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Insulin-like Growth Factor Expression in Malnourished Rat Dental Pulp

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Abstract

This study evaluated the expression levels of IGF-I, insulin peptides, and their respective receptors, IGF-IR and IR, in the dental pulp of rats subjected to protein malnutrition. The nourished group (N) received a diet with 20% casein, while the malnourished group (M) received a diet with 5% casein. The renourished group (R) consisted of M group members maintained on the N group diet from the 22nd to the 60th day of life to evaluate the effects of renourishment. Dental pulps of the maxillary first molars were stained with hematoxylin and eosin, and picrosirius red, and were subjected to immunohistochemical tests. Malnutrition altered the odontoblastic layer and the collagen component of the dental pulp. IGF-I and IGF-IR expression levels were higher in the N group but there were no differences in the insulin expression levels between the groups. IR immunoexpression was higher in the N group compared with groups M and R. The results suggest that protein deprivation changed the constitution of the dental pulp and affected the factors that influence its development. The process of renutrition during puberty was not able to support the recovery of pulp morphology.

Keywords: Protein malnutrition; Wistar rat; dental pulp; IGF-I; Insulin

Introduction

A protein deficiency in rat diets during critical periods of tissue development has been associated with morphological changes in orofacial structures, including facial bones and teeth [1,2]. In dental tissues, malnutrition can cause odontogenic disturbances [3], delayed teeth eruption [4], and qualitative (hypomineralization or hypocalcification) and quantitative (hypoplasia) defects [5]. Population studies [6] and evidence from animal models [7] support the hypothesis that dental morphological changes caused by malnutrition tend to increase the susceptibility to tooth decay. In addition, some studies suggest that the restoration of a proper diet during an early life stage can reverse dental disorders induced by malnutrition [8,9]. Nutritional deprivation also causes changes in endocrine pathways, such as the insulin-like growth factor (IGF) pathways that are essential for tooth formation and are also involved in oral pathological processes [10]. The IGF system has autocrine and paracrine functions that regulate the proliferation and differentiation of dental tissues [11] act in their mineralization, and are involved in resorption-repair processes during tooth movement [12]. Likewise, it is believed that circulating insulin plays a crucial role during early tooth morphogenesis [13] and that the reduction in IGF-I levels can be paralleled by changes in collagen production in food-restricted animals [14]. Thus, the present study investigates the expression levels of IGF-I, insulin peptides, and their respective receptors, IGF-IR and IR, in the dental pulp of rats subjected to protein deprivation during the intrauterine and lactation periods through the end of the pubertal stage, as well as the effects of nutritional recovery during the period from weaning through the 60th day of life.

Materials and Methods

Animals and nutritional protocol

The animal procedures were approved by the Ethics Committee on the Use of Animals at the Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil. Young male and female Wistar rats (200–240 g) were mated for seven to ten days. After conception, the females were placed in individual cages and kept under standard conditions at 21°C with a 12 h light-dark cycle. During pregnancy and lactation, the animals had unrestricted access to water and to a purified diet (AIN-93 G) for rodents [15]. The diets for animals in the control or nourished (N) and the malnourished (M) groups contained casein at 20% and 5%, respectively (Rhoster, São Paulo, Brazil). After weaning, pups from both groups received their respective diets until sexual maturity (60 days).The renourished group (R) consisted of animals from the M group that were maintained on the N group diet from the 22nd to the 60th day of life.

Tissue preparation

After euthanasia in a CO_2 chamber, the maxillae of five male rats from each group, N, M, and R, were dissected en-bloc, fixed in a 10% tamponed formaldehyde solution for 48 hours and washed in running water for 48 h. Then, the specimens were decalcified in EDTA solution, dehydrated in an ascending alcohol series (70% to absolute) and embedded in paraffin. Longitudinal sections of 5 µm obtained from the maxillary first molars and surrounding tissues were stained with hematoxylin and eosin (HE), and with picrosirius red under polarized light to determine the types of collagen fibers [16].

Immunohistochemistry

The immunohistochemical reactions for IGF-I, insulin, IGF-IR, and IR were performed on sections obtained from three animals of each group (N, M, and R). Briefly, the specimens were deparaffinized, hydrated and covered with pepsin working solution for unmask the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections. Pretreated with 0.3% H_2O_2 in methanol (10 min) and washed for 5 min in PBS (0.1 M, pH 7.4). After blocking nonspecific binding (5% normal goat serum for 30 min in a humid chamber), the specimens were incubated for 2 h (22°C) with rabbit anti-IGF-I, anti-IGF Irβ, anti-insulin, and insulin Rβ specific antibodies (H-70, H-60, H-86, and C-19, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:250 dilutions. They were then washed in PBS (4x for 10 min), incubated with a biotinylated anti-rabbit IgG secondary antibody for 30 min and washed in PBS with Tween (0.01%, pH 7.4). The specimens were coated with streptavidin-biotin-peroxidase (ABC VECTAIN) for 30 min, washed in PBS, visualized by staining with diaminobenzidine chromogen (Vector Laboratories, Burlingame, CA, USA), washed in distilled water, dehydrated, immersed in xylene and mounted with coverslips and entelan. A negative control sample, omitting the primary antibody, was included in each assay.

Morphometry and Statistical Analyses

The specimens were examined under an optical microscope (Axioscop, Carl Zeiss, Göttingen, Germany), with a 40x magnification, connected to a computer. The images were captured using a camera (AxioCam) and analyzed with the Axiovision software. Five histological sections from three animals in each group (N, M and R) were selected for morphometric analysis. A 475-point test system was superimposed on the images, computing only the points that focused on the HE-stained nuclei (Tn, total number). Using the same test system, the number of cells expressing IGF-I, insulin, IGF-IR and IR were determined (Pn, partial number). The percentage of immune-reactive cells was determined using the formula (Pn/Tn) × 100. The statistical analyses were performed using an ANOVA and Tukey's test ($p<0.05$).

Results

Histology

On the periphery of the pulp, adjacent to the dentin, odontoblastic layers were observed in all groups. However, the oval-shaped odontoblasts containing granular cytoplasm and eccentric nuclei, present in the N group, were not observed in the M group in which cells were characterized by a fusiform morphology and intense cytoplasmic labeling. In this regard, the pulp cells of the R group resembled those of the N group, although they showed a lower staining intensity. In the N group, the organization of the odontoblast layer exhibited a parallel alignment of cells (in palisade), similar to the distribution pattern of dentinal tubules. There was a change in this pattern in the other groups (M and R). The following was also observed "flaws" in the cellular distribution in M (gaps) (Figure 1).

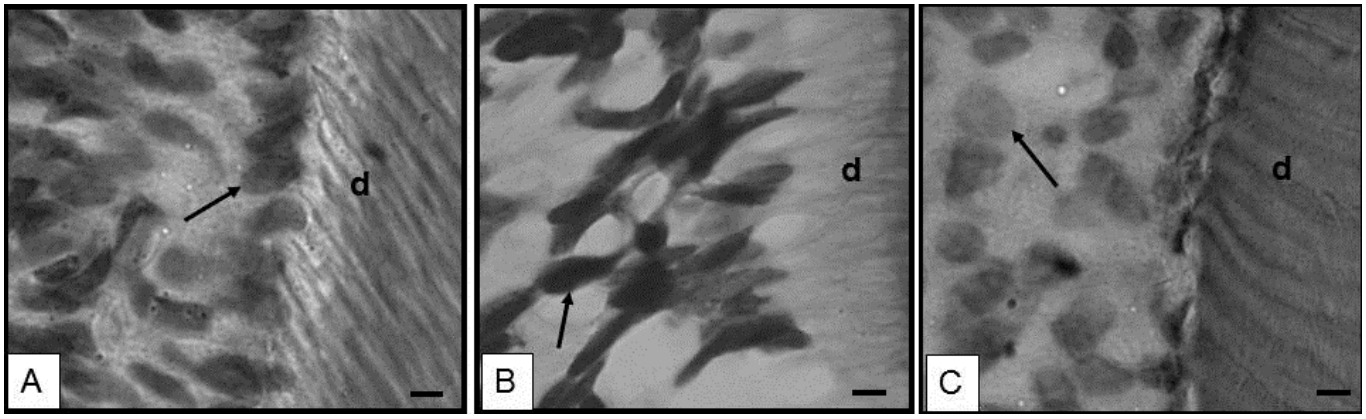

Figure 1: Peripheral region of the dental pulp of rats in the N (A), M (B) and R (C) groups. Note the different aspects of odontoblasts (arrows), as well as the arrangement along the dentinal tubules (d). (HE; Calibration bar: 4 μm).

In the sections stained with picrosirius red and analyzed under polarized light (Figure 2), the pulp tissue of nourished animals (N) was observed to be composed mainly of type I collagen (in red, orange or yellow). In the M and R groups, despite the presence of type I fibers, the tissue was composed predominantly of type III fibers (green).

Quantitative analysis

The statistical analysis showed that the expression levels of both IGF-I and IGF-IR were significantly higher for the N group compared to the M and R groups ($p=0.05$). There were no statistically significant differences ($p<0.033$) between the M and R groups. Although a trend towards a higher mean percentage of immunoreactive cells was detected in the dental pulp of the N group, there was no statistically significant difference between the groups (N, M and R). Additionally, the N group had the highest percentage of IR reactive cells, although it was only statistically different from the M group ($p<0.0002$). Figure 3 illustrates the pattern of cells that exhibited immunostaining for the factors IGF-I and IGF-IR in the dental pulp. Table 1 shows the mean percentages of markers in the different groups.

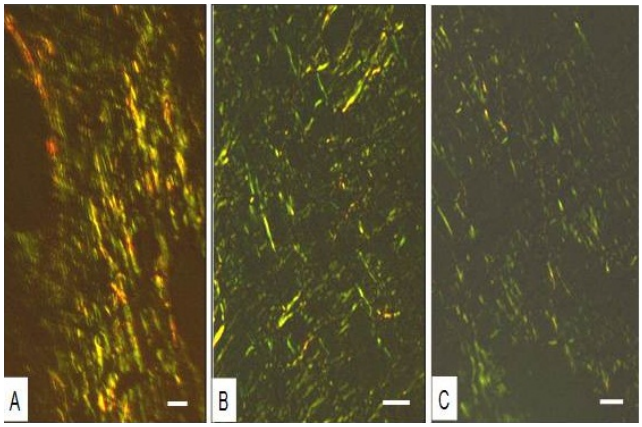

Figure 2: Central area of the dental pulp of rats in N (A), M (B) and R (C) groups. Note the predominance of type I fibers in group N and of type III in animals of groups M and R (picro-sirius red under polarized light; Calibration bar: 10 μm).

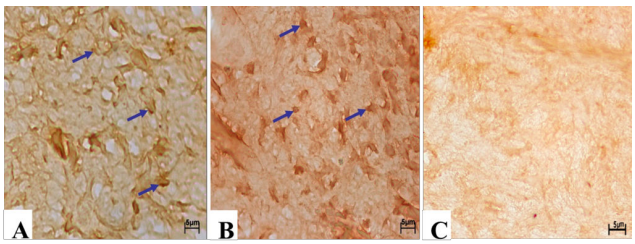

Figure 3: Representative micrograph of immunopositive cells (arrows) in rat dental pulp. IGF-I (A), IGF-IR (B) and negative control (C). (Calibration bar: 5 μm).

	N	M	R
IGF-I	78.4 ± 5.4*	65.3 ± 2.5* 65.29 ±	61.9 ± 7.2
IGF-IR	74.6 ± 16.0*	36.9 ± 12.8*	44.1 ± 10.9
Insulin	59.1 ± 13.2	50.8 ± 9.5	52.7 ±11.3
IR	62.4 ± 8.5*	24.7 ± 1.2*	27.0 ± 0.4*
			ANOVA* P<0.05

Table 1: Percentage means of immunohistochemical expression levels of IGF-I, IGF-IR, Insulin and IR in the dental pulps of nourished (N), malnourished (M) and renourished (R) rats. (Mean ± SD).

Discussion

The methodology used in the present study showed that protein malnutrition had an effect on the structure of dental pulp. Both the cell morphology as well as the organization and peripheral pattern distribution, which changed particularly in the M group, support the hypothesis that nutritional deprivation during the prenatal and postnatal phases leads to tooth pulp morphostatic loss, interfering with predentin production. Therefore, the insufficient morphological recovery observed in the R group may represent an adaptive mechanism of the mesenchymal cells of the dental pulp. Thus, we inferred that the changes detected in these animals are not irreversible since the complete molar development in rats occurs between 100–120 days [17] and our specimens were tested at 60 days. Collagen tissue is important in different organs because it promotes the resistance and firmness needed to support cells, ensuring that the extracellular matrix (ECM) performs its regulatory role in determining cellular shape and activities [18]. In the present used, we used the technique picrosirius that's according to Junqueira and Carneiro [16] is effective for this purpose and still nowadays is widely used in many tissues [1,19,20], including on the dental pulp [21,22]. It is possible that the prevalence of type III fibers in the pulp of malnourished animals (groups M and R) affects these structural functions and confers a degree of immaturity to the ECM. The latter could interfere with the differentiation of mesenchymal cells into odontoblasts, causing changes in dentin formation. This is due to the involvement of the ECM in epithelial-mesenchymal interactions during tooth morphogenesis and differentiation. Additionally, tooth development can be disrupted by mutations in collagen genes. Furthermore, bioengineering has shown that odontoblast differentiation from stem cell culture occurs only in the presence of the ECM system with a three-dimensional configuration from collagen [23]. The M group's odontoblastic layer, cell density, and types of collagen fibers were consistent with previously reported changes resulting from altered nutrition in 21-day-old animals [24]. The presence of IGF-I and IGF-IR in the dental pulp relates not only to embryonic events [25], but also to cell proliferation and differentiation processes present after injuries to the dentinopulpar complex that forms tertiary dentin. These activities affect pulp capping [26] and the treatment of apical perforations [27]. The essential regulator in the expression of IGF-I receptors is the nutritional status of the individual [28], a fact highlighted in the present study. In the N group, immunopositive cells for the peptide and its receptor were detected at similar percentages. Thus, considering the importance of IGF-I in the development of pulp and dentin formation, the function was preserved in the pulp of nourished animals, possibly regulating the mitotic activity or secretion of proteins active in dentinogenesis [29]. In the present study, the IGF-I/IGF-IR double immunopositive cells decreased in the groups exposed to malnutrition (M and R). The most plausible hypothesis takes into account the homology between the receptors of IGF-I and insulin. Thus, the increased immunoreactivity of IGF-IR indicates cross-reactivity with IR through changes in the affinity of the receptor for insulin or IGF-I [30]. In the present work, insulin and its receptor could also be detected in the dental pulp of rats, showing no statistically significant differences between groups. To our knowledge, this is the first study that describes the occurrence of these peptides in the pulp tissue. Considering that insulin is produced locally only in the pancreas, and that the hormone is widely distributed throughout the tissues of the body, we concluded that malnutrition had no effects capable of significantly altering the local hormone concentration. IR expression showed a higher concentration in the N group compared with the M and R groups, suggesting the maintenance of insulin-specific anabolic responses only in nourished animals, and the reduction of their metabolic effects (glucose and amino acids transport, and RNA, protein and glycogen synthesis [31,32] in those exposed to the effects of malnutrition (M and R groups). However, the

lack of association between insulin and IR prevents us from explaining the physiological mechanism involved.

Conclusion

In rat dental pulp, protein malnutrition changes the odontoblast layer and the collagen scaffold, as well as interferes with the expression levels of IGF-I, IGF-IR and IR.

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