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AN IMPROVED METHOD FOR PREPARING MICROVASCULAR CORROSION CASTS OF RAT EMBRYOS

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Abstract

This paper presents an improved method for preparing microvascular corrosion casts of rat embryos (day 16.5-21.5). Special attention was paid on the viscosity of the casting material, the method of mechanical restraint, and the subsequent drying of the casts. The embryos were perfused with Mercox resin diluted with 25% methyl methacrylate monomer followed by undiluted Mercox resin to avoid outflow of resin from the vasculature after perfusion. The specimens were mounted on a stainless steel plate with self-curing resin to prevent flotation, mechanical damage, and collapse of the cast specimens following preparation. After digestion and washing, the cast specimens were freeze dried to prevent deformation of the casts.

This specimen preparation resulted in much better scanning electron microscopic images of the embryonic microvasculature of various oral tissues, free from leakage and insufficient filling.

Key Words: Vascular corrosion cast, Blood vascular supply, Scanning electron microscope, Rat embryos, Prenatal development, Embryology, Dental papilla, Tongue, Secondary palate.

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Introduction

Scanning electron microscopy (SEM) of vascular corrosion casts has become a standard method for studying the fine distribution of blood vessels in many organs and tissues (Taniguchi et al., 1952, 1955; Murakami, 1971, 1978; Nowell and Lohse, 1974; Gannon, 1978; Clark et al., 1979; Hodde and Nowell, 1980; Murakami et al., 1983; Lametschwandtner et al., 1984, 1990; Ohta et al., 1990). However, there are few reports of prenatal microvasculature of experimental animals. This may be due to the extreme difficulty in handling delicate capillaries and the methacrylate resin casts of embryonic animals.

Yoshida et al. (1987) introduced a method for the preparation and handling of corrosion casts of embryonic animals, and the prenatal microvasculature of various oral tissues of rat embryos have been examined with modifications to the original method (Yoshida et al., 1988, 1989; Chiba, 1988; Yoshida, 1991). The present paper introduces details of the improved method for preparing microvascular corrosion casts of embryonic tissues with prenatal rats as the model.

Materials and Methods

Animals

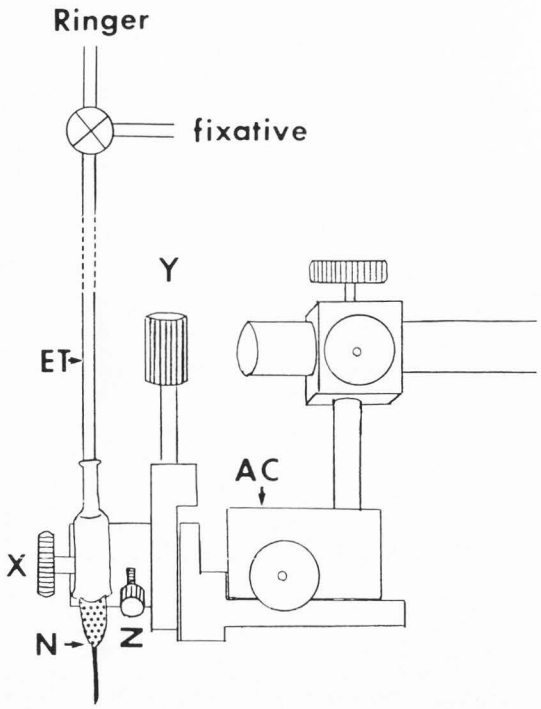
Sixteen embryos from 4 young pregnant female Wistar white rats (day 16.5, 18.5, 19.5, and 21.5, four embryos at each stage) were used in this study.

Microperfusion apparatus

The microperfusion apparatus consisted of five parts: an optical stereomicroscope, a fiber optic light source, an adjustable clamp, an extension tube, and a 27 gauge injection needle (Fig. 1).

Injection of Mercox resin

The pregnant rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Next the embryos were removed from the uterus of the pregnant rats and anesthetized similarly and fixed with iron pins on a cork board. The thoracic cavities were opened and the injection needle for perfusion was carefully inserted into the ascending aorta through the



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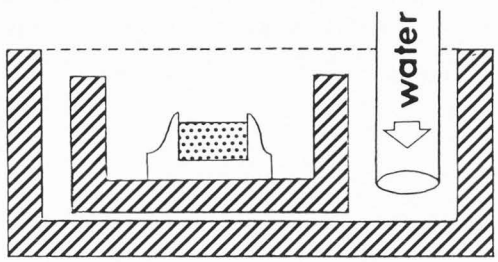

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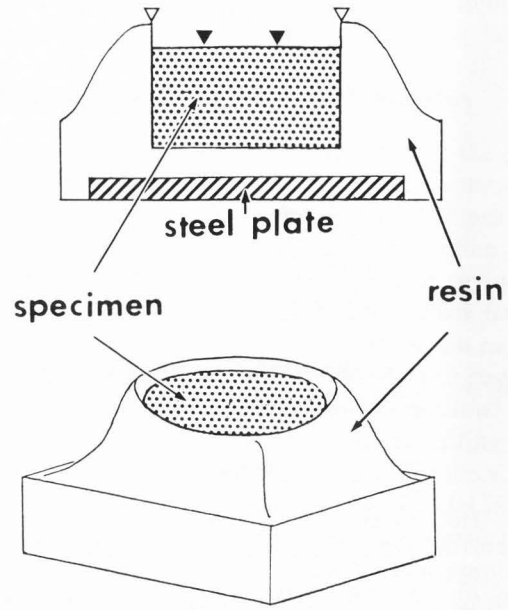

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Fig. 1. Microinjection apparatus for SEM study of prenatal microvascular casts. AC: adjustable clamp, ET: extension tube, N: injection needle. The tip of the needle can be moved three-dimensionally with the three screws (X, Y, Z) of the adjustable clamp. Bar=3 cm

Fig. 2. The specimens are mounted on a thin stainless steel plate with self-curing acrylic resin, to prevent damage during preparations. The top edge of the self-curing acrylic resin (open arrow heads) is higher than the level of the cast specimen (solid arrow heads).

Fig. 3. Cast specimens are washed in double glass bottles to prevent the direct action of the water flow.

left ventricle while controlling the adjustable clamp three dimensionally under the optical stereomicroscope.

The embryos were first perfused with Ringer solution (20-30 ml) and 4% paraformaldehyde in 0.1 M phosphate buffer (15-20 ml, pH 7.4) under a pressure of 30-40 mmHg, and next perfused with Mercox resin (Japan Vilene Co.) diluted with 25% monomeric methyl methacrylate ester (5-10 ml, Ohtani and Murakami, 1978) followed by undiluted Mercox resin (5 ml) under hand pressure (below 100 mmHg). Immediately following this, the neck of each animal was tied with a thick thread to avoid outflow of resin, and the embryos were left at room temperature for 5 hrs to allow the resin to polymerize.

Digestion, Washing and Drying

After polymerization, the tongues and upper jaws were dissected out carefully with a razor blade. The dissected specimens were mounted on a thin stainless steel plate with self-curing acrylic resin (Unifast, GC Japan). The top edge of the self-curing resin was placed 0.5-1.0 mm above the specimens (Fig. 2). They were then digested in 20% KOH solution at 40 °C for 24 hrs in a 50 ml glass container without shaking. The solution was changed 3-4 times during digestion. The resulting casts were washed thoroughly in running tap water in double glass bottles for 12 hrs (Fig. 3) and the water on the casts was removed in a freeze drier (Poralon E-5300).

Microdissection

After drying, the casts were mounted with adhesive tape on specimen holders with a surrounding resin base. To expose the vessels of the dental papilla, upper jaws were microdissected under an optical stereomicroscope with sharpened needles or forceps, after drying. They were coated with gold in an ion sputter coater (Eiko IB-3) and examined under a scanning electron microscope (Hitachi S-2300 or S-4000) at an accelerating voltage of 5-10 kV.

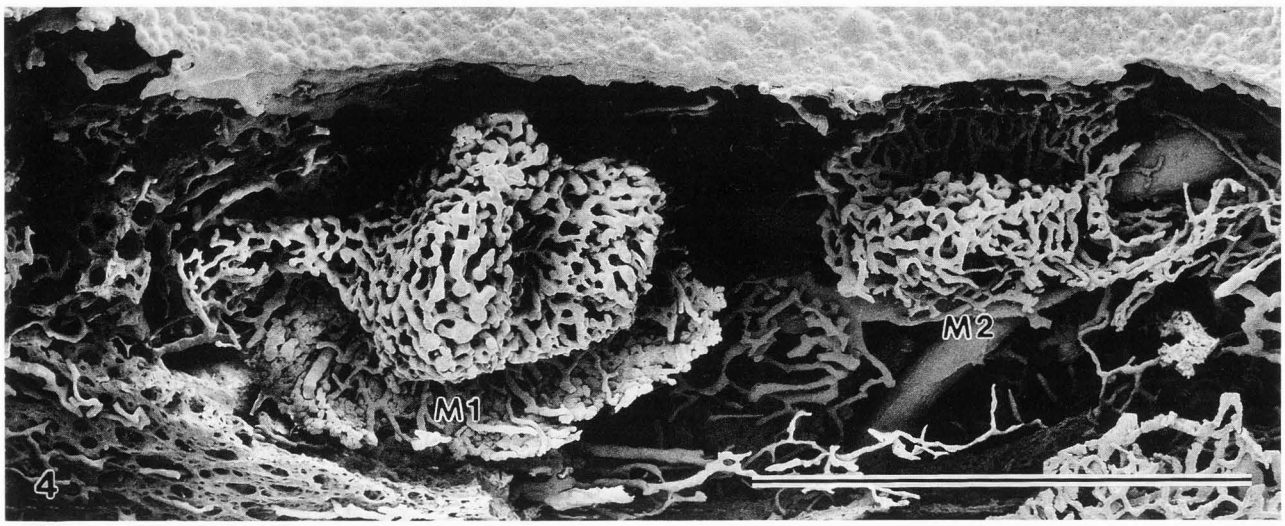


Fig. 4. Dental papilla of a 19.5 day embryo. The capillary networks of the maxillary first (M1) and second (M2) molar tooth germs are completely filled with resin. Bar=1 mm.

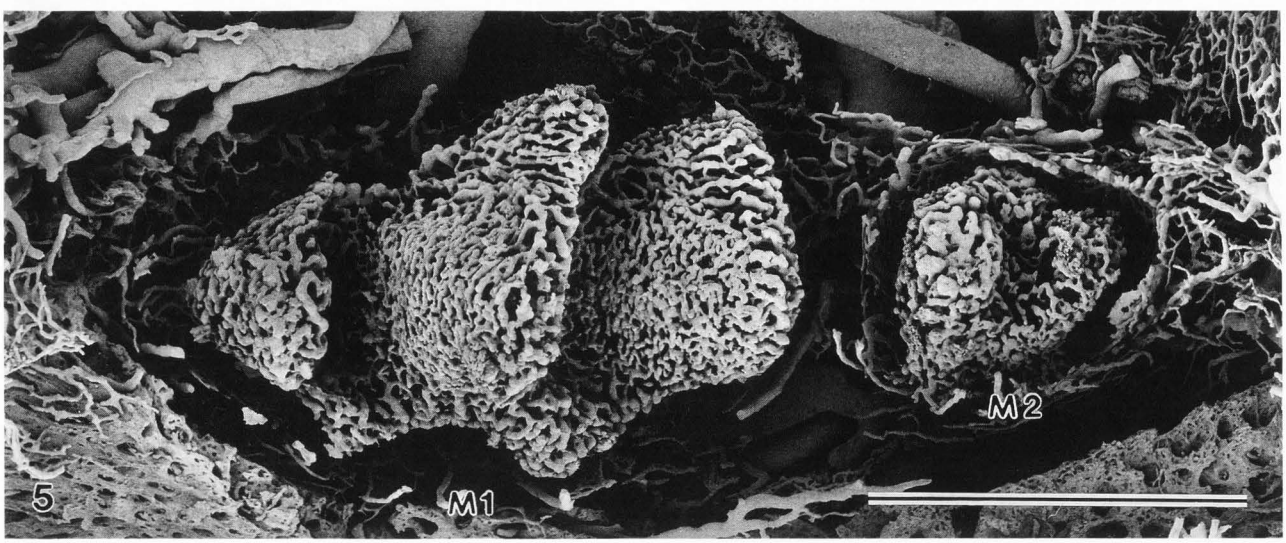


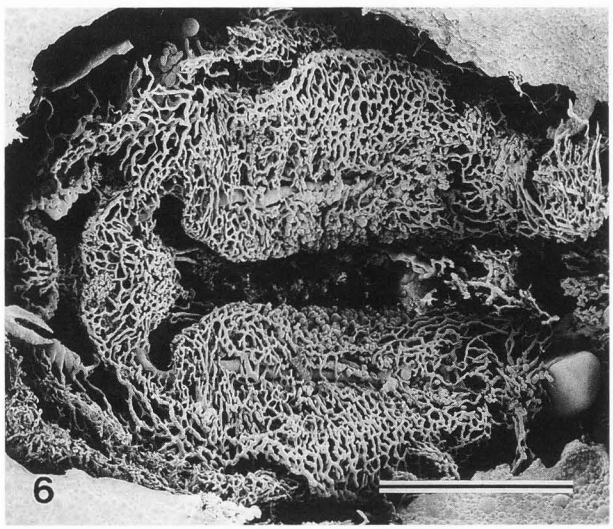
Fig. 5. Dental papilla of a 21.5 day embryo. M1: maxillary first molar, M2: maxillary second molar, Bar=1 mm.

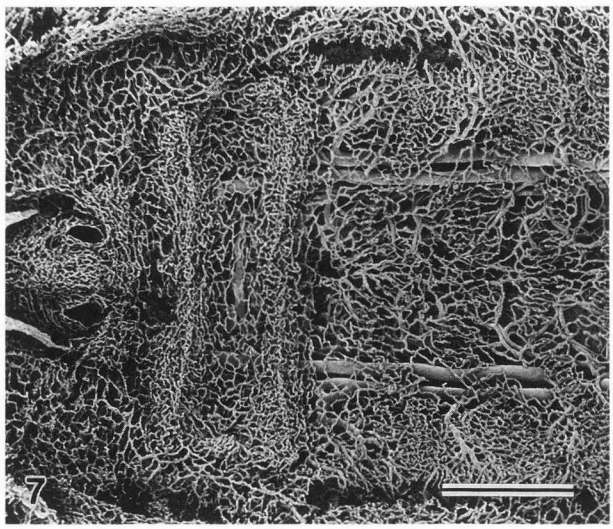
Results and Discussion

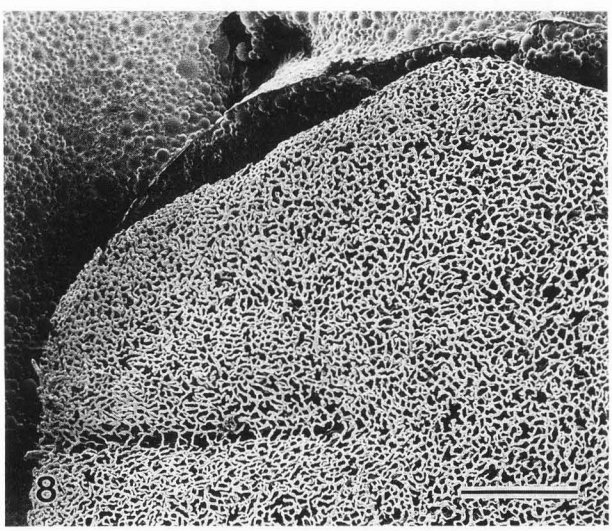
Capillary networks in the dental papilla (Figs. 4, 5), secondary palate (Figs. 6, 7) , and dorsal surface of the tongue (Fig. 8) in rat embryos (day 16.5-21.5) were clearly demonstrated with the preparation method described here.

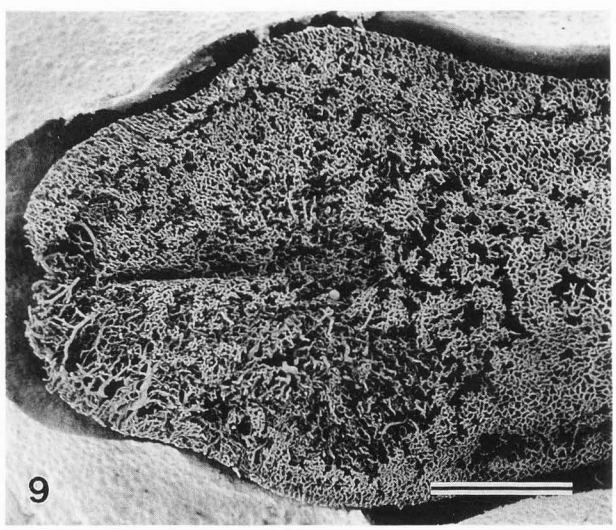
The microperfusion apparatus used here is the same as that in the original method (Yoshida et al., 1987), and it allows easy perfusion of rat embryos. Here, as in previous studies (Yoshida et al., 1988, 1989; Chiba, 1988; Yoshida, 1991), the rat embryos were perfused through the heart, which was thought to be simpler than through the umbilical artery or vein used by other investigators (Pexieder, 1981; Moscoso and Pexieder, 1990). However, it has not been possible to perfuse embryos younger than 15 days in this manner, as their hearts are too undeveloped to allow perfusion.

The original method perfused rat embryos with Mercox resin diluted with 25% monomeric methacrylate monomer, however this occasionally resulted in insufficient perfusion, possible due to resin outflow from the vasculature after perfusion (Fig. 9). To overcome this, embryos were perfused first with diluted Mercox resin, followed by undiluted Mercox resin, and this









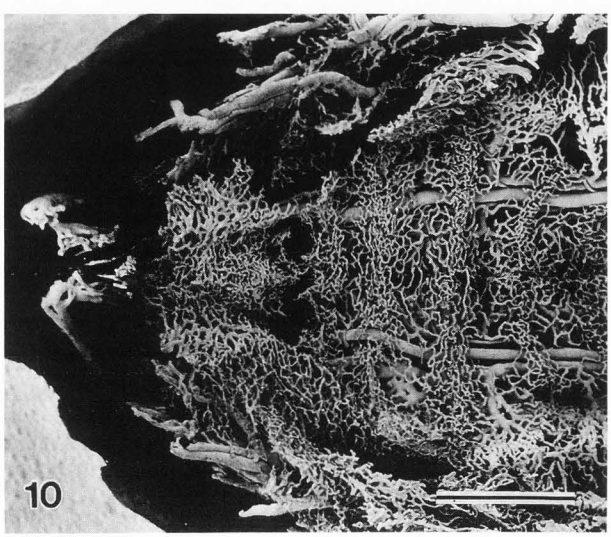


Fig. 6. Secondary palate of a 16.5 day embryo. Bar=1 mm.

Fig. 7. Secondary palate of a 21.5 day embryo. Bar=1 mm.

Fig. 8. Dorsal surface of the tongue of a 21.5 day embryo. Capillary beds of the tongue are completely filled with resin. Bar=500 μm.

Fig. 9. Insufficient filling of the microvessels in the dorsal surface of the tongue of a 19.5 day embryo, possibly due to outflow of the resin after perfusion. Bar=1 mm.

Fig. 10. Damaged cast of microvessels in the secondary palate of a 18.5 day embryo due to insufficient mechanical restraint by the self-curing acrylic resin. Bar=1 mm.

procedure has proven superior.

In our method, the dissected specimens are mounted with self-curing acrylic resin on a thin stainless steel plate (Fig. 2), to help prevent flotation of the specimens during digestion and washing. Self-curing acrylic resin is used to mount the specimens on the stainless steel plate and also to prevent mechanical damage and collapse of the cast specimens. The mechanical restraint is essential for the extremely delicate and fragile cast specimens of embryonic animals. Cast specimens without resin restraint would be destroyed during preparations (Fig. 10). It is recommended to place the top edge of the self-curing resin 0.5-1.0 mm above the cast specimens (Fig. 2) and to wash the cast specimens in double glass bottles to avoid any direct effect of the flowing water (Fig. 3).

Drying, but not necessarily dehydration, is essential for the observations of cast specimens in the scanning electron microscope. With less delicate and fragile casts than the rat embryos here, any drying method could have been acceptable (air drying, CO_2 critical point drying, or freeze drying). We recommend freeze drying with water for the cast specimens of rat embryos, to prevent deformation of the cast specimens; acetone or other organic solvents would dissolve the casts. The water level should be 2 mm or more above the cast specimens and freezing should be rapid.

Even observing these conditions, the delicate casts are occasionally destroyed in the freezing. Using t-butyl ethanol as the medium for freeze drying may overcome this. As methyl methacrylate casts dissolve slowly in t-butyl ethanol, it may take longer than 30 min, such casts should be dehydrated through a series of ethanol, before being placed in absolute t-butyl ethanol and freeze dried.

It is concluded that the changes in specimen preparation resulted in much better scanning electron microscopic images of the embryonic microvasculature of various oral tissues, free from leakage and poor filling.

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Discussion with Reviewers

T. Pexieder: Is it correct that after the pregnant female you have anesthetized also every individual embryo/fetus? If yes, please explain the rationale of doing so.
Authors: Yes, we anesthetized embryos after the removal from the uterus of the pregnant rats, as the anesthetic was considered not to have fully anesthetized the embryos.

T. Pexieder: Why have you selected the pressure of 30-40 mmHg for the perfusion fixation?
Authors: Because the vascular system of rat embryos is very delicate and fragile. In our experience, the 30-40 mmHg pressure is adequate to remove blood from the vasculature of rat embryos.

T. Pexieder: What does freeze drying with water mean?
Authors: It means that we used water as the medium for freeze drying, because acetone or other organic solvents would dissolve the casts.

T. Pexieder: Your explanation of microvasculature filling failure when using concentrated Mercox as a result of "outflow of the resin", is not compatible with the improvement of the filling after diluted Mercox resin followed by undiluted one injection. Please comment.
Authors: We do not say that we failed filling the resin when we used concentrated Mercox but used diluted Mercox alone. We used diluted and then undiluted Mercox to ensure filling.

G. J. Burton: What degree of shrinkage do you observe using the thinned resin followed by Mercox? Are endothelial imprints seen at higher power?
Authors: We did not measure the degree of shrinkage, but Mercox resin is reported to reduce volume by 10-20% when hardening. Endothelial imprints were not really observed in our casts.

G. J. Burton: How do you judge whether there is complete or incomplete filling? Have you embedded the injected specimen before digestion to see whether all the vessels present have been filled?
Authors: We have not observed embedded specimens. We identified the filling only by the continuity of the networks in the casts.

G. J. Burton: Your fixative of 4% paraformaldehyde is very hypertonic for such delicate embryonic tissue. Have you considered using glutaraldehyde or a mixture of two to reduce the osmolarity?
Authors: We do not know if 4% paraformaldehyde is hypertonic for embryonic tissues, but we have experienced difficulty in injecting Mercox after perfusion with a mixture of 1% glutaraldehyde and 1% paraformaldehyde, possibly due to contraction and/or hardening of the microvessels.

G. J. Burton: I am surprised that freeze drying from water does not damage the casts due to cracking of the ice block. Were the specimens illustrated dried in this way?
Authors: Yes, all the specimens illustrated in this paper were freeze dried from water. As described in the discussion, however, some cast specimens were destroyed in the process of freezing. It is necessary to find a better medium for freeze drying of the casts.

Y. Ohta: Could you tell us the viscosity of the finally prepared injected plastic, if you can show any relative values comparing with percentage of the glycerin solutions?
Authors: The viscosity of the undiluted Mercox resin corresponds to a 20-30% glycerin solution and the diluted one to a 10-20% glycerin solution.

Y. Ohta: Is there any danger of damage to casts if t-butyl ethanol is used for dehydration?
Authors: Yes, it is dangerous to use a series of t-butyl ethanol for dehydration, because the casts will dissolve when placed in t-butyl ethanol for a long time. As described in the discussion, t-butyl ethanol should be used only immediately before freeze drying.