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Induced Hypoxia in Rat Pulp and Periapex Demonstrated by ^{3}H-Misonidazole Retention

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Abstract. Cellular hypoxia may be a useful indication of tissue distress in the dental pulp that could be used to investigate the early stages of pulpal responses. Tritiated misonidazole (^{3}H-MISO) is a marker which preferentially labels cells with decreased oxygen tension (hypoxia). The experiments reported here were carried out to determine whether this agent could distinguish between hypoxic and normoxic pulp and periapical tissues. Rats were injected intra-peritoneally with either ^{3}H-MISO, unlabeled MISO, or saline, then divided into normoxic, hypoxic, and control groups. Normoxic animals were maintained at ambient pressure. We induced hypoxia by maintaining animals in a hypobaric chamber at 0.5 atm for 24 hrs. ^{3}H-MISO retention was assessed by quantitative analysis of tissue autoradiographs. ^{3}H-MISO retention rates in normoxic animals showed little variation except for increased retention in mature ameloblasts and immature odontoblasts in the continually erupting incisor. In both incisor and molar pulps, hypobaric hypoxia significantly increased ^{3}H-MISO retention when compared with normoxic controls. Hypobaric hypoxia also resulted in intense ^{3}H-MISO retention in cellular cementum, periodontal ligament, osteocytes, and, occasionally, in molar pulp horn odontoblasts. This study demonstrated that, with standard autoradiographic techniques, ^{3}H-MISO can label induced hypoxic disturbances in the pulp and surrounding periodontium.

Key words: hypoxia, misonidazole, dental pulp, odontoblasts, ameloblasts, male Sprague-Dawley rats.

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Introduction

The dental pulp, when subjected to inflammatory stress, cannot swell, and its interstitial fluid pressure rises (Van Hassel, 1971). One consequence of increased pulpal pressure may be cellular hypoxia. Hypoxia is defined as a decrease in the normal oxygen tension (pO_2) of the cell. Hypoxia may be the first indication in the pulp that cells are being metabolically challenged (Biesterfeld *et al.*, 1979).

Misonidazole, 1-(2-hydroxy-3-methoxypropyl)-2-nitro-1H-imidazole (MISO), has been used in experimental cancer therapy because of its ability to sensitize hypoxic tissue to the free radical effects of ionizing radiation and its ability to bind covalently, selectively, to hypoxic cells. Selective binding of MISO to hypoxic cells has been demonstrated in cell culture (Chapman *et al.*, 1981; Raleigh *et al.*, 1985), in animals (Chapman *et al.*, 1981), and in humans (Urtasun *et al.*, 1986).

For ^{3}H-MISO to bind covalently to a cell, the nitro group must undergo a four-electron reduction to produce a hydroxylamine (Varghese and Whitmore, 1980; Franko, 1986). The bioactivated hydroxylamine covalently binds only in the cell where the reduction occurs (Varghese and Whitmore, 1980; Van Os-Corby *et al.*, 1987). Thus, a radioactive label attached to MISO will be retained in the cell where reduction takes place (Raleigh *et al.*, 1985; Franko *et al.*, 1989).

MISO binds only slightly to normoxic sites due to futile RED/OX cycling, since oxygen limits the amount of reactive MISO derivative produced (Franko, 1986). Cellular hypoxia favors substantial reduction of MISO to hydroxylamine that subsequently binds within the cell (Varghese *et al.*, 1976).

A method for labeling hypoxic cells with tritiated misonidazole (^{3}H-MISO) was described by Chapman *et al.* (1981). This technique was later used to demonstrate the presence of hypoxic cells in tumors (Urtasun *et al.*, 1986) and selected normal tissues (Cobb *et al.*, 1989a).

A possible complication to the use of ^{3}H-MISO as a hypoxic cell marker was the report by Cobb *et al.* (1989a) that certain tissues retained more ^{3}H-MISO than others, even under putative normoxic conditions. Either localized hypoxia was normal and common in sites where labeling occurred, or some undefined chemical reduction mechanism permitted deposition of the tritium label under

normoxic conditions. Therefore, when ^{3}H-MISO is used as a hypoxic cell marker, the normoxic retention rate must first be characterized, and increased retention due to hypoxia must be readily detectable.

Increased hypoxic retention of bioreductive drugs has been tested *in vivo* by a variety of methods to induce hypoxia, including blood vessel clamping (Bremner *et al.*, 1990), administration of flavone acetic acid (Sun and Brown, 1989), 5-hydroxytryptamine (Chaplin, 1986), and hydralazine (Bremner *et al.*, 1990). These methods cause hypoxia by reducing blood flow, which has the disadvantage of decreasing drug delivery to the tissue. In contrast, a hypobaric chamber causes tissue hypoxia by physical reduction of the partial pressure of oxygen (McAleer *et al.*, 1992). Changes in blood flow secondary to hypoxia cannot be completely discounted, but MacManus *et al.* (1989) observed increased MISO binding, and McAleer *et al.* (1992) showed increased anti-tumor effects of several bioreductive drugs with hypobaric hypoxia, suggesting that blood flow was not significantly reduced.

Increased ^{3}H-MISO retention with hypobaric hypoxia has been previously demonstrated in rat incisor pulps by means of a liquid scintillation technique (Baumgardner *et al.*, 1994). This technique surveys the entire pulp organ and, as such, cannot localize sites where increased hypoxic ^{3}H-MISO retention occurs. The aims of this investigation were to determine: (1) normoxic ^{3}H-MISO retention rates in the dental pulp and periapex of rats, (2) whether increased ^{3}H-MISO retention with hypobaric-hypoxia is detectable, and if so, (3) the specific sites where hypoxic ^{3}H-MISO retention occurs.

Materials and methods

Animals

Thirty adult male Sprague-Dawley rats (average weight = 230 grams) were divided into experimental (N = 18) and control (N = 12) groups. The guidelines for the ethical use of animals at the University of Iowa were followed throughout this study.

Drug administration protocol

MISO (Hoffmann-LaRoche, Nutley, NJ) was labeled with tritium (^{3}H) according to the procedure of Born and Smith (1983). All experimental animals were injected I.P. with 75 mg ^{3}H-MISO (specific activity, 4.67 MBq/mg) *per* kg body weight. In the control group, 8 of the 12 animals were injected with 75 mg/kg of unlabeled MISO, while the other 4 were injected with saline. All animals were maintained for 24 hr after drug or sham treatment to allow sufficient time for clearance of unbound circulating MISO (Chin and Rauth, 1981).

Hypobaric hypoxia induction

Both experimental and control animals were divided between the normoxic and hypoxic groups. Animals in the normoxic group were maintained under normal atmospheric conditions, while animals in the hypoxic group were placed in a hypobaric chamber at 0.5 atm. A vacuum pump attached to one end of the chamber reduced the air pressure to 0.5 atm, while a valve at the opposite end allowed for constant fresh air inflow.

Animal death and tissue-processing protocols

After 24 hr, all animals were anesthetized with a mixture of Ketamine HCl (Parke-Davis, Morris Plains, NJ) plus 20% xylazine I.P. (Mobay Corporation, Shawnee, KS) and perfused through the left ventricle with 10% normal buffered formalin.

The maxillary incisors and maxillary and mandibular molars with surrounding periradicular bone were dissected *en bloc* and submerged in 10% normal buffered formalin for a minimum of 48 hr. Samples were decalcified by means of 10% ethylenediaminetetraacetic acid (EDTA) with hydrochloric acid (SP-Decalcifying Solution™, Baxter Scientific Products, McGaw Park, IL). Decalcification was monitored radiographically. After demineralization (about 30 hr), samples were rinsed under running tap water for 2 hr, then processed through graded alcohols to paraffin. We prepared subbed slides by dipping clean slides into a solution of 5.0 gm gelatin and 0.5 gm chromium potassium sulfate *per* 1000 cc distilled water and then air-drying them. Serial sections 4 μm thick were cut from all blocks and placed on the subbed slides. The molar and incisor blocks were sectioned through the entire extent of the pulp along a mesial-distal plane parallel to the long axis of the tooth.

Samples were de-waxed and dipped in Kodak® NTB2 autoradiographic nuclear track emulsion under proper darkroom conditions. The slides were stored in light-tight boxes under refrigeration at approximately 4°C. At appropriate intervals, slides were removed from storage, developed, stained with hematoxylin and eosin, and coverslipped.

At minimum, every tenth sequential serial section was examined by light microscopy, scanned for proper histologic sectioning, and then checked for autoradiographic grain density per unit area with the aid of an ocular grid square. Selected samples were digitized on the Videk Megaplus image acquisition system (Perceptics Corp., Knoxville, TN) and evaluated by computer (Apple® Macintosh IIci, Apple Computer, Inc.). The output of the digital camera was amplified and digitized into a 1024 x 1024 pixel array, such that each pixel had a gray level range of from 0 (white) to 255 (black). For each section that was digitized, an empty field image was also digitized and subtracted from the section image to reduce grey-level variation within the image due to location in the field (Fischer and Bond, 1972).

Statistical estimation of bound ^{3}H-MISO

We estimated bound ^{3}H-MISO by counting grains overlying a minimum of 200 μm^2 of tissue. Autoradiographic development times were chosen based upon the period of exposure which produced maximum grain number with minimum grain overlap (fewer than 120 grains *per* 22.5 μm^2 grid square over the area of greatest activity). Comparisons of normoxic and hypoxic samples were performed under identical development and processing procedures.

Estimates of the minimum number of counts needed for statistical accuracy were patterned after England and Rogers (1970) and England and Miller (1970). In area A, where λ is the

theoretical grain density, the average number of grains is Ave(n) = λ(A). If λ_B is an estimation of background grain density and λ_{S+B} an estimate of sample activity including background, then the estimate λ_S of true sample activity has an average, variance, and coefficient of variation as follows:

$$\text{Ave}(\lambda_S) = \text{Ave}(\lambda_{S+B}) - \text{Ave}(\lambda_B) \quad \text{(Eq. 1)}$$

$$\text{Var}(\lambda_S) = \text{Var}(\lambda_{S+B}) + \text{Var}(\lambda_B) \quad \text{(Eq. 2)}$$

$$CV(\lambda_S) = \sqrt{\frac{1}{\lambda^2_S}\left(\frac{\lambda_{S+B}}{A_{B+S}} + \frac{\lambda_B}{A_B}\right)} \quad \text{(Eq. 3)}$$

The acceptable coefficient of variation for the grain count was set at 0.10. N, the number of grains that must be counted to maintain this coefficient of variation, was derived from:

$$N = \frac{1}{(CV)^2}\left(\frac{\rho^2 + 2\rho + 2}{\rho^2}\right) \quad \text{(Eq. 4)}$$

where ρ was estimated by the ratio λ_S/λ_B (England and Miller, 1970). Typically, the number of grains that needed to be counted for statistical validity approximated 500 grains *per* sample.

Background counts *per* unit area were obtained from the emulsion layer just coronal to the molar or incisal edge, where no underlying tissue was present.

We estimated true grain counts *per* unit area (λ_S) by subtracting existing background counts (λ_B) from the total sample activity, which is in reality λ_{S+B} (Eq. 1). Therefore, the standard deviation of λ_S was calculated with propagation of error by taking the square root of the variance of the total sample activity plus the variance of the background counts (Eq. 2). Sample distributions were analyzed by Bartlett's test for homogeneity of variance. Since all populations were found to be normally distributed, all two-group comparisons were made with the *t* test, and all multiple group comparisons were made with ANOVA, with $p < 0.05$ accepted as significant (England and Miller, 1970). Where appropriate, *post hoc* ANOVA results had further pairwise analysis by Scheffé's F test (Scheffé, 1953).

Results

Both normoxic and hypoxic animals retained ^{3}H-MISO, as demonstrated by the significant increase in autoradiographic grain counts in these animals over the saline and unlabeled MISO controls (Table 1). In addition, hypoxic animals had a significant increase in the amount of ^{3}H-MISO retained when compared with their normoxic counterparts. The degree of ^{3}H-MISO retention in the normoxic animals was stable and consistent in all areas observed in the rat molar and incisor, with the exception of the ameloblasts and odontoblasts in the continually erupting incisor. The normoxic ^{3}H-MISO retention rate varied in these two cell types at different maturation stages.

Incisor ameloblasts

In the enamel organ, the region of greatest autoradiographic grain density was the ameloblast layer. The amount of ^{3}H-MISO retained significantly increased as the cells matured from the apex to the incisal edge (Figs. 1, 2). Although increased retention with cell maturation was seen in both the normoxic- and hypoxic-induced samples, hypoxia significantly increased the amount of ^{3}H-MISO retained at each stage of development when compared with similar morphologic sites in normoxic animals (compare Fig. 1 with Fig. 2 and Table 2). As can be noted from Figs. 1 and 2, increased ^{3}H-MISO retention occurred in the stratum intermedium and papillary layers of the enamel organ, although to a lesser extent than in the ameloblast layer. Because it was difficult to distinguish these two layers distinctly in 4-μm autoradiographs throughout the entire length of the incisor (Warshawsky and Smith, 1974), we quantified only the ameloblast layer (Table 2).

Incisor odontoblasts

In contrast to ameloblasts, odontoblasts retained more ^{3}H-MISO in the immature cell type. Pindborg and Weinmann (1959) divide the rat incisor into 5 morphologic sectors from the apex to the incisal edge. The amount of ^{3}H-MISO retained in the odontoblast layer was significantly higher in sectors 1 and

Table 1. Grain counts in coronal molar pulp

Group	Mean[a] ± SD	
	Normoxic	Hypoxic
Experimental (^{3}H-MISO)	82 ± 16[b] (n = 9)	345 ± 51[b,c] (n = 9)
Unlabeled MISO	2 ± 8 (n = 4)	2 ± 5 (n = 4)
Saline controls	0 ± 7 (n = 2)	0 ± 8 (n = 2)

[a] Sample mean count *per* 100 μm^2 corrected for background [*i.e.*, Sample Ave(λ_S)].
[b] Samples that were significantly different from saline control animals (ANOVA, Scheffé *post hoc* test at $p < 0.05$).
[c] Samples that were significantly different from normoxic animals at $p < 0.05$.

There were no significant differences between saline and unlabeled MISO control groups.

Table 2. Grain counts in incisor ameloblasts

Region	Mean[a] ± SD (n = 9 for each group)	
	Normoxic	Hypoxic
Apical third	38 ± 5[c]	187 ± 31[b,d]
Middle Third	60 ± 8[c]	334 ± 42[b,d]
Incisal Third	124 ± 22[c]	521 ± 61[b,d]

[a] Sample mean count *per* 100 μm^2 corrected for background [*i.e.*, Sample Ave(λ_S)].
[b] Hypoxic samples that were significantly different from similar morphologic normoxic samples (ANOVA, Scheffé's test at $p < 0.05$).
[c] Normoxic regions that were significantly different from each other at $p < 0.05$.
[d] Hypoxic regions that were significantly different from each other at $p < 0.05$.

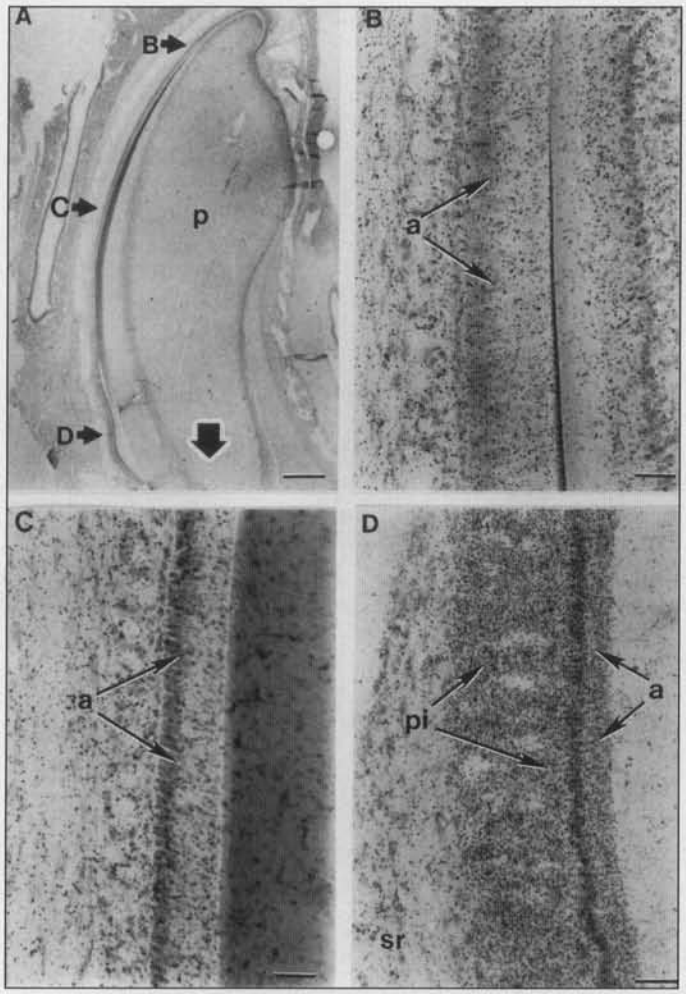

Figure 1. Normoxic rat incisor. (A) Low-power view of the incisor (original mag., 26x). Apical, middle, and incisal thirds of the ameloblast layer are labeled with lettered arrows, enlarged, and shown in B, C, and D, respectively. Large arrow points to direction of eruption, *i.e.*, toward incisal edge. (B) Enlarged view of ameloblasts (a) in the apical third. (C) Enlarged view of ameloblasts (a) in the middle third. (D) Enlarged view of ameloblasts (a) in the inicsal third. Note increased ^{3}H-MISO retention from apical to incisal third (see Table 2) and compare with similar areas in the hypoxic incisor in Fig. 2. There is also increased ^{3}H-MISO in the papillary and stratum intermedium layers (pi) compared with the stellate reticulum (sr). p = pulp; a = ameloblasts; pi = papillary and stratum intermedium layers; sr = stellate reticulum. Scale bar is 380 μm for A and 20 μm for B, C, and D.

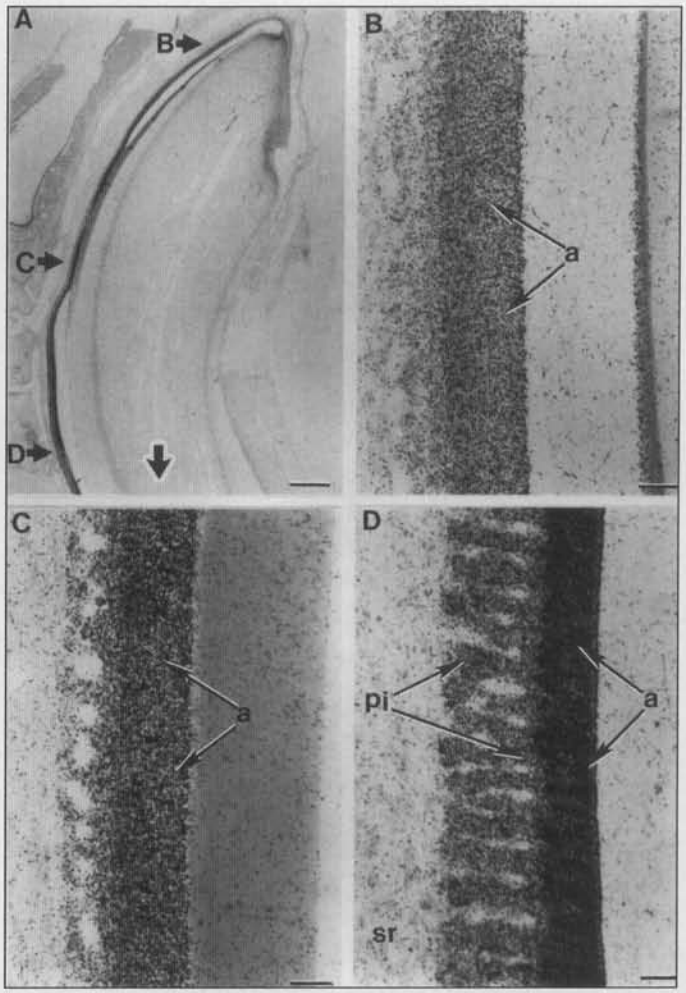

Figure 2. Hypoxic rat incisor. (A) Low-power view of the incisor (original mag., 26x). Apical, middle, and incisal thirds of the ameloblast layer are labeled with lettered arrows, enlarged, and shown in B, C, and D, respectively. Large arrow points to direction of eruption, *i.e.*, toward incisal edge. (B) Enlarged view of ameloblasts (a) in the apical third. (C) Enlarged view of ameloblasts (a) in the middle third. (D) Enlarged view of ameloblasts (a) in the incisal third. Note increased ^{3}H-MISO retention from apical to incisal third. Compare with similar areas in the normoxic incisor in Fig. 1. Hypoxia increased the amount of ^{3}H-MISO retained when compared with corresponding areas in normoxic incisor (see Table 2). a = ameloblasts; pi = papillary and stratum intermedium layers; sr = stellate reticulum. Scale bar is 380 μm for A and 20 μm for B, C, and D.

2, which extend from the apex to the point where enamel formation starts, than in the remaining 3, more incisal, sectors (Fig. 3). Past sector 2, the amount of ^{3}H-MISO retained in odontoblasts abruptly decreased and stayed constant at this lower level throughout the remainder of the incisor. Odontoblasts from hypoxic animals retained significantly more ^{3}H-MISO at each stage of development than odontoblasts from comparable morphologic sites in normoxic animals (Table 3).

Molar pulp and periapex

When morphologically equivalent sites were compared, hypoxic animals always had statistically significant increased ^{3}H-MISO retention over normoxic animals. In addition, specific hypoxic sites retained more ^{3}H-MISO than other hypoxic areas within the same animal. Specifically, regions where intense hypoxic cell labeling occurred were: (1) molar pulp horn odontoblasts (Fig. 4), (2) osteocytes in the periradicular bone (Fig. 5), and (3) cementocytes (Fig. 6) and the periodontal ligament directly surrounding them (Fig. 7). Osteocytes and cementocytes displayed intense ^{3}H-MISO retention in all hypoxic samples examined. In contrast, the pulp horn odontoblasts and periodontal ligament displayed intense hypoxic ^{3}H-MISO retention only sporadically.

^{3}H-MISO retention was uniformly distributed throughout the central and peripheral regions of molar

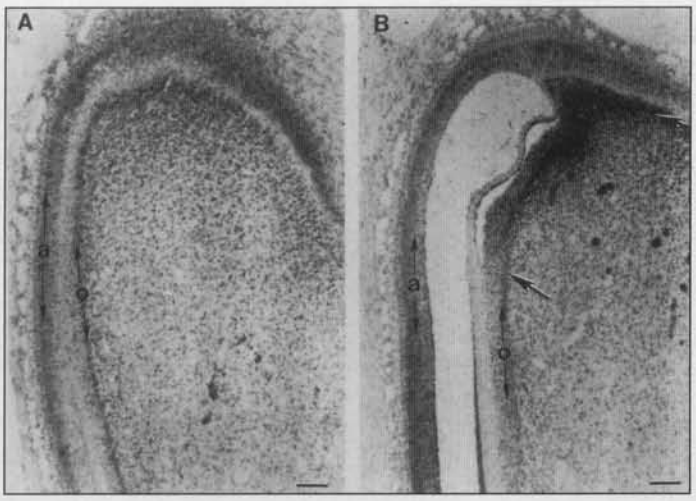

Figure 3. Incisor odontoblasts. (A) Autoradiograph of apical area from normoxic animal. (B) Autoradiograph of apical area from hypoxic animal. Note intense ^{3}H-MISO retention in immature odontoblasts (large arrows). a = ameloblasts; o = odontoblasts. Scale bar is 30 μm for both A and B.

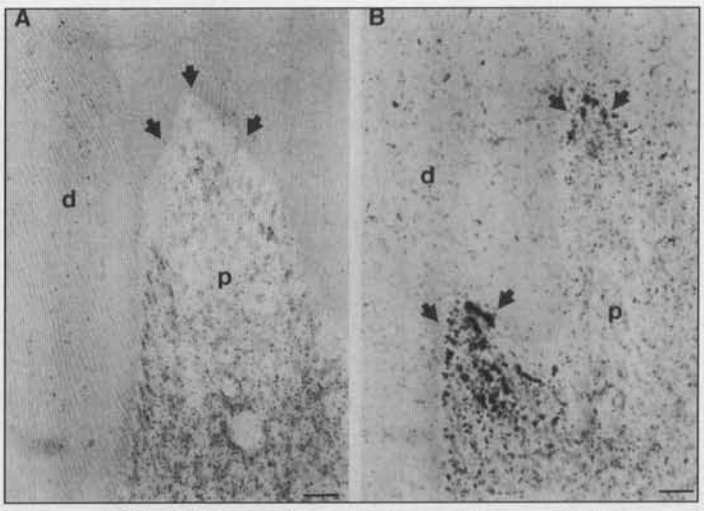

Figure 4. ^{3}H-MISO retention in molar pulp horn. (A) Normoxic molar pulp horn (arrows). (B) Hypoxic molar pulp horn. Note intense labeling compared with normoxic horn (arrows). d = dentin, p = pulp. Scale bar is 20 μm for both A and B.

pulp. The only exception occurred in hypoxic pulps when occasional clusters of pulp horn odontoblasts had intense ^{3}H-MISO labeling (Fig. 4). In normoxic molar pulp, there was no statistical difference in the degree of ^{3}H-MISO retention between odontoblasts and the cells of the central pulp (Table 4). This was also true for hypoxic molar pulp when the intensely labeled hypoxic pulp horn odontoblasts were excluded. There was always a statistically significant increase in ^{3}H-MISO retention from normoxic to hypoxic pulps, regardless of the region examined (Table 4).

Neither cell necrosis nor inflammation was ever observed histologically in any of the samples examined, which supports the conclusions of Cobb *et al.* (1989a) that MISO at a dose of 75 mg/kg does not produce acute cytotoxic effects.

Discussion

MISO was originally thought to bind in significant amounts only in hypoxic tissues, since, in normoxic tissues, oxygen would prevent formation of the reactive species (Franko, 1986). Cobb *et al.* (1989a,b) conducted an autoradiographic survey of normoxic tissues in the mouse and found that a number of tissues accumulated ^{3}H-MISO intensely, even under putative normoxic conditions. The study by Cobb *et al.* (1989a,b) surveyed normoxic retention only and found that (unspecified) cells of the enamel organ retained ^{3}H-MISO at a significantly greater rate than stroma. Data from the present study found that normoxic tissue retains ^{3}H-MISO and that the rate of retention varies in some cells. However, the normoxic retention rate in all regions was always significantly lower than that found in similar regions from hypoxic animals.

In general, the bioreduction of MISO to the hydroxylamine required for cellular binding is dependent upon both the number of reducing equivalents available in the tissue (oxygen-independent binding) and the relative oxygen tension in the cell (oxygen-dependent binding) (Parliament *et al.*, 1992). The experimentally induced hypobaric hypoxia created an environment that facilitated increased MISO binding to the dental pulp and surrounding tissues. Hypoxia induction does not appear to affect

Table 3. Grain counts in incisor odontoblasts

Region	Mean[a] ± SD (n = 9 for each group)	
	Normoxic	Hypoxic
Pre-odontoblasts (from Sector 1)	115 ± 18[c]	518 ± 63[b,d]
Odontoblasts (from Sector 3)	75 ± 10[c]	337 ± 41[b,d]

[a] Sample mean count *per* 100 μm^2 corrected for background [*i.e.*, Sample Ave(λ_S)].
[b] Hypoxic samples that were significantly different from similar morphologic normoxic sample (*t* test at $p < 0.05$).
[c] Normoxic regions that were significantly different from each other at $p < 0.05$.
[d] Hypoxic regions that were significantly different from each other at $p < 0.05$.

Table 4. Grain counts in molar pulp by cell type.

Region	Mean[a] ± SD (n = 9 for each group)	
	Normoxic	Hypoxic
Odontoblasts	88 ± 15	352 ± 58[b]
Central pulp	77 ± 12	341 ± 51[b]

[a] Sample mean count *per* 100 μm^2 corrected for background [*i.e.*, Sample Ave(λ_S)].
[b] Hypoxic samples that were significantly different from similar morphologic normoxic samples (*t* test at $p < 0.05$).

There was no significant difference between odontoblasts and coronal central pulp within the same animal in either the normoxic or hypoxic samples; intensely labeled hypoxic pulp horn odontoblasts were excluded.

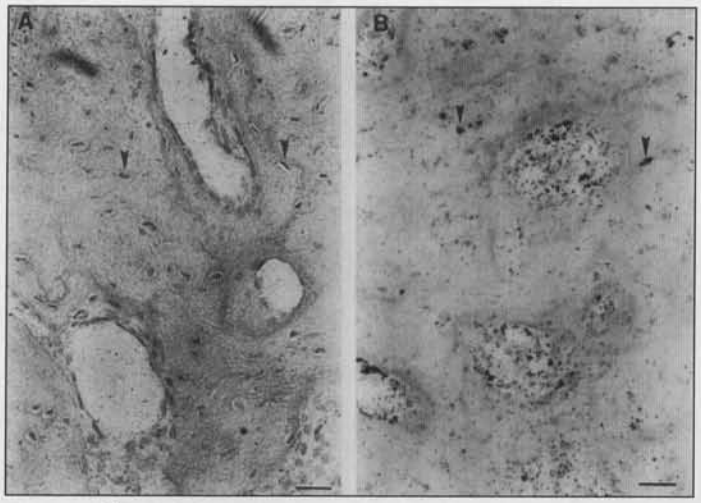

Figure 5. ^{3}H-MISO retention in periradicular osteocytes. Arrows indicate osteocytes in a normoxic animal (A) as compared with a hypoxic animal (B). Note increased ^{3}H-MISO retention with induced hypoxia. Scale bar is 20 μm for both A and B.

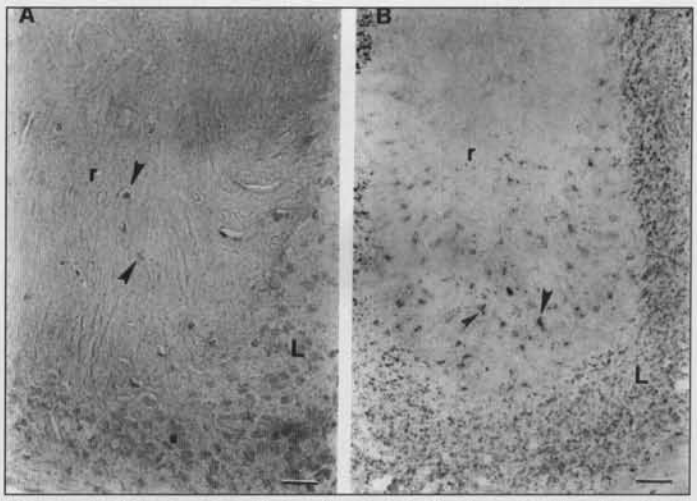

Figure 6. Cellular cementum. Arrows indicate cementocytes in a normoxic animal (A) as compared with a hypoxic animal (B). Note increased ^{3}H-MISO labeling in hypoxic cementocytes and periodontal ligament. r = root; L = periodontal ligament. Scale bar is 20 μm for both A and B.

differences in oxygen-independent MISO retention among tissues due to their different reducing capacities. The observations that hypoxia increased ^{3}H-MISO retention even when the normoxic, oxygen-independent retention rate varied (as in maturing ameloblasts and odontoblasts in the incisor) demonstrate that hypobaric hypoxia mediated oxygen-dependent binding of ^{3}H-MISO. The normoxic ^{3}H-MISO retention level should be considered a baseline value that is cell-type-specific. Differences in baseline normoxic values among tissues may account for the variations in autoradiographic grain densities noted by Cobb *et al.* (1989a) without invalidating MISO's use as a hypoxic cell marker.

The abrupt decrease in ^{3}H-MISO retention from pre-odontoblasts to odontoblasts may be related to their differentiation stage. Odontoblasts have mesodermal origins (Reith, 1968). Mesenchyme, *per se*, characteristically demonstrates low ^{3}H-MISO retention when compared with other tissues (Cobb *et al.*, 1989a; McManus *et al.*, 1989; Baumgardner *et al.*, 1994). The differentiation of odontoblasts may thus proceed along a path favoring lowered ^{3}H-MISO retention.

Factors other than differentiation might be involved with increased ^{3}H-MISO retention in maturing ameloblasts. Ameloblasts in the rat incisor: (1) undergo a phenotypic change to that of sulcular epithelium and (2) become atrophic.

Once enamel formation is complete, ameloblasts and outer layers of the enamel organ atrophy, fuse together into reduced-enamel epithelium, and eventually unite with the sulcular epithelium (Pannese, 1962). Epithelium has been shown (Cobb *et al.*, 1989a) to have a high ^{3}H-MISO retention level; we have noted the same phenomenon (unpublished data). Possibly, increased ^{3}H-MISO retention in maturing ameloblasts is related more to a change in cell function than to stage of differentiation. In this regard, ameloblasts either undergoing atrophy or converting to sulcular epithelium provide conditions which may encourage increased ^{3}H-MISO retention.

Oxygen tension in normoxic, developing bone has a strong correlation with functional needs (Brighton and Heppenstall, 1971). At the age of the animals in the present study, the periradicular bone and cellular cementum were already fully developed (Paynter and Hunt, 1964). Minor variations within samples from the molar periapex were seen primarily in hypoxic bone and cementum, with ^{3}H-MISO retention appearing to be most intense in cells farthest from the arterial blood supply. Based on these observations, increased ^{3}H-MISO retention in the periapex appeared related more to oxygen diffusion than to pre-existing oxygen tension differences based on cell function.

In the molar region of hypoxic animals, ^{3}H-MISO retention was most intense in cementocytes, osteocytes, periodontal ligament, and pulp horn odontoblasts. While cementocytes and osteocytes showed intense ^{3}H-MISO retention in all hypoxic samples, periodontal ligament and pulp horn odontoblasts were more inconsistent. These findings may be related to oxygen diffusion. Cementocytes and osteocytes are both contained within lacunae and derive oxygen and nutrients by diffusion. Periodontal ligament, being closer to the blood supply, is occasionally able to maintain a higher oxygen tension under hypoxic conditions. However, cementocytes and osteocytes, being farthest from the blood supply, were never able to maintain a high oxygen tension in the hypoxic animals.

When molar odontoblasts retained ^{3}H-MISO intensely, it was in hypoxic animals and only in the pulp horn area. However, as was the case with the periodontal ligament, not all hypoxic pulp horn odontoblasts retained ^{3}H-MISO intensely. This inconsistency may lie with their unique and elaborate capillary network. The capillaries range from 4 to 8 μm and are located just medial to the cells (Takahashi *et al.*, 1982). The pattern of intense ^{3}H-MISO retention in pulp horn odontoblasts, when it occurred, followed the distribution of the subodontoblastic capillary plexus (Fig. 4). If this premise is correct, ^{3}H-MISO retention could have occurred as a result of temporary closure of the capillary

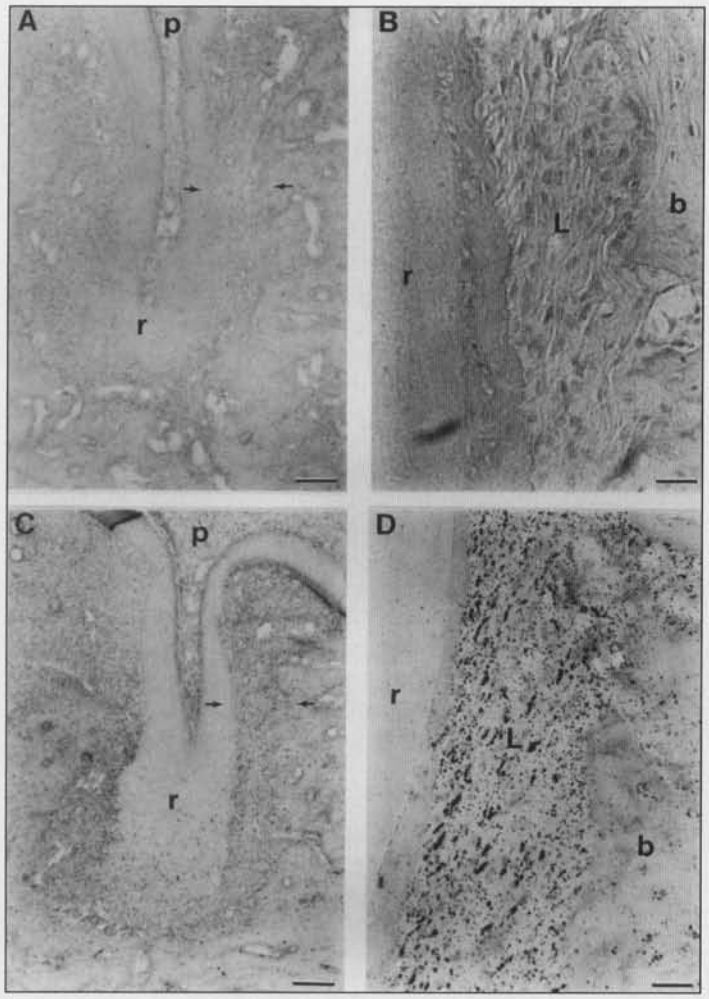

Figure 7. Molar root and periodontal ligament. (A) Molar root and surrounding periodontium from a normoxic animal. Area between arrows enlarged and shown in B. (B) Normoxic periodontium. (C) Similar area as in A but from a hypoxic animal. Area between arrows enlarged in D. (D) Hypoxic periodontium. Note intense ^{3}H-MISO retention as compared with B. p = pulp; r = root; L = periodontal ligament; b = bone. Scale bar is 68 μm for A and C and 20 μm for B and D.

bed, presumably from hypobaric pressure changes.

Brown (1979) has defined two classes of hypoxic cells, acute and chronic. Acutely hypoxic cells exist in tumors because of temporary closure of blood vessels (Brown, 1979) and are scattered randomly throughout the tumor mass (Franko, 1986). Chronically hypoxic cells, however, are localized to areas some distance from blood vessels. These chronically hypoxic cells occurred in tumors as a result of rapid cell growth beyond the ability of blood vessels to keep pace (Tannock, 1968).

Acutely and chronically hypoxic cells have been described for tumor tissues only. By analogy to the present study with the hypobaric-hypoxic system, osteocytes and cementocytes might be considered chronically hypoxic due to their positions distant from nearby vasculature. Minor disturbances in the oxygen supply likely subject these tissues to an immediate and intense hypoxic incident. Pulp horn odontoblasts and periodontal ligament, however, would be acutely hypoxic, labeling only randomly when physiologic conditions of brief vasospasm or constriction coincided with optimum ^{3}H-MISO availability.

In any investigation of this type, once baseline normoxic ^{3}H-MISO retention values of a tissue are established, then comparisons with a hypoxic condition can be made. Using hypobaric hypoxia-inducing techniques similar to those used in this study, MacManus *et al.* (1989) reported a range of 1.8 times more ^{3}H-MISO retained in a hypoxic heart to 2.9 times greater retention in a hypoxic spleen. In dental pulp, hypoxia caused an increased ^{3}H-MISO retention of approximately 1.6 times, when a liquid scintillation counting technique was used (Baumgardner *et al.*, 1994). The variation in increased hypoxic retention among tissues could be due to saturation of that tissue's MISO-reducing capacity. However, differences may result from effective regional blood flow that a particular tissue is able to secure for itself in times of oxygen deprivation.

Effective blood flow is defined as that level necessary to maintain cells in the normoxic state (Baumgardner *et al.*, 1994). Studies on blood flow typically cannot correlate changes in flow with effects on cellular oxygen perfusion. In this regard, ^{3}H-MISO appears useful for the examination of dynamic changes in cellular oxygen perfusion under hypoxic conditions. With the techniques developed in this paper, it may now be possible to examine conditions which create change in blood flow (cavity preparation, inflammation, trauma, etc.) and then correlate the effects of these changes on the effective blood flow to that region.

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