Studies on Hypoxia: II. Autoradiographic Quantitation of Proline-³H Incorporation by Connective Tissue Cells of the Neonatal Hamster

DONALD M. SMITH* and SEONG S. HAN

Departments of Oral Biology and Pedodontics, School of Dentistry, and Department of Anatomy, Medical School, The University of Michigan, Ann Arbor, Michigan

Quantitative evaluation of autoradiographic grains from anoxia-treated and control neonatal hamster indicated that anoxia impairs collagen synthesis and significantly suppresses the incorporation of proline-³H in the odontoblast, fibroblast, and osteoblast by 24 hours after the insult. However, the rapid and apparently total recovery of the dental and paradental connective tissue cells suggests that anomalies of dentition, heretofore attributed to brief spells of anoxia at birth, may not be due to such a hypoxic insult during the perinatal period.

Hypoxic and anoxic experiences of the fetus have been considered as etiologic factors in the malformation of body structures that becomes manifest during the subsequent development of the organism. Many developing and mature organs and tissues have been studied experimentally under conditions of low oxygen pressure to determine the various physiologic,[1-9] metabolic[10-26] and morphologic[27-33] alterations that result.

However, little has appeared in the literature concerning the immediate effects of anoxia on the connective tissue cells and, more specifically, on the synthesis of collagen, which is intimately involved in the matrix formation of dentin, bone, and other connective tissues.[34-36]

With regard to protein synthesis, Turner and Turner[37] reported decreased protein synthesis in certain glandular tissues after exposure to low oxygen tension. Morawa and Han,[38] in a recent survey of histologic changes after a prenatal insult by hypoxia, observed that epidermal cells were poorly differentiated and that subcutaneous fibroblasts were reduced in size and cytoplasmic basophilia. They hypothesized possible detrimental effects of hypoxia on protein biosyntheses.

The purpose of the present study was to determine, by means of quantitative autoradiography, the effect of anoxia on the synthesis of collagen and related proteins of dental and paradental connective tissues in the neonatal hamster. Tritiated proline was selected as the precursor of choice, since it is one of the unique amino acid constituents of collagen[39] and has been used in many previous studies[35,42,43] dealing with the biosynthesis of connective tissue matrixes.

Materials and Methods

ANIMALS.—A total of 14 newborn golden hamsters (*Mesocricetus auratus*) from three litters was used for this study. The hamster was used because of its short gestation period (16¼ days) and rapidly developing dentition and because, in the first molar, the morphologic features and level of development at birth are similar to that of the human being.[40] Also, the body size of the hamster minimized the amount of isotope required.

All adult hamsters were maintained on a stock ration* and tap water ad libitum. The young were raised by their respective dams for the duration of the experiment.

RADIOISOTOPE.—Proline, tritiated in the form of L-proline, 4-³H (proline-H³),† was the radioactive precursor given. The specific activity was 5 curies per millimol. The pre-

* Mott Foundation Clinic, Flint, Mich.

* Ralston-Purina, St. Louis, Mo.

† New England Nuclear Corp., Boston, Mass.

cursor was diluted in physiologic saline solution and injected into the neonatal hamsters.

EXPERIMENTAL DESIGN.—Fourteen neonatal hamsters were allotted equally to control and experimental groups. The experimental animals were put in an open container under a bell jar that was flushed with 100% N_2 for 30 minutes. The hamsters were removed from the bell jar, immediately given injections of the precursor ($5\mu c$/gm of body weight), marked for identification, and returned to their respective dams. The control hamsters were treated identically, except that during the period within the bell jar an adequate supply of fresh room air was available for normal respiration. One each of the control and experimental hamsters was decapitated at 15 minutes; at 1, 6, and 24 hours; and at 3, 7, and 14 days after proline-H³ was given.

PREPARATION OF TISSUES.—The head of each hamster was sectioned into quarters by a slice through the midsagittal plane and the transverse plane between the jaws. In the smallest specimens, the transverse slice was made through only the right half of the head, leaving the left half intact. The specimens were immediately fixed in Bouin's solution for 48 hours. After fixation, each was decalcified in a 0.5M solution of versene adjusted to pH 7.5 with HCl at room temperature. The time in the versene varied with the age of the hamster.

After decalcification, specimens were washed overnight in running tap water and processed for embedding as described by Culling.[41] They were vacuum-embedded in paraffin so that sections could be cut in a plane through the developing upper molars. Serial sections, 8μ thick, were made on a rotary microtome and fixed to microscope slides that previously had been cleaned and treated with a subbing solution of distilled water (100.0 ml), pure gelatin (0.5 gm), and chromium potassium sulfate (0.05 gm).

AUTORADIOGRAPHY.—Slides were allotted to three sets by taking every third slide of the serially sectioned materials. Thus, slides 1, 4, 7, and so on, comprised the first set, while the second and third sets were made up of slides beginning with slide 2 and slide 3, respectively. Slides in the first set were used for autoradiography. These slides were cleared of embedding material by passing them through baths of xylene, were hydrated through a series of graded alcohol, and were

held in distilled water. In complete darkness, each slide was dipped for five seconds in emulsion* (preheated to 45C), was placed on end at a 60° angle, and was allowed to dry for 30 minutes in an oven maintained at 45C. After drying, the slides were removed from the oven and placed in a regular plastic slide box that contained small packs of moisture inhibitor.† The box was then double wrapped in sheet lead, wrapped again in brown paper, and sealed in a plastic bag containing inhibitor packs. This was placed in a refrigerator (4C) for four to six weeks for autoradiographic exposure.

After the exposure period, the slides were removed from the plastic box and developed in Dolmi (2,4 diaminophenol dihydrochloride developer) without safelights.

The developing schedule was as follows: (1) Dolmi developer at room temperature, 1 minute; (2) two rinse baths of distilled H_2O, 1/2 minute each; (3) two baths in fixative, 1 minute each; and (4) tap water rinse, 15 minutes.

The slides were then stained in Harris' hematoxylin and acidic eosin Y and mounted.

The radioactive grain counts were made (1) of odontoblasts of the region where the cells had fully differentiated and dentinogenesis had been initiated, as indicated by the deposition of the matrix, (2) of randomly selected pulp fibroblasts of the first molars, and (3) of osteoblasts from the alveolar bone adjacent to the teeth. The average number of grains was obtained by counting silver grains in approximately 20 or more cells of each type per sample, and standard deviations were calculated.

The second set of slides was stained with hematoxylin and eosin, and the last set was stained with Masson's trichrome.

Results

Unlike the prolonged developmental effect after prenatal hypoxia, various connective tissue cells in this study showed only limited modifications. Especially difficult was the evaluation of possible effects on fibroblastic structures, as fibroblasts varied greatly in appearance and in the amount of cytoplasmic basophilia in both the control and experimental hamsters. In odontoblasts and osteoblasts, however, certain changes tended to

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* Kodak NTB-3, Eastman Kodak Co., Rochester, NY.

† Drierite, W. A. Hammond Drierite Co.,

support data from quantitative autoradiography. These changes were seen within hours after the insult and will be discussed later.

FIFTEEN MINUTES.—Fifteen minutes after termination of the insult and injection of proline-³H (given immediately after the insult), dental tissues of the experimental hamsters appeared similar to those of the controls. The size of pulp capillaries and the appearance of odontoblasts in the experimental hamsters could not be distinguished from those of the controls. The same was true with fibroblasts and osteoblasts. Grain counts in both groups were low and did not differ.

ONE HOUR AND SIX HOURS.—The pulp capillaries of the anoxic hamsters tended to contain more red blood corpuscles, but there was no apparent enlargement of the lumens. The negative image of the Golgi apparatus of the odontoblasts in the anoxic hamsters was smaller, and there were fewer in hamsters killed after one and six hours (Fig 1, 2). Difference could not be ascertained in other types of cells.

The average numbers of grains in the odontoblasts, fibroblasts, and osteoblasts, however, were lower in the anoxic hamsters than in the controls (Fig 3–5). Although the number of grains six hours after proline-³H injection increased in all three types of cells, there was a greater (but still insignificant) difference between experimental and control hamsters only in odontoblasts and fibroblasts. No difference was observed in the osteoblasts.

TWENTY-FOUR HOURS.—The pulp of the experimental group had large and dilated capillaries engorged with red blood corpuscles. The nucleus of the odontoblast was smaller in experimental hamsters than it was in the control, and the cell body was thinner. The negative Golgi image continued to be less noticeable in the anoxic hamsters. The cytoplasm of odontoblasts and ameloblasts was somewhat less dense, and the cells in an adjacent region often were more separated from one another. The only change in fibroblasts was a reduction in size of the negative image of the paranuclear Golgi apparatus, which was not too clear.

For the first time, the numbers of grains were significantly less in all three cell types in the anoxic hamsters group (Fig 3–5). The grain number in odontoblasts in the experi-
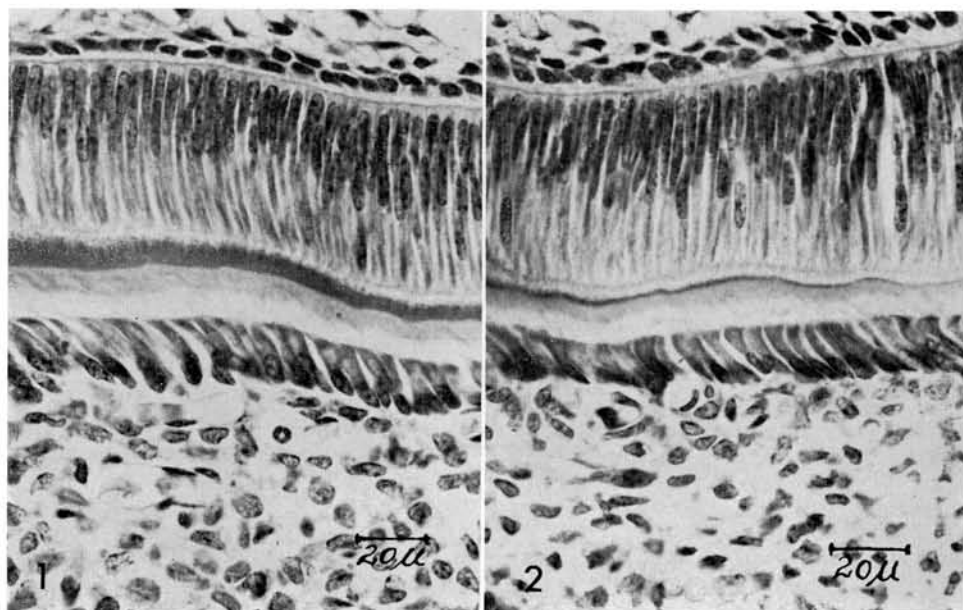

FIG 1.—Cross section through dental tissues of a developing first molar in a control hamster (H&E stain, orig mag ×535).

FIG 2.—Cross section through dental tissues of a developing first molar in an experimental hamster six hours after anoxic insult (H&E stain, orig mag ×535).
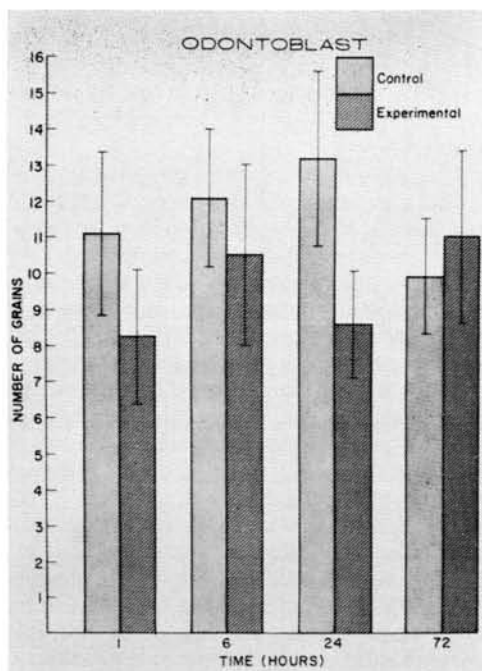
Fig 3.—Average number of grains per cell in experimental and control odontoblasts at varying periods after proline-³H injection. The range indicates the standard deviation of arithmetic means.
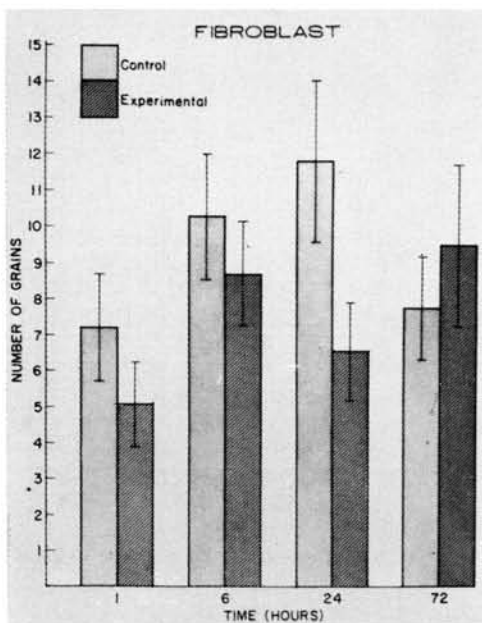

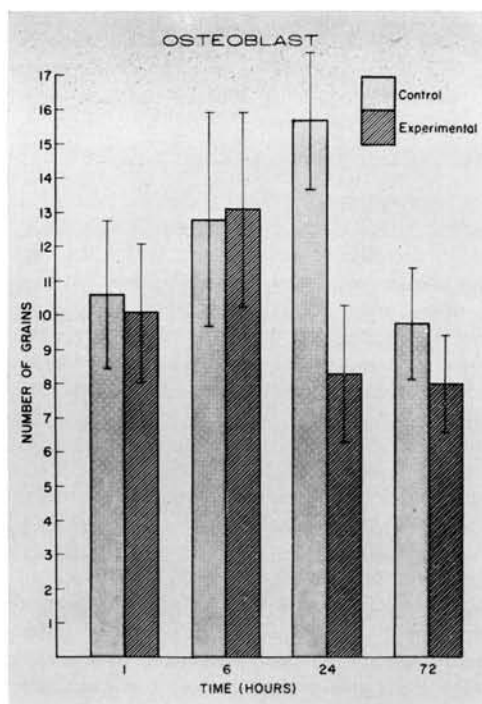Fig 5.—Average number of grains per cell in experimental and control osteoblasts.

Fig 4.—Average number of grains per cell in experimental and control fibroblasts.

mental group was about 40% lower (8.6 per cell) than the average grain count of the control (13.2 per cell). Fibroblasts also showed a similar difference, in that the average number in the control hamsters was 11.8 per cell; whereas, the experimental hamster had only 6.5 grains per cell. The greatest difference was seen in osteoblasts, which had an average grain number of 15.7 per cell for the controls as opposed to 8.29 per cell for the experimental hamsters. In all cell types, therefore, there was a significant difference between the control and experimental groups as well as a definite absolute decrease in grain counts in the experimental group at 24 hours when compared to the counts at six hours. The control values continued to increase.

THREE DAYS.—Because both groups were histologically similar, no further description is given.

The grain counts at three days did significantly differ between the experimental and control groups. This was reflected by an overall increase, or at least no further decrease, in grain numbers of the experimental group.

The total counts in the control group, however, decreased to a level comparable to that of the experimental group. This similarity between the two groups was maintained after 7 to 14 days.

Discussion

Quantitation of silver grains appearing in autoradiographs provides a unique opportunity for studying functional and biochemical modifications of tissues at the level of individual cells. Coupled with histologic or cytologic observation, the result obtained from such quantitative efforts may represent the best reflection of functional modifications in a given cell in situ. For instance, this study demonstrated a statistically clear difference in grain counts between the control and experimental hamsters. Cytologic changes were minimal.

Previously, two separate studies of the available proline-^{3}H levels in the blood of the hamster, made by liquid scintillation technics,[42,43] indicated that the level of blood ^{3}H remains at a peak during several hours after the injection of proline-^{3}H and declines gradually during the days that follow. In view of such changes in ^{3}H levels, the interpretation of quantitative data from autoradiographic works should be made with respect to the changing levels of available precursors. Thus, the differences among control animals killed at different intervals might be regarded as directly or indirectly reflecting changes in the blood level of ^{3}H. It is also possible that the gradual increase in grain counts of the cells in control animals might be due partly to the limited resolution of light microscopic autoradiography; ie, the precise determination of the cell border is almost impossible. Hence, some of the grains included in counting could belong to already secreted proteins in the immediate vicinity of the cells.

Keeping these consideration in mind, the relative difference between experimental and control hamsters, which was most pronounced and significant 24 hours after the anoxic insult and the injection of radioactive precursors, may be taken as evidence for a detrimental effect of the insult on the capacity of various connective tissue cells to synthesize fibrous proteins, specifically, collagen. The insignificant difference observed on the third day, and thereafter, suggests the temporary nature of such effect. If this is true (ie, if the statistically significant effect occurs

during the early period and a full recovery is achieved by day 3), then one must differentiate the specific influence of the anoxia from the stress effect that is related to the changing levels of adrenocorticosteroid hormones.

Direct information remains to be gained from future research. However, the theory that the effect probably is not due to modified adrenocortical functions can be reasoned because (1) previous in vitro works and perfused liver studies have also shown a significant reduction in protein synthesis, and (2) the adrenal cortex in the neonatal hamster is not functioning at the same level as in a mature hamster.

It is significant that there were differential effects of the insult on the three types of connective tissue cells. After 24 hours, the osteoblast was most affected and the odontoblast was least affected by anoxia. This might reflect the different rates of collagen synthesis that occur in these cell types. It is tempting to speculate further that such differential impairment of protein biosyntheses in various cells, at a crucial time during cellular differentiation, might be responsible for the metabolic interference occurring in fetal tissues during prenatal hypoxia, as the normal differentiation and growth of cells are implemented by rapidly evolving and changing patterns of enzyme systems, which are proteinaceous.

These, along with the hypothesis proposed by Morawa and Han,[38] can be taken as indicative that hypoxia-related malformations might be due partially to disruptions in the normal pattern of protein syntheses necessary for a controlled sequential evolution of various protein moieties in development. In this sense, the present results offer an alternative to previous views (expressed by Grabowski[29] and others), that the accumulation of lactic acid results in the chemical disturbance of metabolism or mechanical interference with tissue growth. These concepts may not necessarily contradict each other, but rather may be considered as ideas that represent some of the many complicated facets of anoxic effects which are yet to be exploited.

The quick recovery of temporary impairment in protein synthesis should be considered in view of the classical consensus that some of the hypoplasia and anomalies of permanent dentition are traced and as-

cribed to the difficulties in breathing for a few minutes during the perinatal period. Considering the direct dependence of the brain and nervous tissues on the presence of oxygen, any perinatal episode of anoxia might be more readily expressed as disturbed nervous functions than as disturbances in the growth and differentiation of the dental structure.

Biochemical studies on the adverse effect of anoxia have been exploited in many different situations, with variable results.[10-26] With respect to protein synthesis, studies have shown a differential effect of anoxia on various tissues.[17,18] The pancreas had increased amino acid incorporation; whereas, in other tissues, an inhibition was noted. In an in vivo study, Turner and Turner[37] described an increased depression of amino acid incorporation into pancreatic protein fractions. This decrease paralleled a decrease in environmental oxygen tensions. Turner and Turner[37] concluded that this might be due to the diminished oxidative phosphorylation that is necessary for rapid amino acid incorporation by gland cells.

Sanders et al,[22] studying the respiratory metabolism and protein synthesis in rat tissues during hypoxia, reported that the efficiency of oxidative phosphorylation was not altered as the result of hypoxia, although there was decreased ATP concentration and decreased rate of incorporation of radioactive leucine into liver proteins. They suggested that even a moderate decrease in ATP content of cells might have serious effects on some cell functions. Based on a polarographic study of oxidative enzymes, Sanders et al[22] concluded that there was no lasting damage to the mitochondria. Villee et al,[26] after an in vitro study with human tissues, concluded that the resistance of fetal tissues to effects of anoxia might be due to a combination of several alterations in metabolic patterns, the summation of which results in an economy of oxygen.

Recent morphologic approaches to the acute effect of anoxia have been centered around the fine structural changes of the liver, muscle, and nervous tissues.[27,28,30-33] The increase in lysosome[27,28,32] and transient swelling of mitochondria[30-33] have been commonly observed changes in such tissues as the liver, heart, and brain.

Although attempts were not made in the present study to detect any possible ultra-structural changes in the cytoplasmic structure, an overall consideration of the previous works suggests that one might expect to see fine structural modifications related to the diminished incorporation of proline-^{3}H. In fact, preliminary electron microscopic evaluation of tissues in a similar experiment indicates that profound changes occur in the fine structure of fibroblasts and in pancreatic acinar cells of the neonatal animals subjected to acute anoxia.[44]

Conclusions

Results of this study demonstrated that the synthesis of connective tissue intercellular matrix (especially that of collagen) by odontoblasts, osteoblasts, and fibroblasts of dental and paradental regions is impaired within 24 hours after a single anoxic insult in the neonatal animal. However, the impairment is transient, and total functional recovery occurs within three days after the insult.

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