A scanning electron microscopy study of disturbances in the developing rat molar induced by cyclophosphamide

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Näsman M, Hultenby K, Forsberg C-M. A scanning electron microscopy study of disturbances in the developing rat molar induced by cyclophosphamide. Acta Odontol Scand 1997;55:186–191. Oslo. ISSN 0001-6357.

Scanning electron microscopy was used to study the effect of cyclophosphamide (Cy) on molar development in 18 Sprague–Dawley rats from 15 to 48 days of age after birth. Doses of 30 mg/kg body weight of Cy dissolved in 1 ml 0.9% NaCl were given to the rats at 10 and 13 days of age. Eighteen control rats had injections of 1 ml 0.9% NaCl at the same ages. The most obvious changes in the experimental teeth were found in the developing roots of the first and second molars and in both the crown and roots of the third molar. The roots of the first and second molars were short and showed apical closure in the experimental rats. In addition to the disturbances in crown and root formation, the third molars were also significantly reduced in total size as compared with the third molars in the control rats. ☐ *Antineoplastic agents; tooth abnormalities; tooth root*

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As the survival rate for childhood cancer has increased, more children are likely to suffer any late sequelae of the disease itself and the anti-neoplastic therapy. The dental sequelae of chemotherapy in the growing individual are irreversible. Reported dental defects subsequent to such treatment in young children are microdontia, arrested root development, and disturbances in enamel formation (1–8).

Cyclophosphamide (Cy), an N-mustard derivative, is an alkylating drug widely used as an anti-tumor agent and first described and recommended as an anti-cancer agent in 1958 (9). It reacts with nucleophilic sites in biologic material, above all the guanine bases in double-stranded DNA. The resulting cross-linkings of the double helix inhibits cell division or leads to mutation (10–12). These characteristics make Cy a potent immunosuppressive drug, and as such it is used also to prolong the survival of allografts and in the treatment of autoimmune diseases (13).

The effects of Cy on developing dental tissues have been examined by various methods using the continuously growing incisor of the rat as model organ (14–20). The extent of injury in odontogenesis has proved to be related to the dose of the drug.

In a histologic study using molars from young rats, 15 to 48 days of age, Näsman & Hammarström (21) showed that the most obvious effects of Cy could be seen in the developing pulp of the third molar and in the apical regions of the developing roots of the first and second molars.

The aim of the present investigation was to examine the effects of Cy on molar size and morphology in young rats with the aid of scanning electron microscopy (SEM).

Materials and methods

Six litters of Sprague–Dawley rats of both sexes, with six siblings in each litter, were used for the study. At 10 days of age, 18 rats from 3 litters (mean weight, 26.3 g) received an intraperitoneal injection of 30 mg/kg body weight of cyclophosphamide (Cyclofosfamid®, Lääkefarmos/Farmos, Åbo, Finland) dissolved in 1 ml 0.9% NaCl. Before the injection the rats were anesthetized with a subcutaneous injection of 0.05 ml fentanylfluanisone (Hypnorm®; Vet. Leo, Helsingborg, Sweden). Eighteen control rats (mean weight, 25.7 g) were injected with 1 ml 0.9% NaCl intraperitoneally. A second injection of 30 mg/kg b.w. Cy was given to the experimental groups of rats at 13 days of age. The control rats were injected with 1 ml 0.9% NaCl at the same age. Two, 6, 12, 18, 26, and 35 days after the last injection the rats were weighed, and their furs examined (Fig. 1). After each of these controls three randomly selected experimental and control rats were killed by decapitation, after CO_2 asphyxiation (4 min). The heads were then fixed in Histofix® (buffered 10% formaldehyde). For histological and SEM studies each head was sagittally divided in half following the median suture of the maxilla. The left maxilla was used for a histologic study, which has been published previously (21). The molars of the right maxilla were used in the present SEM investigation.

Preparation for scanning electron microscopy

Right maxillary molars from experimental and control groups were dissected free and were fixed for 24 h in a mixed solution of 2.5% glutaraldehyde and

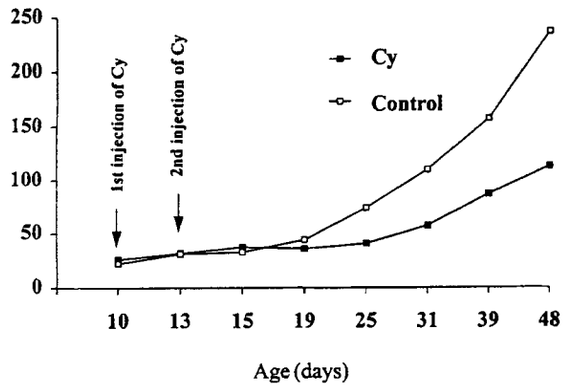

Fig. 1. Graphic presentation of body weights and time for 1st and 2nd injection of the experimental rats (Cy) and the control rats at 10, 13, 15, 19, 25, 31, 39, and 48 days of age.

4% paraformaldehyde dissolved in phosphate-buffered saline (PBS) buffer at pH 7.4. The specimens were rendered partially anorganic by treatment for 60–90 min, using 0.08% pronase (Sigma Chemical Co., St. Louis, Mo., USA) in 0.9% NaCl supplemented with 0.25% $CaCl_2$, 2 H_2O (pH 7.3) at 40°C. After enzymatic treatment, some organic remnants were observable on the root surface, preventing the desired examination of the root surface by SEM. The specimens were therefore treated with 10% sodium hypochlorite for a maximum of 10 min. Longer treatment could result in complete disappearance of the newly formed enamel, dentin, and cementum. The teeth were then rinsed in distilled water, dehydrated with increasing concentrations of ethanol, and air-dried for 24 h.

The specimens were mounted on aluminum stubs with the buccal surface facing upwards. They were coated with 20 nm Au-Pd alloy (Polaron Equipments Ltd, E 5150) and examined in a scanning electron microscope (Philips 501) at an accelerating voltage of 15 kV and tilt angles between 0° and 30°.

The distances from the cementoenamel junction (CEJ) to the apical ends of the mesial root of the first and the distal root of the third molars were measured (Fig. 2a,b).

The approximate total size of the third molar was estimated by comparing experimental and control teeth of the same age and magnification on SEM photographs (Figs. 6a, 7a).

Results

General findings

Two days after the last injection of Cy, the experimental animals began to lose hair. After another 4 days they had lost all their fur. Gradually, however, normal fur was restored. The fur of the control animals was unaffected.

The experimental rats showed only minimal weight gain during the first 2 weeks after the Cy injection. During the remaining period of investigation a stable weight increase was recorded in the Cy-treated rats, but they did not reach the size and weight of the control animals (Fig. 1).

SEM findings

The present investigation was mainly focused on the development of the mesial root of the first maxillary molar and the crown and distal root of the third maxillary molar.

First molar. The morphology of the crowns of the first and second molars was unaffected by the Cy treatment. The preparation of the specimens of these teeth for SEM with pronase and sodium hypochlorite did not have any visible effect on the crowns either. This indicates that the enamel maturation of the crowns of the first and second molars was completed in all rats at the start of the experiment.

Two days after the last injection the formation of the radicular furcation in the control rats gave the roots a definitive and distinct outline with funnel-shaped endings. At this stage of development the root length was still less than the crown height. In the experimental rats the roots of the first molars were shorter than those of the control rats. Furthermore, the affected roots were thinner than normal and tapered in the apical area.

Six days after the last injection the root formation in the control rats had advanced, but the roots were still shorter than the crowns, and the apical foramina were open with no signs of closing. In the experimental rats the apical foramina of the first molar roots were in the process of closing (Fig. 4a,b).

Twelve days after the last injection the root length in the control rats was now approximately the same as the crown height, and the roots were straight with open apices. In the experimental rats the roots were shorter than in the control rats, and the apices were closed.

Eighteen days after the last injection the root length exceeded crown height in the control rats. In the experimental rats the roots were still shorter than the height of the crown.

Table 1. Distances in millimeters (mean values from three measurements of each tooth) from the cementoenamel junction to the apical end of the mesial root of the maxillary first molar in four experimental rats and four control rats of different ages

Age (days)	Experimental group	Control group	Difference (%)*
19	0.60	0.85	70
25	0.94	1.40	67
31	0.90	1.59	57
39	1.23	1.96	63

* Root length in the experimental group in percentage of the root length in the control group.

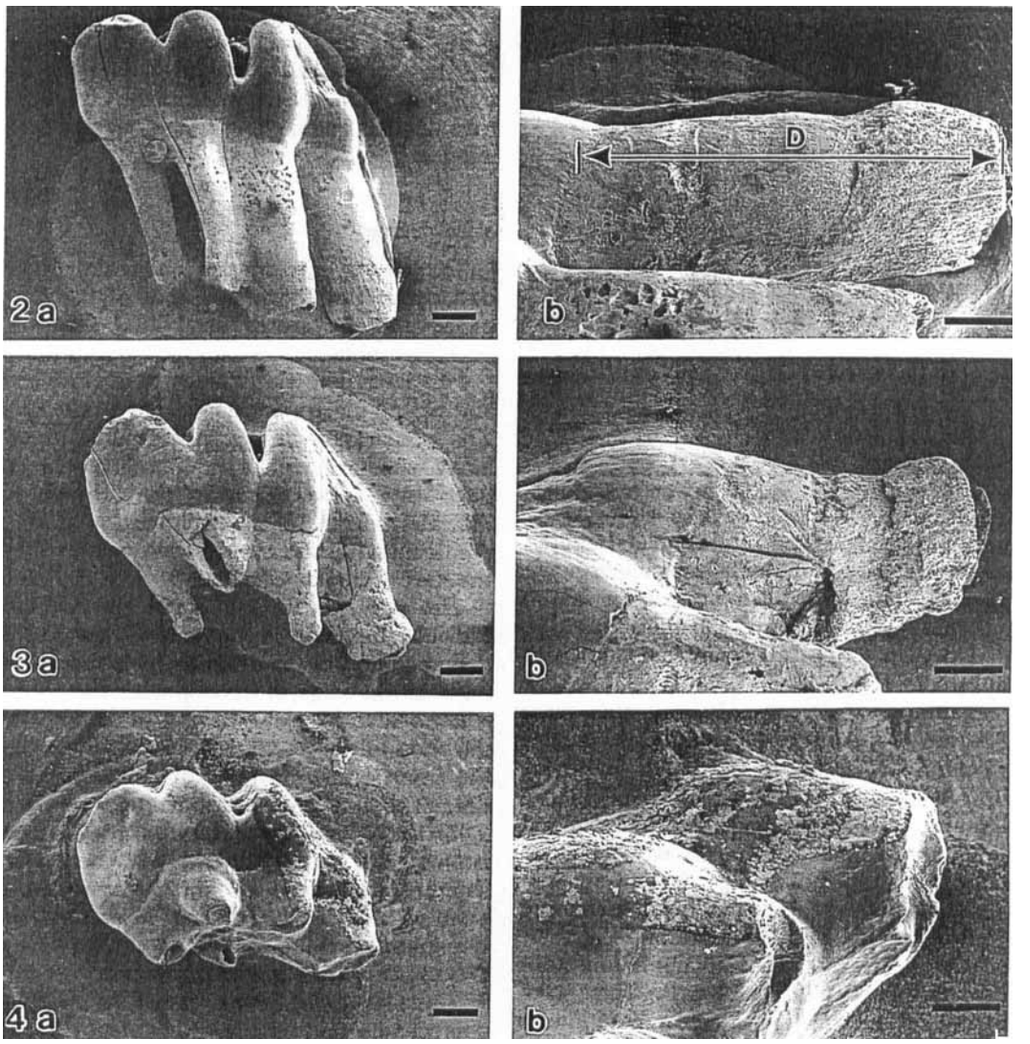

Fig. 2a. Scanning electron microscopy (SEM) overview of the maxillary first molar of a 39-day old control rat. The development of the tooth is completed and morphology is normal. Bar = 0.5 mm. 2b. SEM detail of the mesial root of a maxillary first rat molar (Fig. 2a). D = distance between cementoenamel junction and apical edge of root. Bar = 0.3 mm.
Fig. 3a. Scanning electron microscopy (SEM) overview of the maxillary first molar of a 39-day-old experimental rat. Note that the crown is unaffected. The roots are short and tapered in the apical area. Bar = 0.5 mm. 3b. SEM detail of the mesial root of a maxillary first molar from a 39-day-old experimental rat. The surface structure resembles that of the corresponding tooth from a control rat (Fig. 2b) with the cellular cementum showing a rough surface at the apical part of the root. Bar = 0.3 mm.
Fig. 4a. Scanning electron microscopy (SEM) overview of the maxillary first molar of a 19-day-old experimental rat. Note that morphology and size of the crown are unaffected. Bar = 0.5 mm. 4b. SEM detail of the mesial root of a maxillary first molar from a 19-day-old experimental rat. Note the short, tapered roots. Bar = 0.3 mm.

Twenty-six and 35 days after the last injection no additional findings could be recorded either in the control rats (Fig. 2a,b) or in the experimental rats (Fig. 3a,b).

In the control rats the distance from the cemento-enamel junction to the apical end of the mesial root of the first molar increased from 0.85 mm at 19 days of age to 1.96 mm at 39 days of age (Fig. 2a,b). The corresponding values in the Cy-treated rats increased from 0.60 mm at 19 days of age to 1.23 m at 39 days of age (Table 1).

Third molar. At the time of the injections, formation of dentin and enamel had just begun in the occlusal part of the third molars. Third molars obtained from the rats that were killed 2 days after the last injection were still in such an early stage of development that the necessary preparation for SEM could not be successfully performed on these teeth.

Six days after the last injection crown formation with cusps and a root trunk could be seen in the control rats. In the experimental rats the crown of the third molar

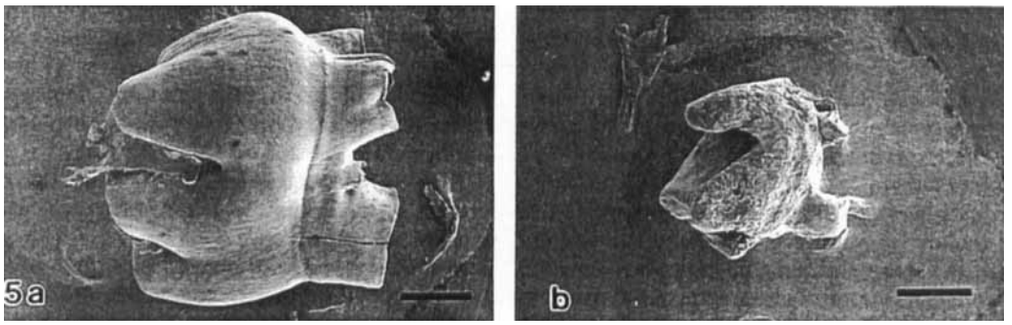

Fig. 5a. Scanning electron microscopy (SEM) overview of the maxillary third molar of a 31-day-old control rat. The smooth surface extending from the cusp tips to the cervical margin indicates complete maturation of enamel. The root formation has started. Bar = 0.5 mm. 5b. SEM overview of the maxillary third molar of a 31-day-old experimental rat, showing a general size reduction as compared with the control tooth (Fig. 5a). The crown shows abnormal morphology with hypoplastic areas and diverging cusp positions. Note the small and curved roots. Bar = 0.5 mm.

was relatively small, and its shape was irregular with no distinct cusps. The formation of a root trunk had started.

Twelve days after the last injection the initial single opening of the root trunk in the control rats had developed into three openings with epithelial diaphragms and cuffs. In the experimental rats the region of the epithelial diaphragm showed three tongue-like extensions. The experimental third molars were smaller than the third molars of the control rats.

Eighteen days after the last injection three roots could be seen in both the control and experimental teeth. The roots of the experimental teeth were small and curved. In addition to a general size reduction, the crowns of the

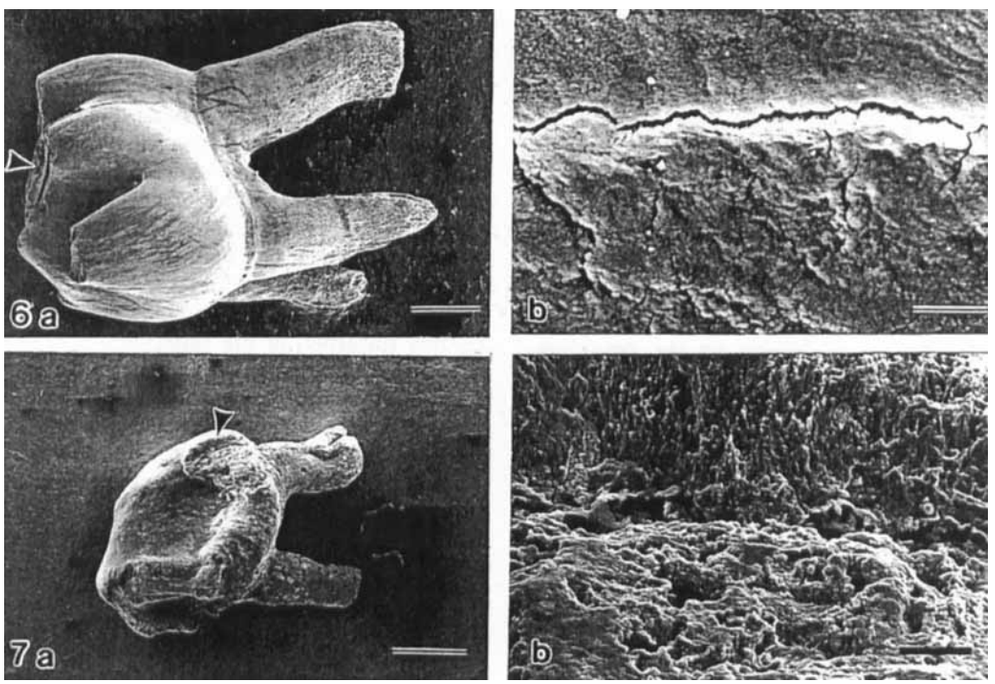

Fig. 6a. Scanning electron microscopy (SEM) overview of the maxillary third molar of a 48-day-old control rat. The development of the tooth is complete with three diverging roots. The arrowhead indicates the tip of the cusp referred to in Fig. 6b. Bar = 0.5 mm. 6b. Detail of cusp tip indicated by arrowhead in Fig. 6a. Note the relatively smooth and compact layer covering the tip of the cusp. Bar = 0.3 mm.
Fig. 7a. Scanning electron microscopy (SEM) overview of the maxillary third molar of a 48-day-old experimental rat. Note the reduced size as compared with the control tooth (Fig. 6a), the poor root development, and the abnormal morphology of the crown with hypoplastic areas and diverging cusp positions. Bar = 0.5 mm. 7b. Detail of cusp tip indicated by arrowhead in Fig. 7a from a 48-day-old experimental rat. The dentin surface has a very porous appearance. Bar = 0.3 mm.

experimental rats also showed abnormal morphology with hypoplastic areas and diverging cusp positions (Fig. 5a,b).

Twenty-six days after the last injection root length and crown height were the same in the control rats. The findings in the experimental group were the same as those described above.

Thirty-five days after the last injection the development of the control molars was complete (Fig. 6a). A projection of the tips of the cusps showed that they were covered with a relatively smooth and compact layer, in appearance like afibrillar acellular cementum (Fig. 6b). The experimental third molar was small with an atypical morphology (Fig. 7a). The shape and orientation of the cusps were abnormal, and the tips of the cusps had an uneven and porous surface (Fig. 7b).

The distal root length of the third molar increased from 0.26 mm at 31 days to 0.68 mm at 48 days of age in the control rats. The corresponding dimension in the experimental teeth increased from 0.13 mm at 31 days of age to 0.32 mm at 48 days of age (Table 2).

Discussion

Previous studies on the effect of Cy on continuously growing adult rat incisors have shown that the effects of the drug were restricted to undifferentiated epithelial and mesenchymal cells, and that the extent of the injury to the odontogenesis was related to the dose of the drug. The advantage of studying the effects of Cy on developing rat molars is that the process of crown and root formation of these teeth in general is similar to that found in human dental development. However, the tolerance level for Cy is comparatively low in young, growing rats, and this may constitute a disadvantage, as only comparatively low doses of Cy may be administered. On the other hand, the results of a previous study of young rats receiving relatively low doses of Cy (21) showed the same types of effects on the developing molars as have been found to occur in incisors after markedly higher doses of Cy had been administered to adult rats (14–20).

The dental specimens studied in the present investigation are contralateral to those used in a previous histologic investigation of the effects of Cy on rat molar development (21). Whereas the previous study focused on structural changes of the tissues involved in the formation of teeth, the present investigation has concentrated on dimensional and morphologic dental abnormalities caused by Cy treatment.

Table 2. Distances in millimeters (mean values from three measurements of each tooth) from the cementoenamel junction to the apical end of the distal root of the maxillary third molar in three experimental rats and three control rats of different ages

Age (days)	Experimental group	Control group	Difference (%)*
31	0.13	0.26	51
39	0.27	0.65	41
48	0.32	0.68	47

* Root length in the experimental group in percentage of the root length in the control group.

The SEM examination of the teeth showed that the crowns of the first and second molars, the formation of which had already been completed at the time of the Cy injection, showed both normal morphology and normal size when compared with the corresponding teeth of the control animals. Severe developmental disturbances were found, however, in the roots of the experimental molars. The root lengths measured as the distance from the CEJ to the apex of the mesial root, never reached more than about two-thirds the normal length (Table 1). Not only did the roots of these teeth fail to reach normal lengths, but closing of the apices also occurred. The reduced root lengths may be explained by the general detrimental effect of Cy administration on the mitotic activity in the root proliferation zone (Hertwig's epithelial root sheath or the preodontoblasts). The closing of the apices is probably caused by the formation of osteodentin in this area (21).

The occurrence of reduction in root size and deviations from normal root morphology have also been established in humans treated at young ages with Cy for different types of cancer. In a study based on planimetric measurements of mandibular teeth on panoramic radiographs, Näsman et al. (8) showed that such patients had significantly reduced root sizes in comparison with healthy control children. With regard to morphology, arrested root development resulting in short v-shaped roots and apical closure were relatively common findings in the Cy-treated children. It is reasonable to assume that these abnormalities are due to disturbances in cell activity similar to those seen in the rats given Cy in the present study.

The development of the third maxillary molars was severely disturbed in the experimental animals. At the time of administration of Cy, the tooth germs of these teeth had reached the morphologic differentiation stage named the bell stage. The characteristics of this stage are that the general outline of the crown and a part of the enamel of the third molars has been formed. The administration of Cy results in the replacement of normal pulp tissue by a structureless necrotic area without cells (21). The formation of normal mantle dentin ceases under the influence of the drug, particularly noticeable in the distal part of the tooth, and areas of osteodentin appear instead. At lower doses of Cy mantle dentin may be formed, but in reduced amounts and at reduced speed (22). Although the mesenchymal cells may resume differentiation to odontoblasts after cessation of Cy therapy, the dentinogenesis is irreversably disturbed, and the roots of these teeth will be reduced in size or even rudimentary. A comparison between the control group and the

experimental group with regard to third molar root length showed that the roots in the latter group only attained half the normal size (Table 2) during the period of investigation.

Even the crowns of the third molars in the experimental group showed major morphologic abnormalities. Furthermore, in the Cy-treated rats the dentin areas of the cusps had a porous appearance (Fig. 6d) and seemed to constitute an inadequate barrier against invasion of microorganisms into the pulp. This may explain the fact that periapical osteitis and pustules may appear in the third molars after early treatment with Cy (21). If the dentin of teeth that are under formation during Cy therapy in humans develop to the same inferior quality as the ones seen in developing rat molars after administration of Cy, there is obviously a considerable risk that carious lesions reaching the dentin may rapidly lead to pulpal infections.

In conclusion, although the dental development in humans and rats cannot be directly compared, the present results nevertheless indicate that administration of Cy may explain some of the effects seen on human teeth after Cy therapy.

Acknowledgements.—This study was supported by a grant from the Swedish Medical Research Council (Project 06001) and the School of Dentistry, Karolinska Institutet, Stockholm, Sweden.

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Received for publication 15 October 1996
Accepted 30 January 1997