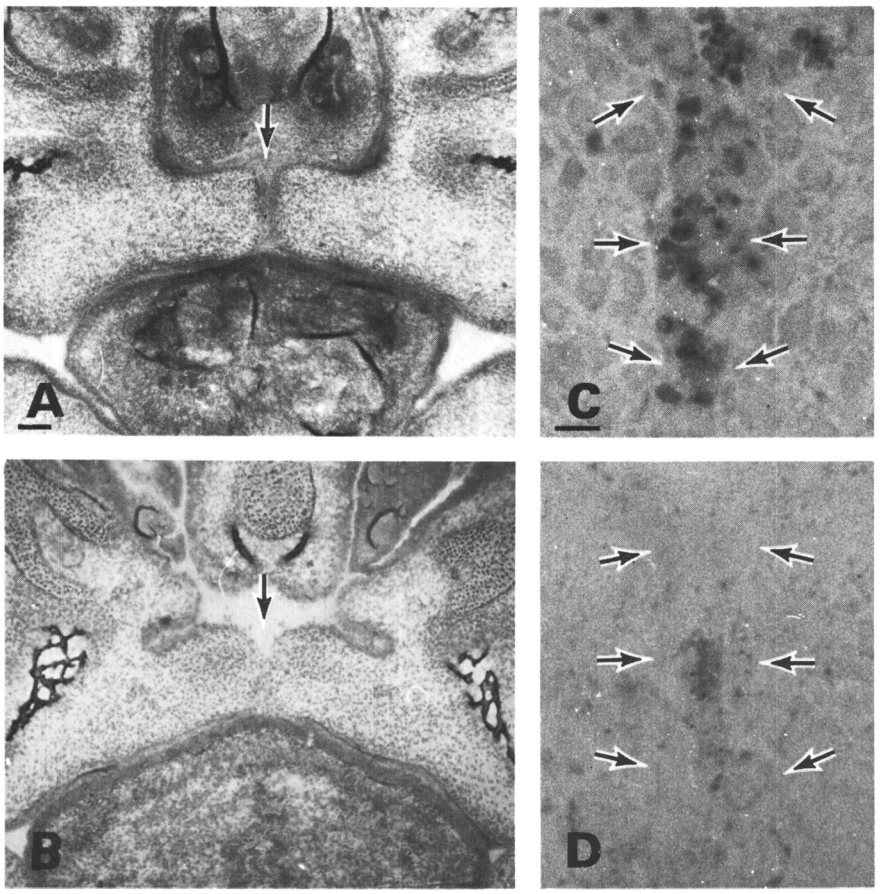

FIG. 3. Frontal sections of frozen heads of fetuses from control and steroid-treated A/J mice. The control section (A) shows the palatal shelves meeting in the midline (arrow) of a 15-day, 2-hr fetus. The section of a steroid-treated fetal head (B) shows shelf contact (arrow), after a steroid-promoted delay in elevation, in a 16-day, 8-hr fetus. (A) and (B) typify the arrangement of the shelves along the middle (anterior–posterior) third of the palate (see Materials and Methods). (C) and (D) are views of the palatal midline from control and steroid-treated fetuses at slightly later stages. These sections are stained for acid phosphatase and show that enzyme activity is distributed throughout the medial edge epithelial seam (between arrows) in controls (C) and is apparently reduced and restricted to superficial cells of the medial edge epithelia (between arrows) in fetuses exposed to steroid (D). Palatal shelves from A/J controls make contact within a 5-hr period beginning at 15 days 2 hr, and then apparently fuse. Cortisone-treated A/J heads with shelves in contact appear from about 15 days 16 hr, when they account for 24% of the total to as late as 16 days 8 hr (3%). Throughout this period, the remaining steroid-treated heads have shelves positioned either vertically (15 to 26%) or horizontally but not touching (52 to 82%); the latter category includes elevating shelves as well. There are no heads with fused shelves. The abnormal distribution of acid phosphatase activities in the medial edge epithelia (D) typifies the altered development of the lysosomal enzyme in shelves in contact throughout this period of steroid delay. These studies are based on the examination of 140 control and cortisone-treated fetal heads. The bars in Figs. 3A and 3C represent 100 μm and 10 μm respectively. The magnification for (A) and (B) is the same; similarly the magnification for (C) and (D) is the same.

steroid-promoted clefting. Furthermore, this possibility is consistent with the findings from our *in vitro* model which suggest that the inhibition of epithelial breakdown by cortisol very likely contributes, at least in part, to the failure of fusion. In any event, the inhibition of programmed cell death, like the delay of shelf elevation, clearly correlates with susceptibility to steroid-induced cleft palate.

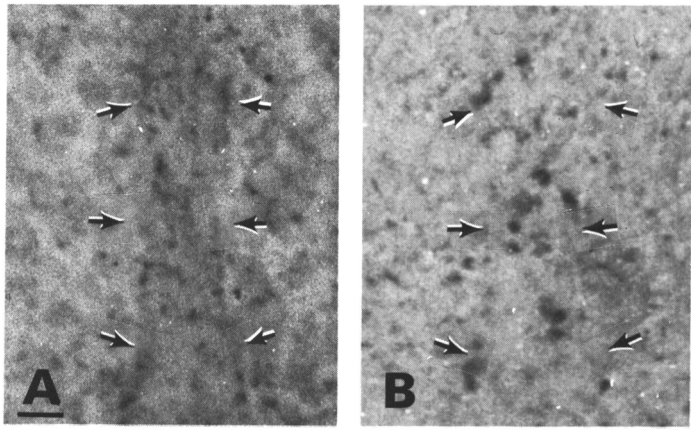

FIG. 4. Frontal sections of the palatal midline in frozen heads from 14-day, 18-hr B10 mice. Acid phosphatase activities are distributed throughout the medial edge epithelia (betweeen arrows) in both controls (A) and fetuses exposed to steroid (B). These preliminary studies are based on the examination of 41 control and cortisone-treated fetal heads. The magnification for (A) and (B) is the same; the bar in (A) represents 10 μm.

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