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A computerized histomorphometric study of the effects of intoxication with Vitamin D_3 or 1,25 $(OH)_2D_3$ on growth and dentin production of impeded and unimpeded rat incisors

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Summary. The effects of Vitamin D_3 and 1,25$(OH)_2D_3$ intoxication on growth and dentinogenesis were studied in impeded and unimpeded rat incisors. Animals were intoxicated either with Vitamin D_3 (2 mg/kg/day) or 1,25$(OH)_2D_3$ (400 ng/kg/day) for 19 days. Undecalcified transverse sections were obtained from all lower incisors. The outlines of all dental tissues were traced and fed into a computer which depicted the changes in all variables measured (circumference and cross-sectional area of tooth, pulp and dentin, width of dentin, CEJ [cemento-enamel junction] diameter and maximal labiolingual diameter). Eruption rates, which had been recorded previously every 48 h, were used to compute the physiological age of each tooth site, so that only areas of similar age be compared.

Growth. External dimensions of all intoxicated teeth decreased as the experiment progressed in contrast with control incisors which continued to increase in size. This effect was more pronounced in unimpeded incisors and may be due to the inhibitory influence of 1,25$(OH)_2D_3$ on cellular proliferation at the apical progenitor end.

Dentinogenesis. 1,25$(OH)_2D_3$ intoxication had no significant effect on the rate of eruption and dentin formation in both *impeded* and *unimpeded* incisors. The same was true for *impeded* incisors intoxicated with Vitamin D_3 but, both eruption and dentin production rates were impaired in Vitamin D_3 *unimpeded* incisors.

Key words: Cholecalciferols – Rat incisor – Histomorphometry – Dentin

Introduction

Within the past decade, our understanding of the metabolism and function of Vitamin D_3 has increased considerably (Haussler and McCain 1977;

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DeLuca 1979; DeLuca 1982; Bikle 1982; Norman 1982). Simultaneously, several of its metabolites have been synthesized and applied clinically (Haussler and McCain 1977; Kanis et al. 1977; Reeve 1979; Pierides 1981; Norman 1982). Indiscriminate use, long-term administration and the relatively large doses that are sometimes used, however, result in some patients becoming intoxicated (Kanis et al. 1977; Davies and Adams 1978; Paterson 1980). This fact has caused renewed interest in the effects of Vitamin D intoxication.

Studies on the effects of overdoses of Vitamin D (D_2 or D_3) (Weinmann 1929; Schour and Ham 1934; Becks 1942; Becks et al. 1946a, b; Irving et al. 1949; Fahmy et al. 1961; Bernick et al. 1971) or of its analogues and metabolites (Ziskin et al. 1943; Ratcliff and Itokazo 1964; Moskow et al. 1964; Pitaru et al. 1982) on dental tissues report conflicting and often contradictory results. Furthermore, most of these investigations were carried out several decades ago, long before our understanding of the Vitamin D endocrine system reached its present level.

Some have described an increase (Ziskin et al. 1943) in dentin formation in rat incisors, accompanied by disturbances such as hypoplasia and osteodentin formation (Pitaru et al. 1982). Others, however, report normal dentin formation in rat incisors (Schour and Ham 1934; Irving et al. 1949), rat molars (Ratcliff and Itokazo 1964; Pitaru et al. 1982) and in dogs (Becks 1942; Becks et al. 1946a, b). All the studies quoted which have examined dentin formation in rat incisors studied isolated areas of the teeth only and did not take eruption rate into consideration – an essential factor in order that only areas of similar physiological age be compared. In addition, no information concerning growth of these teeth under Vitamin D_3 intoxication is available.

For an accurate and reliable description of changes occurring both in growth and dentin formation in the rat incisor, a three-dimensional time related analysis is needed. Such a computerized three-dimensional histomorphometric method to examine all tooth constituents was recently developed (Steigman et al. 1983), based on integration of morphometric data extracted from ground sections of the whole incisor by means of a computer. This method was further used to describe both the normal (impeded) rat incisor and that with doubled eruption rate (unimpeded, resulting from repeated cutting the tooth out of occlusion) (Michaeli et al. 1982).

We think this method is well suited to elucidate the effects of Vitamin D_3 and $1,25(OH)_2D_3$ intoxication both on growth and on dentin formation rate by studying the morphology of the continuously erupting rat incisor. Such is the purpose of this investigation.

Materials and methods

Thirty male rats of the Charles River strain weighing 191 ± 17 gm were allotted to three equal groups. Rats in the first group were subcutaneously injected every 24 h with 400 ng/kg $1,25(OH)_2D_3$ (Calcitriol) and those in group two, with 4 mg/kg Cholecalciferol every 48 h. The third group served as controls in which the rats were injected daily with the same volume (0.1 ml) of the solvent, propylene glycol, only.

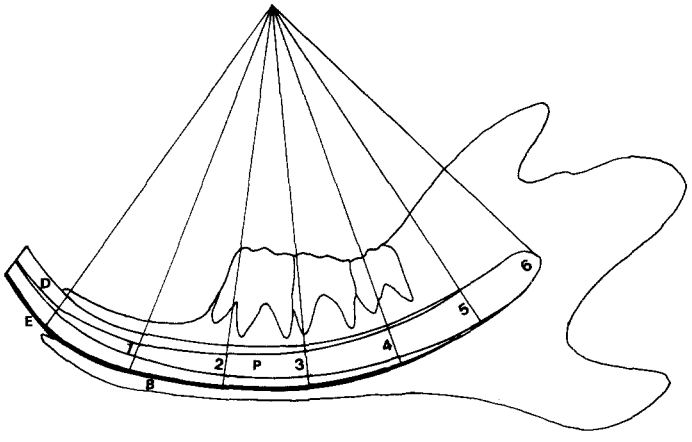

Fig. 1. Schematic representation of a lateral radiograph of the right mandible showing the division of the incisor into six equal segments (1 to 6). *B*, alveolar bone; *D*, dentin; *E*, enamel; *P*, pulp

Each group was further subdivided into two subgroups. In one group, the rats had all their incisors in normal occlusion, and thus in the normal impeded state of eruption. In the other group, the lower right incisor of each animal was cut out of occlusion every 48 h resulting in a continuous state of accelerated unimpeded eruption of these teeth.

Growth of all animals was monitored throughout the experiment by weighing each rat every 6 days.

Eruption and attrition rates were determined every 48 h by a method described earlier (Michaeli and Weinreb 1968; Michaeli et al. 1975). Throughout the experiment, all animals were given 30,000 μ/kg/day Benzathin Penicillin G (IM) to prevent possible respiratory tract infections caused by the frequent ether anesthesia necessary to perform measurements of eruption and attrition.

The experiment lasted for 19 days. After sacrifice, two incisors from each group were decalcified in 10% EDTA (pH = 7) for three weeks, embedded in paraffin wax and cut mid-sagittally for histological examination. All sections, 6 μ thick each, were stained with hematoxylin and eosin.

An orthoradial lateral radiograph was taken of each of the remaining right mandibles and the outlines of the incisors were traced from the radiographs onto an acetate sheet, where the center of the arc formed by the labial border of the incisor was geometrically determined. From there, seven radii were drawn, 15° apart, starting from the posterior border of the alveolar bone and dividing most of the incisor into six equal segments (Fig. 1). The actual length of each segment was determined.

Each right mandible was placed on its tracing and embedded in clear polyester to fix its position. The polyester block containing the mandible was mounted on an Isomet low speed saw (Buhler Ltd) and three consecutive undecalcified transverse sections, 100 μm thick each, were obtained from segments 2–5 (Fig. 1), starting at their apical end and progressing in an incisal direction (Fig. 2a). (This method prevents distortion caused when performing serial sectioning on a circle-shaped tooth.)

Each ground section was viewed through a Reichert Visopane microscope (× 53) and the outlines of the incisor tooth were traced on an acetate sheet. The following anatomical variables were measured on each tracing by a sonic digitizer (Summagraphics) and stored in a micro PDP-11 computer (Fig. 2b):

1. Perimeter of the tooth and its cross-sectional area.
2. Cross-sectional area and external perimeter of the dentin.
3. Perimeter and cross-sectional area of the pulp.
4. Mesio-lateral tooth width between the two cemento-enamel junctions (line 1-1′).

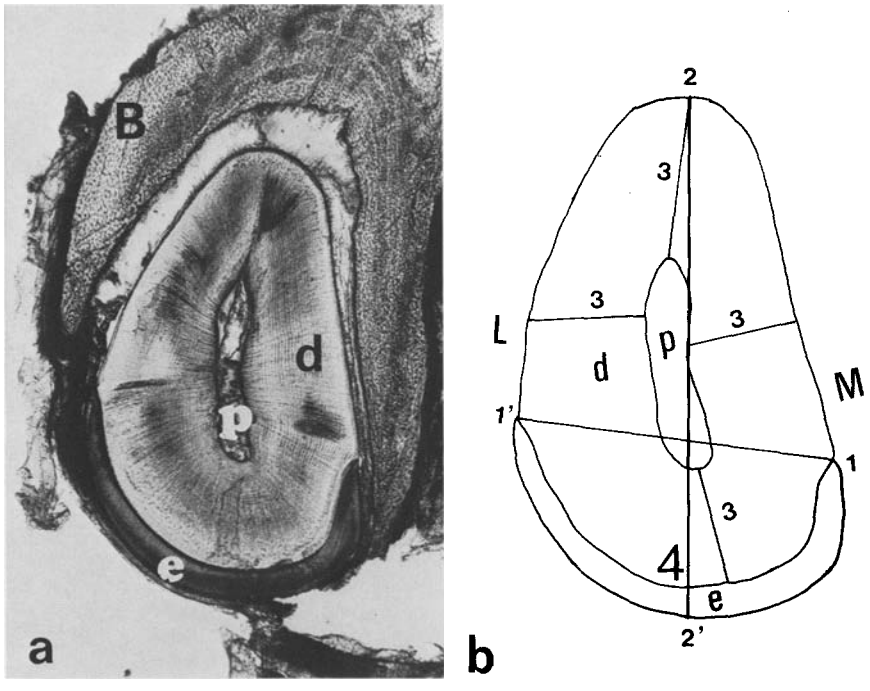

Fig. 2a, b. Typical undecalcified cross-section of the rat incisor (**a**), and its tracing showing the location of lines used in the measurements (**b**). *B*, alveolar bone; *d*, dentin; *e*, enamel; *p*, pulp; *M*, medial; *L*, lateral. 1-1′-CEJ width; 2-2′-labio-lingual tooth diameter; 3-dentin width; 2-4-labio-lingual dentin diameter

5. Maximal labio-lingual tooth width: from point 2 (the most distant on the lingual cementum from the midpoint of line 1-1′), to point 2′ (the most distant on the enamel from point 2).
6. Maximal labio-lingual diameter of the dentin (line 2-4).
7. Width of the dentin on the four pulpal walls: mesial, labial, lateral and lingual (3).

All data for each variable were plotted against the respective distances from the posterior alveolar bone (abscissa) and fitted with a second-order polynomial. In order to compare teeth having different eruption rates, the location of an area in the tooth cannot serve as an adequate measurement, only the physiological age of each tooth area must be used. Therefore, all polynomials of y (tissue parameter) dependent upon the distance of the tissue from the tooth origin ($\times$) were replaced by ones with y dependent upon the physiological age of each site in days.

The following variables of tooth external dimensions were used to determine the growth of the incisor: outer perimeter of the dentin, labio-lingual diameter of the dentin and medio-lateral diameter of the tooth. The latter was derived from the area occupied by the outer dentin boundary and the labio-lingual diameter by approximating the dentin cross-section to an elippse and using the equation $S=\frac{3.14\times A\times B}{4}$ where S = cross-sectional area occupied by dentin perimeter; A = major axis of ellipse (labio-lingual dentin diameter) and B = minor axis of ellipse (medio-lateral tooth diameter). The mean width of the dentin wall (being the average of the widths of the 4 pulpal walls – medial, lingual, lateral and labial) served to determine the rate of dentin production.

In order to evaluate the statistical significance of the inter-group differences in each variable, the mean and standard deviation were calculated for each week preceding sacrifice and subjected to a student's *t*-test.

Results

General effects

Both experimental groups exhibited signs of intoxication such as poor physical condition and statistically significant loss of weight, hypercalcaemia and hypophosphataemia. While loss of weight in 1,25$(OH)_2D_3$ rats was less pronounced than in Vitamin D_3 rats, the former displayed more severe hypercalcaemia and hypophosphataemia.

Eruption rates

A. *Impeded incisors.* Control teeth erupted 540 μ/day; those from the 1,25$(OH)_2D_3$ and Vitamin D_3 groups erupted at a slightly lower rate: 510 μ/day and 520 μ/day respectively. (No differences were significant).

B. *Unimpeded incisors.* Upon repeated shortening of the teeth, their eruption rate gradually increases to reach, after 48–96 h, a new steady state of maximal unimpeded eruption rate. Thus, control unimpeded incisors erupted 830 μ/day in the first two days and then, for the remainder of the experiment, 930 μ/day (mean unimpeded eruption rate). Similarly, 1,25$(OH)_2D_3$ incisors erupted 870 μ/day for two days and thereafter 915 μ/day (not being significantly different from controls). On the other hand, Vitamin D_3 teeth were significantly effected by the intoxication: they erupted 745 μ/day for two days, then reached a peak of 1,040 μ/day in the next two days but declined

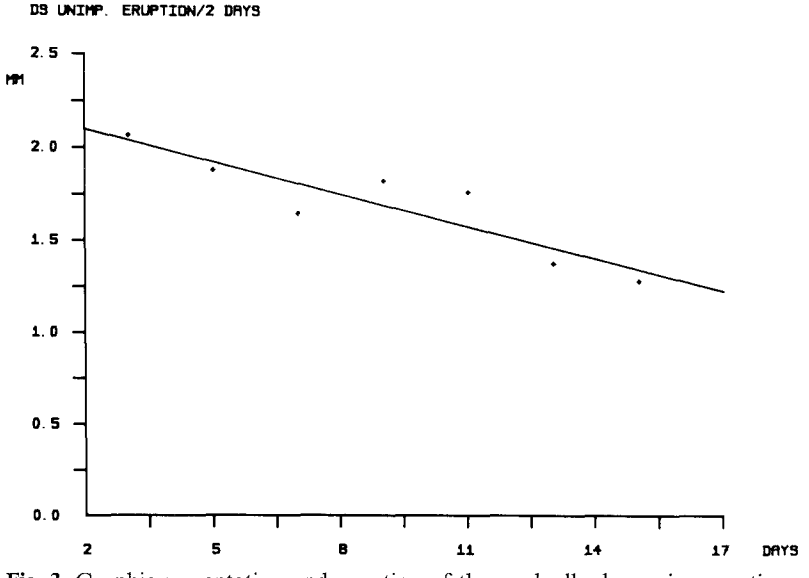

Fig. 3. Graphic presentation and equation of the gradually decreasing eruption rate (mm per 48 h, ordinate) of unimpeded rat incisors given daily Vitamin D_3 overdose. *Abscissa:* duration of the experiment in days. $y = 2.219 - 0.059x$

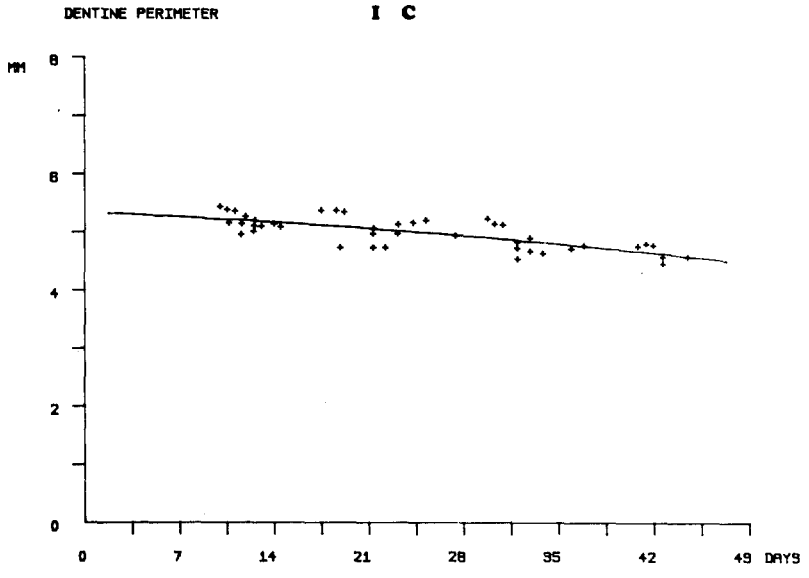

Fig. 4. Graphic illustration of the changing outer circumference of the dentin in control incisors (*mm*, ordinate) with the physiological age of tooth areas in days (*abscissa*). Higher values (*left*) represent areas produced more recently and demonstrate growth of teeth with time. *I*, Impeded; *C*, Controls

thereafter gradually to reach a low rate of 600 μ/day at the end of the experiment. In order to calculate the physiological age of each area along the teeth in the Vitamin D_3 unimpeded group, a straight line was fitted to these data (Fig. 3).

External tooth dimensions

A. *Control group.* Figure 4 depicts the changes in the outer perimeter of the dentin in *impeded* incisors. The declining curve indicates an increase in the perimeter with time, since the high values represent the more recently formed part of the tooth (apical), while the oldest part (incisal) shows a smaller perimeter. This is an expression of the continuous growth of the tooth in size which accompanies the general growth of the rats.

Similarly, other parameters of the external shape, namely CEJ width (the distance between the two cemento-enamel junctions) (1-1′ in Fig. 3), medio-lateral tooth diameter and maximal labio-lingual diameter of the dentin (2–4) exhibited a decreasing curve when plotted against the age of tooth segments. Control *unimpeded* incisors showed a similar picture.

B. *Experimental groups.* Figure 5 presents age adjusted variables for both experimental groups together with those of controls. As opposed to controls, teeth in both experimental groups ceased to increase in size and even diminished as the experiment progressed. This was true both for impeded and

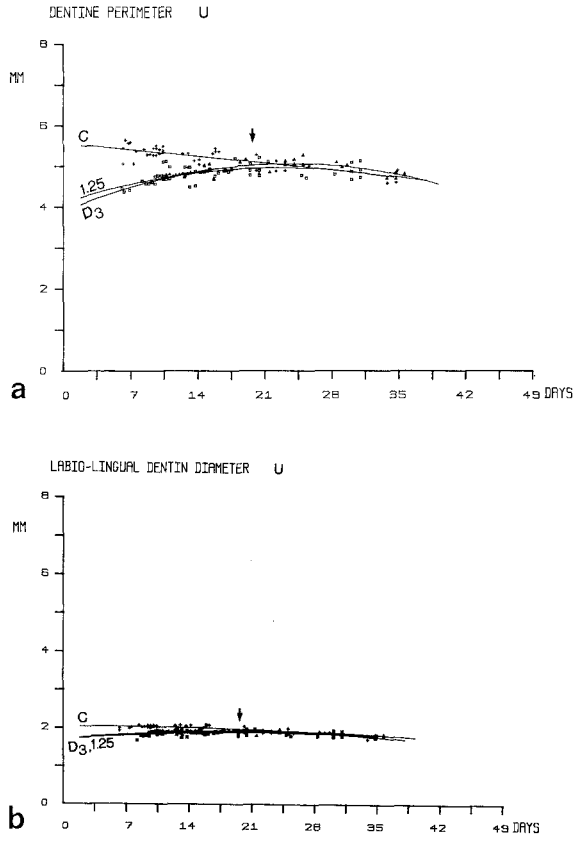

Fig. 5a–c. Graphic presentation of the diminution of **a** outer dentin perimeter, **b** labio-lingual dentin diameter, and **c** medio-lateral tooth diameter of unimpeded incisors caused both by Vitamin D_3 and 1,25$(OH)_2D_3$ intoxication (*mm*, ordinate) related to the physiological age of tooth sites (abscissa). Crosses – controls; squares – 1,25$(OH)_2D_3$; triangles – Vitamin D_3. *U*, Unimpeded. Arrow, onset of experiment

unimpeded incisors although it was much more pronounced in the latter. Figure 5a shows the changes in the external perimeter of dentin in the three unimpeded groups. Beyond the age of 19 days (which corresponds to the beginning of the experiment – see arrow), no differences can be

Table 1. The effect of $1,25(OH)_2D_3$ or Vitamin D_3 intoxication on the outer circumference of dentin of unimpeded rat incisors (mm, mean ± SD)

Group	Weeks before sacrifice			
	1	2	3	4
Control	5.34 ± 0.14	5.22 ± 0.19	5.03 ± 0.10	4.76 ± 0.17
$1,25(OH)_2D_3$	*4.77 ± 0.19	*4.92 ± 0.13	4.89 ± 0.22[a]	4.90 ± 0.18[a]
Vitamin D_3	*4.80 ± 0.03	**4.94 ± 0.16	5.09 ± 0.11[a]	4.91 ± 0.18[a]

* $P < 0.001$ (vs. control); ** $P < 0.005$ (vs. control)

[a] Not significant (vs. control)

Table 2. The effect of $1,25(OH)_2D_3$ or Vitamin D_3 intoxication on the labio-lingual dentin diameter of unimpeded rat incisors (mm, mean ± SD)

Group	Weeks before sacrifice			
	1	2	3	4
Control	2.05 ± 0.04	2.02 ± 0.09	1.91 ± 0.04	1.80 ± 0.06
$1,25(OH)_2D_3$	*1.86 ± 0.07	*1.88 ± 0.05	1.88 ± 0.09[a]	1.86 ± 0.07[a]
Vitamin D_3	*1.89 ± 0.04	**1.92 ± 0.06	1.94 ± 0.05[a]	1.87 ± 0.06[a]

* $P < 0.001$ (vs. control); ** $P < 0.02$ (vs. control)

[a] Not significant (vs. control)

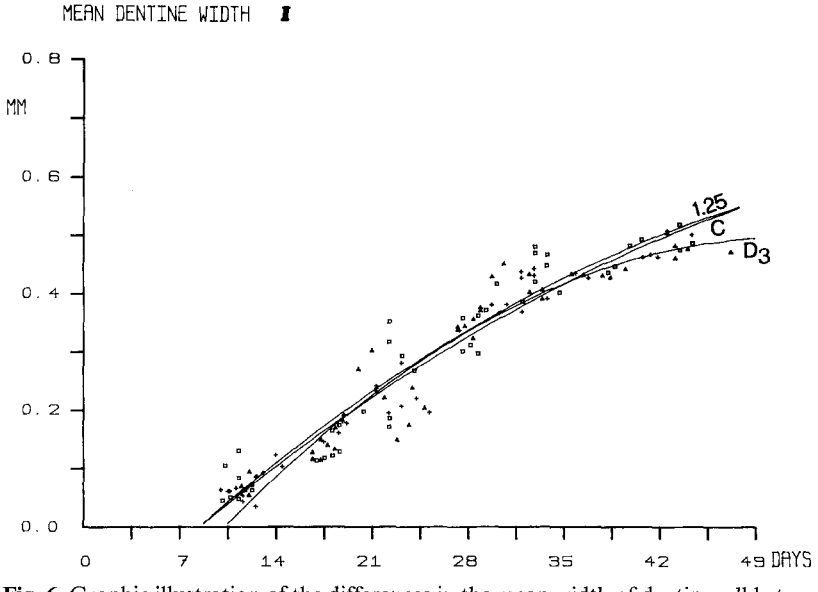

Fig. 6. Graphic illustration of the differences in the mean width of dentin wall between impeded incisors of control (*crosses*), $1,25(OH)_2D_3$ (*squares*) and Vitamin D_3 (triangles) groups with physiological age. *I*, Impeded

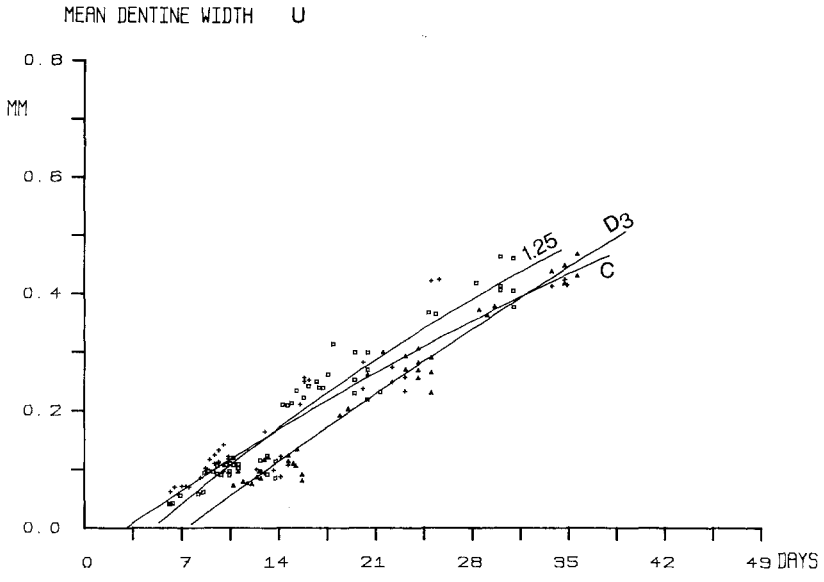

Fig. 7. Graphic illustration of the differences in the mean width of dentin wall between unimpeded incisors of control (*crosses*), 1,25$(OH)_2D_3$ (*squares*) and Vitamin D_3 (*triangles*) groups with physiological age. *U*, Unimpeded

Table 3. The effect of 1,25$(OH)_2D_3$ or Vitamin D_3 intoxication on the medio-lateral tooth diameter of unimpeded rat incisors (mm, mean $\pm$ SD)

Group	Weeks before sacrifice			
	1	2	3	4
Control	1.25 ± 0.08	1.20 ± 0.08	1.19 ± 0.05	1.11 ± 0.05
1,25$(OH)_2D_3$	$*1.07 \pm 0.08$	$**1.14 \pm 0.05$	1.12 ± 0.03[a]	1.15 ± 0.05[a]
Vitamin D_3	$*1.07 \pm 0.06$	$**1.11 \pm 0.08$	1.18 ± 0.04[a]	1.14 ± 0.07[a]

* $P < 0.001$ (vs. control); ** $P < 0.05$ (vs. control)

[a] Not significant (vs. control)

detected between the experimental groups and controls. However, in the apical area produced during intoxication, the results differ markedly. While untreated incisors continued to increase in size, experimental ones decreased (see Table 1). This was true for all other parameters of the external shape namely, labio-lingual dentin diameter, (Fig. 5b, Table 2) and medio-lateral tooth diameter (Fig. 5c, Table 3).

Dentin production

A. *Control group.* Since dentin is produced inward along the entire incisor, the pulp diminishes and the dentinal wall increases in thickness as one proceeds incisally.

Table 4. The effect of $1,25(OH)_2D_3$ or Vitamin D_3 intoxication on the mean width of dentin wall of unimpeded rat incisors (mm, mean ± SD)

Group	Weeks before sacrifice			
	1	2	3	4
Control	0.11 ± 0.02	0.20 ± 0.08	0.31 ± 0.09	0.42 ± 0.01
$1,25(OH)_2D_3$	0.10 ± 0.02[a]	0.25 ± 0.03[a]	0.32 ± 0.08[a]	0.42 ± 0.03[a]
Vitamin D_3	*0.09 ± 0.02	**0.14 ± 0.06	0.28 ± 0.02[a]	0.41 ± 0.04[a]

* $P<0.05$ (vs. control); ** $P=0.05$ (vs. control)

[a] Not significant (vs. control)

B. *Experimental groups. 1,25(OH)$_2$D$_3$ animals:* Figures 6 (impeded) and 7 (unimpeded) show that $1,25(OH)_2D_3$ intoxication has no significant effect on dentin production which progresses at the same rate as in controls.

1. In impeded incisors, the curves depicting the mean dentin wall thickness with time are identical for $1,25(OH)_2D_3$ and control incisors (Fig. 6).

2. In unimpeded ones, the dentin wall in the incisal region of $1,25(OH)_2D_3$ intoxicated incisors is wider than that of controls, but not significantly (Fig. 7, Table 4).

Vitamin D_3 animals. 1. Impeded incisors: No statistically significant differences could be observed in the mean width of the dentin wall as compared to controls (Fig. 6).

2. Unimpeded incisors: This group was the only one to differ significantly from control. In the apical (youngest) region, the mean wall thickness was decreased (Fig. 7, Table 4). As we proceed incisally, dentin production rate returned to normal so that mean wall thickness equalled that of controls in the incisal region (Table 4).

Discussion

Growth

As the rat incisor is continuously formed, it is the rate of the constant proliferation of the odontogenic epithelium and of the odontoblasts which will finally determine the external dimensions of the tooth.

The computerized histomorphometric method used in this study revealed a gradual increase in all external dimensions of all control teeth. This is proof that male rats, 7 weeks of age, are still rapidly growing and that the external dimensions of rat incisors cannot indiscriminately be assumed to be constant.

On the other hand, a gradual decrease in external dimensions was noticed in all experimental animals. This change started at the onset of the experimental period (arrows in Fig. 5), and was true both for $1,25(OH)_2D_3$ and Vitamin D_3 animals with no significant difference between these two groups

(Tables 1–3). This fact strongly suggests that a marked decrease in the proliferation rate of basal, formative cells had taken place and led to diminution in tooth size. Several studies that examined the influence of $1,25(OH)_2D_3$ on cell proliferation have demonstrated an inhibitory effect: $1,25(OH)_2D_3$ was found to inhibit the proliferation of prechondroblasts in the mandibular condyle of young mice (Silbermann et al. 1983; Mirsky and Silbermann 1986) and in the epiphyseal plate of young rabbits (Plachot et al. 1982) and adult rats (Bernick et al. 1971). Also, it was found to inhibit the proliferation of human leukaemia cells (Matsui et al. 1984), human melanoma cells (Colston et al. 1981) and mouse thymocytes (Ravid et al. 1984). We have also examined the mandibular condyle of rats used for the present study and found a significant decrease in the size of the progenitor compartment of the cartilage (Weinreb and Weinreb, submitted).

It can therefore be assumed that it was the inhibitory action of $1,25(OH)_2D_3$ that led to the gradual decrease in tooth size in both experimental groups. This assumption is further strengthened by the finding that the decrease in external dimensions was much more pronounced in unimpeded teeth. Previous experiments have established that unimpeded teeth exhibit a doubled proliferation rate of both odontogenic epithelium (which produces ameloblasts) (Bar-Lev et al. 1976) and odontoblasts (Weinreb, Jr. et al. 1985). It is thus reasonable to expect a more severe disturbance of cell proliferation when a much higher turnover is present. However, this assumption should be further validated by other methods such as ^3H thymidine incorporation and autoradiography.

Dentin formation

A. *Justification of the variables used for the final analysis.* When dentin production is to be analyzed, several variables may come to mind, i.e., the pulp perimeter and cross-sectional area, the cross-sectional area of the dentin wall and its width. However, when comparing controls to experimental animals, several problems arise. The parameters of pulp shape cannot properly indicate dentin production rate because, in addition to their correlation with the amount of dentin which has been laid down, they depend upon the starting point from which dentin production started. Since this point (outer dentin perimeter) differed markedly between control and experimental groups, one has to analyze parameters pertinent to dentin alone. Of these, the cross-sectional area occupied by the dentin wall too, cannot be used for comparative purposes, because it is also dependent on the dentin perimeter: the same wall thickness would yield smaller cross-sectional area values in teeth with a diminished external circumference. Thus, the mean dentin wall thickness was chosen as a true reflector of dentin production rate.

B. *Rate of dentin formation.* Previous studies on dentin production in Vitamin D intoxication have used impeded incisors only. Furthermore, in these investigations, the results obtained were not adjusted for age of the produc-

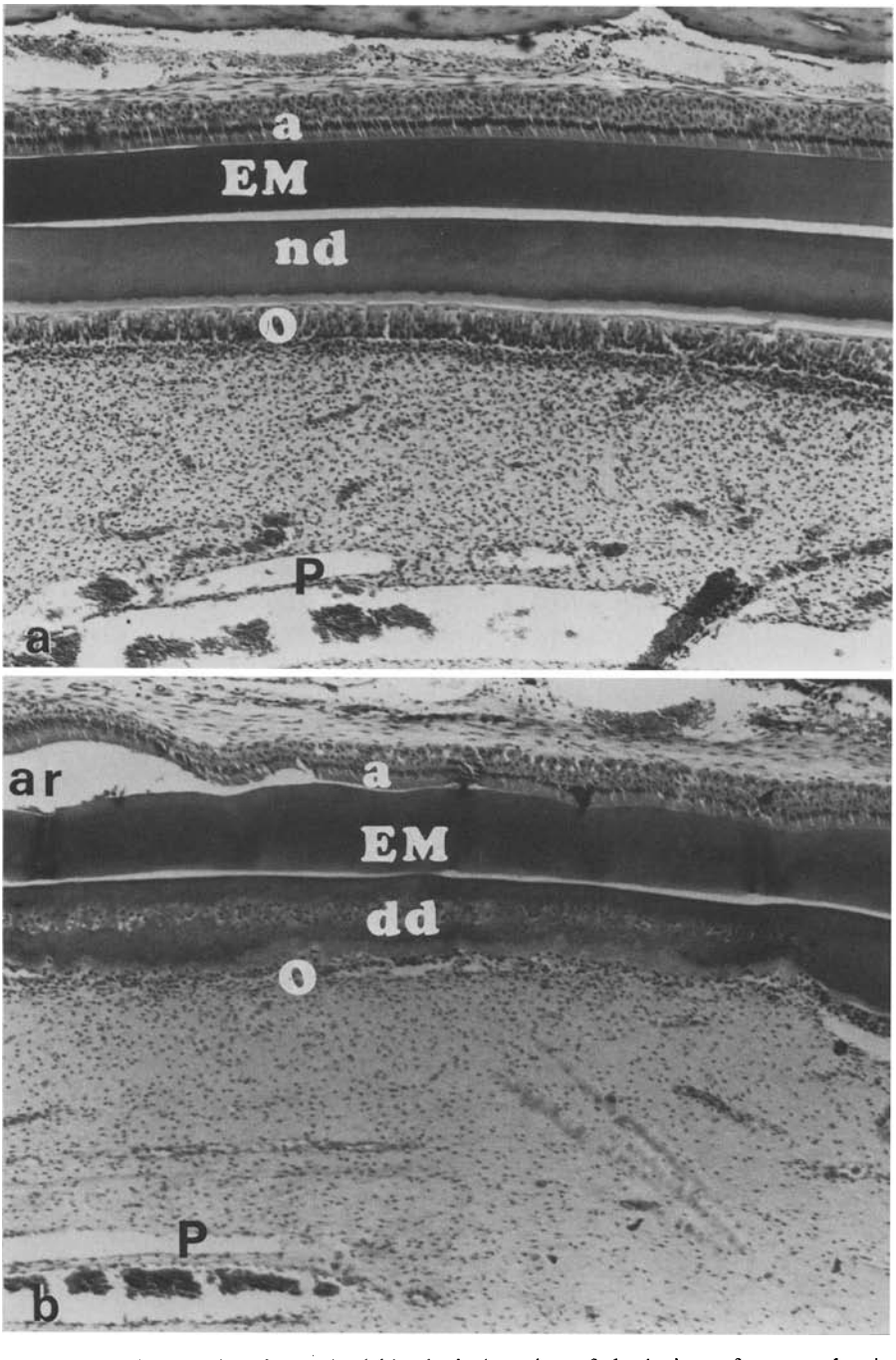

Fig. 8a, b. Micrographs of a sagittal histological section of the incisor of **a** control animal and **b** a Vitamin D_3 intoxicated animal showing severe disturbance of dentin formation at the apical end of the latter. H&E ×90; *a*, Ameloblasts; *EM*, Enamel matrix; *dd*, Disturbed dentin; *O*, Odontoblasts; *P*, Pulp; *ar*, artefact; *nd*, normal dentin

ing cells, nor did they take into account the normal variability in the production rate of dentin during the life-span of the odontoblasts. (Histomorphometric studies of normal rat incisors have shown that the rate of dentin apposition in normal teeth varies during the life-span of the tooth being greatest in the middle third of the incisor – Michaeli et al. 1981; Michaeli et al. 1982; Steigman et al. 1983).

Ziskin et al. (1943) administered large daily doses of Vitamin D analogue (Ertron) and found an increase in the total amount of dentin produced during the experimental period when compared with an equal pre-experimental (control) period.

Pitaru et al. (1983) compared the width of dentin at the same distance from the apex in both controls and $1,25(OH)_2D_3$ intoxicated incisors and found a significant increase in wall thickness in the experimental animals. These results were not adjusted, however, for possible changes in the rate of eruption.

The present study compared age-adjusted dentin wall thickness along the entire incisors of control, $1,25(OH)_2D_3$ and Vitamin D_3 intoxicated animals, taking into account the normal variability of dentin formation at different locations in the incisor.

From Figs. 6 and 7, and Table 4, it can be seen that a $1,25(OH)_2D_3$ overdose (existing in both experimental groups) does not significantly increase the rate of dentin production by rat incisor odontoblasts. This conclusion is in agreement with a recent autoradiographic investigation by Clark et al. (1985) in which specific receptors for $1,25(OH)_2D_3$ were found in rat incisor pulp fibroblasts, but not in odontoblasts in the same teeth. Also, recent immunocytochemical studies (Taylor 1984; Taylor et al. 1984) have shown Vitamin D-dependent calcium-binding protein immunoreactivity to be localized in rat incisor ameloblasts only and not in odontoblasts in these teeth. All these studies imply that $1,25(OH)_2D_3$ has no direct effect on secretory odontoblasts.

The only significant effect observed in the experimental incisors is a diminution in dentin production rate in the apical (youngest) region of Vitamin D_3 intoxicated unimpeded teeth (Fig. 7, Table 4). This result is in close correlation with the histology of the apical end of these teeth where distinct disturbances, both of apposition and mineralization of dentin, could be detected (Fig. 8b). These were totally absent in $1,25(OH)_2D_3$ intoxicated incisors. After the early stages of dentinogenesis, dentin production rate by Vitamin D_3 intoxicated incisors slowly recuperated so that mean wall thickness of these teeth equalled that of controls in the incisal regions (Fig. 7, Table 4). The reason for this difference between $1,25(OH)_2D_3$ and Vitamin D_3 overdoses cannot be determined with the present method alone, and demands the use of other methods of investigation. A possible involvement of other Vitamin D_3 metabolites should, however, be taken into consideration.

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