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Fluoride alters connexin expression in rat incisor pulp

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ABSTRACT

Objective: Connexins (Cxs) are important to control growth and cell differentiation of dental tissues. The aim of the present study was to assess the impact of chronic exposure to sodium fluoride (NaF) on Cxs expression and alkaline phosphatase (ALP) activity in dental pulp, and on morphometric parameters of adult rat mandible and incisors.

Design: Three groups of male Wistar rats (22 days-old) were given water containing: (a) 0.3 mg/L (Control), (b) 10 mg/L and (c) 50 mg/L of NaF for eight weeks. Incisor pulp homogenates were prepared for determination of Cx32, Cx43 and Cx45 gene expression, using semi-quantitative RT-PCR, and of ALP activity. Morphometric parameters of mandible and incisors were determined on radiographs.

Results: Cx43 gene expression increased with exposure to NaF in a dose-dependent manner. Cx32 mRNA levels were higher than controls in the 10 mg/L NaF group only; Cx45 mRNA levels were lower in groups given 10 and 50 mg/L of NaF than in controls. ALP activity was higher in both high-NaF dose groups compared to the control group ($p < 0.05$). Lower incisor diameter was lower in the 50 mg/L NaF than in the control group ($p < 0.01$). None of the mandibular growth parameters were affected by NaF treatment.

Conclusion: Our results showed that fluorotic alterations in rat incisor were associated with increased Cx43 expression and ALP activity, as well as with changes in the expression pattern of different Cxs in pulp tissue. The observed changes may have a stimulating effect on dentin mineralization.

1. Introduction

Fluoride contributes to the maintenance of bone health and protects teeth from tooth decay. However, when the level of fluoride in drinking water is above 1.5 mg/L, dental fluorosis can occur. Fluorotic enamel exhibits an irregular mineralization pattern, which results in subsurface porosity and brown stains. In very severe cases, the fluorotic enamel contains relatively less mineral and more proteins than normal, and it is susceptible to surface damage.[1,2] As occurs in other pathologies, severe fluorosis, which results in enamel hypomineralization, causes hypermineralization of dentin.[3]. Dentin hypermineralization and hardness correspond to a structural pattern of sclerotic dentin, indicating the alteration of dentin formation.[4]

Whereas the effects of fluoride on enamel formation have been studied extensively, less is known about the impact of fluoride on dentin and pulp tissue. Normal dentinogenesis is a

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sequential process that starts with a coordinated series of events leading to the differentiation of ectomesenchymal pulp cells into odontoblasts, which are responsible for the secretion of an extracellular matrix that is subsequently mineralized.[5] Several molecules are involved in the process of dentin formation and in the regulation of this process. Alkaline phosphatase (ALP), an enzyme expressed in the early stages of dentin mineralization, is a marker of dental pulp cell viability.[6]

Cell–cell communications are important to control growth, development, and homeostasis of various tissues, including teeth.[7,8] Connexins (Cxs) are a family of transmembrane proteins that can be distinguished on the basis of their molecular weight. They are expressed in different tissues and are known for their assembly into intercellular channels, called gap junctions.[9] The latter allow the direct exchange of ions and small regulatory molecules through channels that interconnect the cytoplasm of neighbouring cells.[10] Electron microscopy studies have shown expression and localization of Cx43 and Cx32 in rat incisor pulp cells[11] and Cx45 expression in cultured dental pulp cells.[12] A recent study demonstrated gap junction-independent functions of Cxs in cell growth and differentiation.[13] Furthermore, Cx43 has been found to play a role in pulp cell differentiation and viability.[14] Connexins appear to be important for tooth development and homeostasis. Nevertheless, the changes in the expression patterns of these molecules in response to pulp tissue exposure to fluoride remain to be clarified.

We hypothesized that an excess of fluoride in drinking water can modify the expression pattern of the different connexins in pulp tissue, and that this change could be part of the mechanism involved in the altered mineralization of dentin, which is characteristic of dental fluorosis. Therefore, the aim of our study was to investigate the effects of chronic exposure to high doses of sodium fluoride on the genic expression of connexins in incisor pulp tissue, and on other functional and morphometric parameters in mandibles and incisors of rats.

2. Material and methods

2.1. Experimental animals and fluoride treatment

Male Wistar rats (*Rattus Novergicus*), aged 22 days, were housed in groups of four animals per cage and kept on a 12 h:12 h light–dark cycle, with water and food (commercial diet) *ad libitum*. The animals were randomly divided into three groups ($n = 8$ per group) and given drinking water containing different concentrations of sodium fluoride (NaF): tap water 0.3 mg/L (Control), 10 mg/L and 50 mg/L, during eight weeks. Water intake was assessed daily; all the animals were weighed at the beginning and at the end of the experiment. At the end of treatment, the rats were anaesthetized with a combined dose of ketamine and xylazine (80 and 12.8 mg/kg of body weight respectively) and euthanized by cervical dislocation. The upper and lower incisors and the mandibles were resected and processed. The animal protocol was approved by the Bioethics Committee of the School of Medicine of the National University of Córdoba (Argentina), and is in keeping with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the European Convention for the Protection of Vertebrates Animals used for Experimental and other Scientific Purposes (Council of Europe N 123, Strasbourg 1985). All efforts were made to minimize the number of animals used and their suffering.

2.2. Determination of urine fluoride concentrations

Urine samples were collected from anaesthetized animals by direct puncture of the bladder, prior to euthanasia. After centrifugation at 2000 × *g* for 10 min, urine fluoride levels were measured by direct potentiometry using an ion-selective electrode (Oakton Fluoride Combination Electrode Mod. 35802) as described by Singer et al.[15]

2.3. Pulp tissue isolation

The dental pulp tissue was carefully removed from the upper incisors of each animal by mechanical means. The samples were aliquoted, quickly frozen, and stored at −20 °C until processing.

2.4. Alkaline phosphatase activity

Alkaline phosphatase (EC 3.1.3.1) (ALP) activity was assayed in pulp tissue homogenates (1:20) using 3 mmol/L p-nitrophenyl phosphate as substrate in 0.5 mmol/L diethanolamine buffer, pH 9.8. Specific ALP activity was expressed in IU/mg of protein/min.[16]

2.5. RNA isolation and expression of connexins by RT-PCR analysis

Total RNA was isolated using a MasterPure™ kit (Epicenter Biotechnologies, USA) following the manufacturer's instructions. To avoid contamination with genomic DNA, RNA was treated with DNAse. RNA concentration and purity were determined by spectrophotometry at 260/280 nm. cDNA was synthesized by using 400 units of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in buffer (50 mmol/L Tris–HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 20 mmol/L dithiothreitol), containing each deoxynucleotide triphosphate at 2 mmol/L, 40 units RNase inhibitor (RNaseOUT, Invitrogen Carlsbad, CA, USA), and 500 ng random primers. PCR amplification was carried out on cDNA using 1.25 units Taq polymerase (Promega) in a buffer containing 50 mmol/L KCl, 20 mmol/L Tris–HCl (pH 8.4) and 2 mmol/L $MgCl_2$, with 0.8 mmol/L of each deoxynucleotide triphosphate and 0.8 μmol/L each of two oligonucleotide primers. Conditions for PCR amplification were as follows: denaturation at 94 °C for 45 s, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min, for a total of 35 cycles, and a final extension step of 10 min at 72 °C. Primer sequences for PCR amplification were as follows: Cx43 (NCBI NM012567.2) Fw (5′-AGCAAGCTAGCGAGCAAAAC-3′) and Rv (5′-AGTTCATGTCCAGCAGCAA-3′), which amplify a 152 bp region located between 1254 and 1103 bp; Cx45 (NCBI NM001085381.1) Fw (5′-GGTGCCATCGAGGAACTCAA-3′) and Rv (5′-GTGGTGGAATTGGTTTGCCC-3′), which amplify a 116 bp region located between 407 and 292 bp; Cx32 (NCBI AH003192.2) Fw (5′-TTTCCCCATCTCCCATGTGC-3′) and Rv