

## Insulin-like Growth Factor Expression in Malnourished Rat Dental Pulp

Aline Gonçalves<sup>1\*</sup>, Sílvia C Boldrini<sup>2</sup>, Marcelo A Cavalli<sup>1</sup>, Joice NB Pereira<sup>1</sup>, Jodonai B Silva<sup>1</sup> and Edson A Liberti<sup>2</sup>

<sup>1</sup>Department of Surgery, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo 05508900, São Paulo, Brazil

<sup>2</sup>Department of Anatomy, Biomedical Science Institute, University of São Paulo, Laboratory of Functional Anatomy Applied To Clinical And Surgery, São Paulo, Brazil

\*Corresponding author: Department of Anatomy, Biomedical Science Institute, University of São Paulo, São Paulo 05508900, Laboratory of functional anatomy applied to clinical and surgery, São Paulo, Brazil, Tel/Fax: +55-11-30917226; E-mail: [aline.goncalves@usp.br](mailto:aline.goncalves@usp.br)

Rec date: Jun 09, 2015; Acc date: Jul 28, 2015; Pub date: Jul 30, 2015

Copyright: © 2015 Gonçalves A, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### 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 22<sup>nd</sup> to the 60<sup>th</sup> day of life to evaluate the effects of renourishment. Dental pulps of the maxillary first molars were stained with hematoxylin and eosin, and picosirius 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 immunoeexpression 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 60<sup>th</sup> 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 (Rhostrer, 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 22<sup>nd</sup> to the 60<sup>th</sup> day of life.

#### Tissue preparation

After euthanasia in a CO<sub>2</sub> 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 picosirius 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<sub>2</sub>O<sub>2</sub> 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 Ir $\beta$ , anti-insulin, and insulin R $\beta$  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

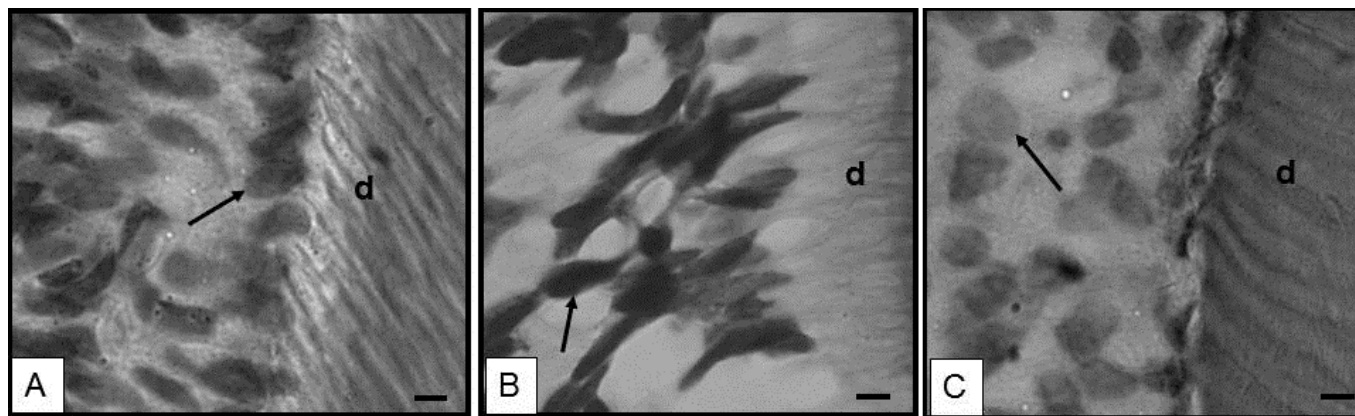
The specimens were examined under an optical microscope (Axioscop, Carl Zeiss, Göttingen, Germany), with a 40x magnification, connected to a computer. The images were captured using a camera

(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 immunoreactive cells was determined using the formula  $(Pn/Tn) \times 100$ . 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).



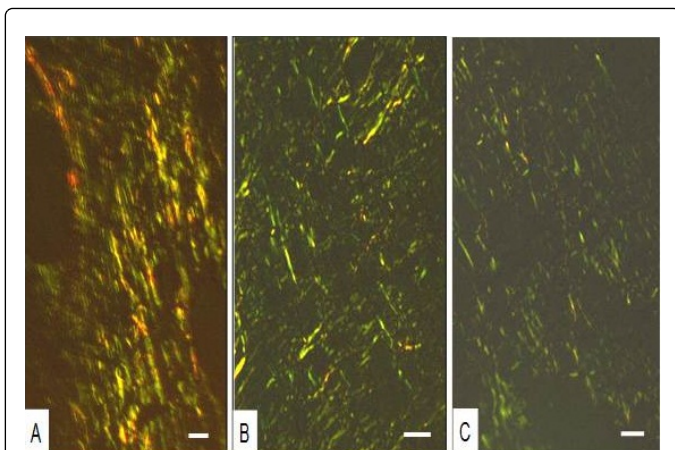
**Figure 1:** Peripheral region of the dental pulp of rats in the N (A), M (B) and R (C) groups. Note the different aspects of odontoblasts (arrows), as well as the arrangement along the dentinal tubules (d). (HE; Calibration bar: 4  $\mu$ m).

In the sections stained with picosirius 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).

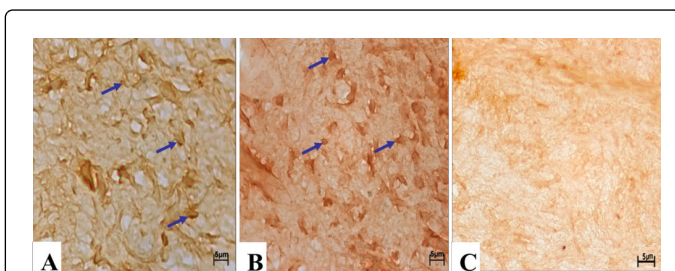
## Quantitative analysis

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  $\mu$ m).



**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  $\mu$ m).

	N	M	R
IGF-I	78.4 $\pm$ 5.4*	65.3 $\pm$ 2.5* 65.29 $\pm$	61.9 $\pm$ 7.2
IGF-IR	74.6 $\pm$ 16.0*	36.9 $\pm$ 12.8*	44.1 $\pm$ 10.9
Insulin	59.1 $\pm$ 13.2	50.8 $\pm$ 9.5	52.7 $\pm$ 11.3
IR	62.4 $\pm$ 8.5*	24.7 $\pm$ 1.2*	27.0 $\pm$ 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  $\pm$  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 dentinopulpal 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.

## References

1. Cavalli MA, Gonçalves A, Pereira JN, da Silva JB, Boldrini Sde C, et al. (2015) Evaluation of protein undernourishment on the condylar process of the Wistar rat mandible correlation with insulin receptor expression. *J Appl Oral Sci* 23: 135-144.
2. Ligh RQ, Fridgen J, Saxton C (2011) The effect of nutrition and diet on dental structure integrity. *J Calif Dent Assoc* 39: 243-249.
3. Maciejewska I, Adamowicz-Klepalska B, Kmiec Z, Dziewiatkowski J (2000) Influence of diet and fluoride on dentin and enamel deposition and maturation in rats. *Folia Morphol (Warsz)* 59: 131-136.
4. Almonaitiene R, Balciuniene I, Tutkuviene J (2010) Factors influencing permanent teeth eruption. Part one--general factors. *Stomatologija* 12: 67-72.
5. Katge F, Poojari M, Sajjani A (2011) Malnutrition and its effects on oral tissues and dentition. *Ind J Dent Educ* 4: 45-47.
6. Ferrini FR, Marba ST, Gavião MB (2008) Oral conditions in very low and extremely low birth weight children. *J Dent Child (Chic)* 75: 235-242.
7. Huumonen S, Larmas M (2005) Effects of protein deficiency induced by raw soy with and without sucrose on dentine formation and dentinal caries in young rats. *Arch Oral Biol* 50: 453-459.
8. Punyasingh JT, Hoffman S, Harris SS, Navia JM (1984) Effects of vitamin A deficiency on rat incisor formation. *J Oral Pathol* 13: 40-51.
9. Lozupone E, Favia A (1994) Morphometric analysis of the deposition and mineralization of enamel and dentine from rat incisor during the recovery phase following a low-calcium regimen. *Arch Oral Biol* 39: 409-416.
10. Werner H, Katz J (2004) The emerging role of the insulin-like growth factors in oral biology. *J Dent Res* 83: 832-836.
11. Caviedes-Bucheli J, Muñoz HR, Rodríguez CE, Lorenzana TC, Moreno GC, et al. (2004) Expression of insulin-like growth factor-1 receptor in human pulp tissue. *J Endod* 30: 767-769.
12. Götz W, Kunert D, Zhang D, Kawarizadeh A, Lossdörfer S, et al. (2006) Insulin-like growth factor system components in the periodontium during tooth root resorption and early repair processes in the rat. *Eur J Oral Sci* 114: 318-327.
13. Umpierrez GE, Goldstein S, Phillips LS, Spanheimer RG (1989) Nutritional and hormonal regulation of articular collagen production in diabetic animals. *Diabetes* 38: 758-763.
14. Partanen AM, Thesleff I, Ekblom P (1984) Transferrin is required for early tooth morphogenesis. *Differentiation* 27: 59-66.
15. Reeves PG, Nielsen FH, Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 123: 1939-1951.
16. Junqueira LC, Cossermelli W, Brentani R (1978) Differential staining of collagens type I, II and III by Sirius Red and polarization microscopy. *Arch Histol Jpn* 41: 267-274.
17. Schour I, Massler M (1963) The teeth. In: Farris EJ, Griffith JQ (eds.) *The rat in laboratory investigation* (2nd edn.) Hafner Publishing Co., New York.
18. Alberts B, Bray D, Lewis J, Raff M, Roberts K, et al. (2011) *Fundamentos da Biologia Celular* (3rd edn.) Artmed. Rio de Janeiro.
19. de Oliveira BC, de Oliveira F, Martini DT, Prisco CR, da Silva Riguetti MM, et al. (2010) The relative effects of severe burn injury and pre- and post-natal protein deprivation on mandibular condyle morphology. *Histol Histopathol* 25: 45-54.
20. Ranjan J, Boaz K, Srikant N, Nandita KP, Lewis A, et al. (2015) Collagen characterization in different patterns of bone invasion by OSCC: A histochemical study with picosirius red and polarizing microscopy. *J Oral Maxillofac Surg Med Pathol*. 27: 258-262.
21. Yamauchi N, Nagaoka H, Yamauchi S, Teixeira FB, Miguez P, et al. (2011) Immunohistological characterization of newly formed tissues after regenerative procedure in immature dog teeth. *J Endod* 37: 1636-1641.
22. Pereira T, Dodal S, Tamgadge A (2014) Analysis of collagen fibres in human dental pulp using picosirius red stain and polarised microscopy. *J of Pierre Fauchard Acad* 28: 73-77.
23. Prescott RS, Alsanea R, Fayad MI, Johnson BR, Wenckus CS, et al. (2008) In vivo generation of dental pulp-like tissue by using dental pulp stem cells, a collagen scaffold, and dentin matrix protein 1 after subcutaneous transplantation in mice. *J Endodont* 34: 421-426.
24. Deng M, Shi J, Smith AJ, Jin Y (2005) Effects of transforming growth factor beta1 (TGFbeta-1) and dentin non-collagenous proteins (DNCP) on human embryonic ectomesenchymal cells in a three-dimensional culture system. *Arch Oral Biol* 50: 937-945.
25. Gonçalves LA, Boldrini SC, Capote TS, Binotti CB, Azeredo RA, et al. (2009) Structural and ultra-structural features of the first mandibular molars of young rats submitted to pre and postnatal protein deficiencies. *Open Dent J* 3: 125-131.
26. Lovschall H, Fejerskov O, Flyvbjerg A (2001) Pulp-capping with recombinant human insulin-like growth factor I (rhIGF-I) in rat molars. *Adv Dent Res* 15: 108-112.
27. Kim M, Kim B, Yoon S (2001) Effect on the healing of periapical perforations in dogs of the addition of growth factors to calcium hydroxide. *J Endod* 27: 734-737.
28. Werner H (2009) For debate: the pathophysiological significance of IGF-I receptor overexpression: new insights. *Pediatr Endocrinol Rev* 7: 2-5.
29. Sudhakar U, Ramakrishnan, Anand PB (2006). Emerging role of the insulin like growth factors in pulp healing, reparative dentinogenesis and periodontal regeneration. *J Conserv Dent* 9: 78-80.
30. Rechler MM, Nissley SP (1985) The nature and regulation of the receptors for insulin-like growth factors. *Annu Rev Physiol* 47: 425-442.
31. Laron Z (2008) Insulin--a growth hormone. *Arch Physiol Biochem* 114: 11-16.
32. LeRoith D, Werner H, Beitner-Johnson D, Roberts CT Jr (1995) Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr Rev* 16: 143-163.