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Zellweger Syndrome Amniocytes: Morphological Appearance and a Simple Sedimentation Method for Prenatal Diagnosis

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ABSTRACT. Zellweger syndrome is the prototype of a growing group of genetic diseases caused by an absence or deficiency of peroxisomes. The defect causes the enzyme catalase to remain in the cytosol instead of being packaged into peroxisomes. This mislocalization can be easily detected by sedimentation analysis. Amniocytes were homogenized and then centrifuged to pellet organelles. Catalase was found to sediment with the peroxisomes in the homogenates of normal cells, but to remain in the supernatant with Zellweger syndrome amniocyte homogenates. This striking difference is unambiguous and reproducible, and provides a simple method for prenatal diagnosis. Moreover, it allows one to differentiate diseases in which peroxisomes are deficient from other peroxisomal diseases in which the organelle is intact, but one enzyme is defective. Electron microscopic observations support the biochemical determinations. Normal amniocytes contain small peroxisomes in which a weak cytochemical reaction for catalase may be demonstrated. Zellweger amniocytes appear to lack these organelles, although some cells have rare structures that might be residual or abnormal peroxisomes. (*Pediatr Res* 24:63–67, 1988)

Abbreviation

ALD, adrenoleukodystrophy

Recently several sets of methods have been developed for the prenatal diagnosis of the Zellweger cerebro-hepato-renal syndrome, an autosomal, recessive, fatal disorder associated with multiple congenital defects and CNS dysfunction (1). Most of these diagnostic methods depend either on the demonstration of impaired plasmalogen synthesis (2–5) or of abnormally high levels of very long chain fatty acids (6, 7). Both of these abnormalities are thought to be secondary to deficiencies of enzymes normally located in the peroxisome (8, 9). Peroxisomes have been shown to be greatly diminished or absent in the Zellweger syndrome (10) and a related disorder, neonatal adrenoleukodystrophy (11). These findings have led to the hypothesis that there exists a group of peroxisomal disorders, analogous to the better known lysosomal disorders. Evidence is developing that the peroxisomal disorders are heterogeneous: some, such as X-linked ALD, may involve the deficient activity of a single peroxisomal enzyme (12–14), whereas in others several peroxisomal enzymes are deficient, usually in association with the absence or deficiency of peroxisomal structures (6, 11). In addition, there are several recent reports of patients with some aspects of the Zellweger syndrome phenotype but in whom liver peroxisomes were normal in size and structure (15–17). In one of these patients, two peroxisomal oxidases were initially reported to be deficient (16) and subsequently a thiolase deficiency was found (17). The existence of this heterogeneity presents a challenge to the prenatal diagnosis of peroxisomal disorders, because it is possible that in some affected fetuses one or more of the current prenatal assays might not reveal abnormalities.

Herein we present an approach to the prenatal diagnosis of Zellweger syndrome that is based on a different principle than most of the existing analytical or enzyme assays. This approach measures by sedimentation analysis the subcellular distribution of catalase, an enzyme that normally is located mainly in the peroxisomal matrix but in Zellweger syndrome is mainly cytosolic. A related approach was employed by Wanders *et al.* (18), who determined the subcellular location of catalase in amniocytes by assaying its latency as a function of digitonin concentration. The procedure described herein provides a convenient and readily quantifiable chemical measure of membrane-bounded catalase. Over and above its diagnostic value, this technique may prove of general interest for the study and understanding of peroxisomal disorders. We also investigated the ultrastructural appearance of peroxisomes in amniocytes, which has not been previously described except for a brief symposium report (19). In an attempt to correlate our biochemical data with ultrastructure, we carried out electron microscopic cytochemistry of peroxisomes on all the amniocyte samples.

METHODS

Tissue culture methods. Amniotic fluid cells, obtained from pregnancies at risk for Zellweger's syndrome or X-linked ALD, were diagnosed by their high content of very long chain fatty acids (C26/C22) (6). Control cells were from routine amniocentesis of pregnancies with advanced maternal age which showed normal cytology. The amniocytes, which had been stored in liquid nitrogen after 2 to 4 passages, recovered well.

Monolayer cultures of amniocytes were grown to ~90% confluency in Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum in 80 cm^2 tissue culture flasks at 37° C in a humidified environment with 5% CO_2. Cells were detached from the flasks by trypsinization, suspended in phosphate-buffered saline containing trypsin inhibitor (GIBCO, Grand Island, NY), and washed three times by centrifugation.

Homogenization and fractionation of cells. Cells from 1 to 3 flasks were homogenized in 500 μl of 0.25 M sucrose, 5 mM

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imidazole, pH 7, and 0.1% ethanol by means of 50–100 strokes of a Dounce homogenizer with a tight-fitting pestle. One flask of cells would be more than sufficient for routine diagnoses. In one experiment, cells were suspended in the above medium and gently sonicated at 4° C in an L & R sonicating water bath 21 times for 15 s each (with 10-s rests in between).

Half of the sample was centrifuged for 30 min at 10,000 rpm in a Sorvall HB-4 rotor with adapters for microfuge tubes. The supernatant was pipetted off and the pellet was resuspended in the starting volume of buffered sucrose.

Assays. Catalase was assayed by the disappearance of H_2O_2 at 0° C (20). The sample (20 μl) was first mixed with an equal volume of 2% Triton X-100 in order to disrupt membranes. The reaction was started by the addition of 1 ml of ice cold substrate, consisting of 2 mM H_2O_2 in 20 mM imidazole buffer, pH 7, with 1 mg/ml of bovine serum albumin. The reaction was stopped after 15 min with 1 ml of $TiOSO_4$ in 1.3 N H_2SO_4 which forms a yellow complex with the remaining H_2O_2. After 10 min or more of color development at room temperature, the yellow complex was measured spectrophotometrically at 410 nm. The reaction has first order kinetics and a Q_{10} of 1.1. One unit of activity is defined as the amount of enzyme that causes a 10-fold decrease in the H_2O_2 concentration/min at 0° C in a volume of 50 ml (after the old "Kat. f." unit) (20). Thus: U/ml = $\log_{10}$(initial A_{410}/final A_{410}) × (reaction volume/50 ml) × (sample volume^{-1}) × (reaction time^{-1}). The initial A_{410} was determined with water in place of sample. The reaction time may be varied from 1 min to 1 h and the sample volume may be varied from 5 to 50 μl (always using an equal volume of detergent). The $TiOSO_4$ was prepared by stirring 7 g of $TiOSO_4$ (ICN K + K Laboratories, Plainview, NY) in 1 liter of 2 N H_2SO_4 on a heating stirrer until the suspension reached the boiling point. After gradual cooling and settling of undissolved material, the supernatant was filtered (if not perfectly clear) and diluted with 0.5 liter of 2 N H_2SO_4 (21).

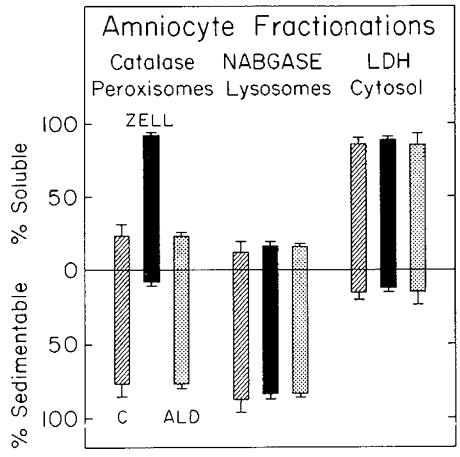

Fig. 1. Subcellular localization of catalase in amniocytes. Cells were homogenized and centrifuged as described in "Methods." Catalase and marker enzymes were assayed on homogenates, pellets, and supernatants; the recoveries are given in Table 1. *NABGASE*, N-acetyl-β-glucosaminidase, a lysosomal marker; *LDH*, lactate dehydrogenase, a cytosol marker; *C*, control; *Zell*, Zellweger; *ALD*, X-linked ALD.

N-acetyl-β-glucosaminidase was assayed by measuring the liberation of *p*-nitrophenol from *p*-nitrophenyl-2-acetyl-β-D-glucosamide (22). The reaction mixture contained, in a final volume of 1 ml, 1.5 mM substrate, 0.2 M sodium citrate, pH 4.5, and 0.01% Triton X-100. The reaction was started by the addition of amniocyte sample (5μl) and was incubated for 60 min at 37° C in a shaking water bath. The addition of 1 ml of 0.5 M sodium hydroxide stopped the reaction and the absorbance was measured at 410 nm (E = 17 M^{-1} cm^{-1}).

Lactate dehydrogenase was assayed at 37° C in the reverse direction by following the oxidation of NADH at 340 nm with pyruvate as substrate. The 1-ml reaction mixture contained 0.5 mM pyruvate, 30 mM phosphate buffer, pH 7.4, 0.14 mM NADH, 0.01% Triton X-100, and 5 μl of amniocyte sample. E = 6.4 mM^{-1} cm^{-1}. Protein was determined according to Lowry *et al.* (23) with bovine serum albumin as standard.

Morphology and cytochemistry. Cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 15–30 min at 4° C, and then washed with cacodylate buffer. They were incubated in the 3,3′-diaminobenzidine medium for the cytochemical demonstration of catalase (24, 25) as described previously (26) except that incubation was for 1 h at a temperature of 45° C as recommended for human samples (27). Subsequent processing for electron microscopy was as described (26) except that the sections were viewed either unstained or lightly contrasted with lead citrate.

Table 1. *Enzyme activities in amniocytes broken by homogenization**

	n	Catalase (mU/mg protein)	N-acetyl-β-glucosaminidase† (mU/mg protein)	Lactate dehydrogenase (mU/mg protein)
Control	5	3.2 ± 1.8	59 ± 42	1.5 ± 0.2
Zellweger	6	5.9 ± 1.4	48 ± 14	1.6 ± 0.3
ALD	3	2.9 ± 1.0	33 ± 7	1.8 ± 0.2
Recoveries‡		112 ± 13%	111 ± 13%	117 ± 12%

* Mean specific activities ± SD of homogenates; total protein ranged from 1–4 mg. Companion data to Figure 1.

† $n = 4$ for control and Zellweger samples.

‡ (Pellet + supernatant)/homogenate × 100%; average for all amniocytes.

Table 2. *Centrifugation of amniocytes broken by sonication*

	Soluble (nonsedimentable) activity*			
	Catalase (peroxisomes) (%)	Lactate dehydrogenase (cytosol) (%)	N-acetyl-β-glucosaminidase (lysosomes) (%)	Catalase/lactate dehydrogenase (%)
Control	7	64	6	11
ALD 1	29	88	23	33
Zellweger 1	61	73	15	84
Zellweger 2	64	69	7	93

* Percent of soluble plus sedimentable activities. Recoveries were 70–131%.

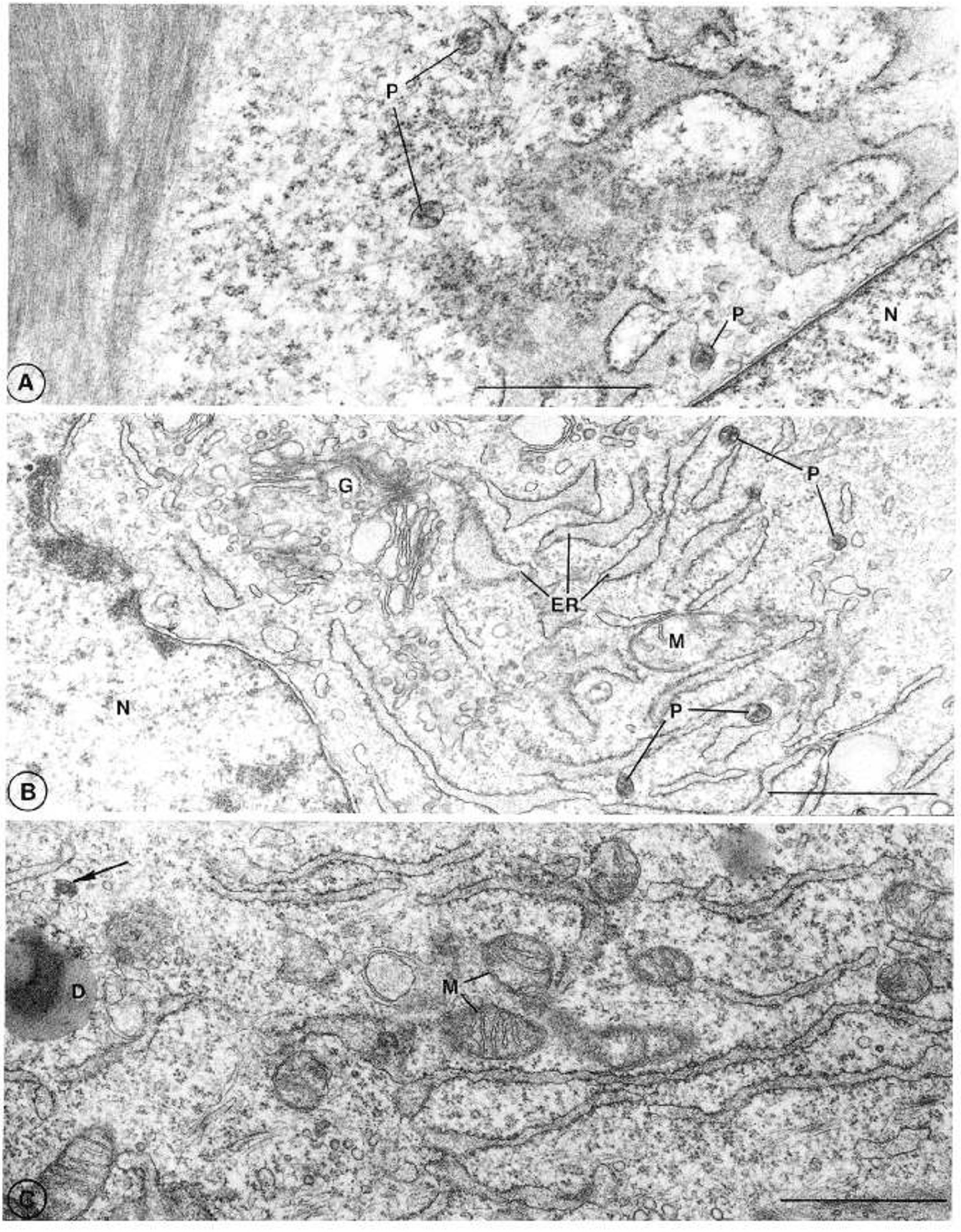

Fig. 2. Electron microscopic cytochemistry. *A*, control amniocyte. *B*, X-linked ALD amniocyte. *C*, Zellweger syndrome amniocyte. The cytochemical procedure to demonstrate catalase activity was carried out as described in "Methods." *P*, peroxisome; *M*, mitochondrion; *N*, nucleus; *G*, Golgi apparatus; *ER*, endoplasmic reticulum; *D*, lipid droplet. *Arrow* indicates an unknown structure in the Zellweger amniocyte that contains some electron-dense material which may be diaminobenzidine. Magnification, 31,000; *bar*, 1 μm.

RESULTS

Biochemical studies. Amniocytes were homogenized and centrifuged in order to pellet nuclei, mitochondria, peroxisomes and lysosomes (as well as any unbroken cells). Control measurements of the cytosolic marker enzyme, lactate dehydrogenase, demonstrated that at least 85–88% of this enzyme was recovered in the supernatant fraction (Fig. 1) indicating that most of the amniocytes were broken by the homogenization procedure. Control assays of the lysosomal marker enzyme, N-acetyl-β-glucosaminidase, verified that the lysosomes were mostly not damaged by the homogenization, and were recovered to the extent of 84–88% in the sediments in the several amniocyte groups (Fig. 1). The recoveries of these enzymes were satisfactory (Table 1). There was no difference in the behavior of these enzymes between the control and disease groups. Thus these experimental conditions are appropriate to evaluate the presence or absence of peroxisomes.

Catalase, a marker enzyme for peroxisomes, was 77 ± 8% sedimentable in the five controls, but was 92 ± 2% soluble in the six Zellweger samples. In the Zellweger homogenates, sedimentable catalase (8 ± 2%) was less than or equal to sedimentable lactate dehydrogenase (12 ± 3%); thus the small amount of sedimentable catalase can be accounted for by unbroken cells or by residual supernatant remaining over the pellet. The sedimentability of catalase in three X-linked ALD amniocyte samples was indistinguishable from the controls (Fig. 1). The recoveries were 112 ± 13% (Table 1).

Total catalase activity in the Zellweger amniocytes was nearly twice that in the control and X-linked ALD samples ($p < 0.05$) (Table 1). This suggests that catalase in the cytosol is degraded more slowly than catalase within peroxisomes, which normally turn over by autophagy (28). The specific activities of the other enzymes measured were similar in all three groups.

One group of amniocytes was subjected to sonication, which under appropriate conditions has been shown to open fibroblasts without excessive damage to cell organelles (29). The sonicated amniocytes were centrifuged in the same fashion as the homogenized amniocytes. As shown in Table 2, sonication released the bulk of the cytosolic marker enzyme whereas most of the lysosomal marker sedimented. Catalase was largely soluble in the Zellweger samples and mostly sedimentable in the control and X-linked ALD samples.

Morphological observations. Peroxisomes were present in control amniocytes (Fig. 2*A*). They were generally 0.1–0.2 μm in diameter and had a coarsely granular content. They were identified by a positive cytochemical reaction for catalase, which produced patches of electron-dense diaminobenzidine oxidation products within the organelle. There were approximately 20 peroxisomes/1000 μm^2 of cytoplasm. The peroxisomes were frequently found in clusters, such that some cell sections showed several whereas many micrographs had none. These clusters often occurred in the vicinity of the endoplasmic reticulum, although connections between peroxisomes and endoplasmic reticulum were not seen.

Amniocyte peroxisomes differ from those of rat liver in that they are smaller, much less abundant, lack crystalloid cores, and give a cytochemical reaction for catalase that is considerably less intense and does not always fill the organelle. In these respects, the peroxisomes of amniocytes resemble those of adrenal cortex (30), heart (31), intestine (32), and cultured skin fibroblasts (29, 33).

Peroxisomes in the amniocytes of X-linked ALD patients (Fig. 2*B*) were similar to those in amniocytes of normal individuals in size and in number. Organelles with the morphological and cytochemical characteristics of the peroxisomes seen in ALD and normal individuals were not found in Zellweger amniocytes (Fig. 2*C*). However, some possibly diaminobenzidine-positive structures were observed: they were generally smaller than the typical amniocyte peroxisomes and were much less numerous. Because of their rarity, small size, and generally less intense staining, they could not be identified unambiguously. In control cells, ALD cells, and Zellweger cells, autophagolysosomes were observed that contained electron-dense material (diaminobenzidine?) (not shown). Such autophagolysosomes appeared to be more numerous in Zellweger cells.

DISCUSSION

We have found a simple and reproducible method of assessing the presence of peroxisomes in cultured amniocytes. Catalase, a peroxisomal enzyme, is mislocalized to the cell cytosol when the organelle is missing. Hence when a homogenate is centrifuged in order to pellet peroxisomes, the catalase is not sedimentable but rather remains in the supernatant (Fig. 1). This provides an easy means for the prenatal diagnosis of Zellweger syndrome. It should be equally applicable to other diseases where peroxisomes are deficient, such as neonatal ALD and infantile Refsum's disease.

There are several advantages to this method. First, catalase is a stable enzyme, in contrast to some others that have been used for prenatal diagnosis. The only necessary precaution is to include 0.1% ethanol in the homogenization medium; the ethanol is known to maintain catalase in active form (34). Catalase is simple to measure and requires no radioactive substrates, lipid extractions, or high pressure liquid chromatography. The assay used herein is an easy colorimetric endpoint assay, and does not require the oxygen electrode and polarograph used elsewhere (18). The homogenization itself is easy to perform and in our experience the sedimentability of catalase in homogenates provides a reliable measure of the presence of peroxisomes in cells. Assay of the two marker enzymes, lactate dehydrogenase (cytosol) and N-acetyl-β-glucosaminidase (lysosomes), is an important control to ensure that the homogenization has ruptured the cells but not the organelles. With these controls one can be confident of the results on an individual amniocyte sample.

The electron microscopy documents the presence of a modest number of small, coreless peroxisomes in normal and X-linked ALD amniocytes. The low intensity of the cytochemical reaction probably reflects the fact that the specific activity of catalase in normal amniocytes (3.2 mU/mg) is 100 times less than that in rat liver. The morphological deficiency of peroxisomes in Zellweger amniocytes agrees with the biochemical results on catalase. The morphological results are consistent with similar reports of a deficiency of peroxisomes in fibroblasts cultured from the skin of Zellweger patients (29, 33). The rare small structures in Zellweger amniocytes that might contain diaminobenzidine reaction product could represent residual aberrant peroxisomes, but this could not be decided unequivocally. Because the cytochemical reaction product in the normal amniocyte peroxisomes is of low intensity (compared to rat liver) and has patchy distribution, and because the organelles occur in clusters (such that many micrographs show none), we would not attempt to do prenatal diagnosis by electron microscopy.

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