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Communication

Gene expression of collagen type I alpha 2 and its relationship with dental fluorosis.

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Abstract: Objective: To compare the gene expression levels of collagen type I alpha 2 (COL1A2) in children with and without dental fluorosis. Methods: Cross-sectional study involving 92 children between 5 and 12 years of age. Socio-demographic characteristics, the presence of dental fluorosis by means of the Thylstrup-Fejerskov index, and gene expression analysis of COL1A2 in peripheral blood samples by reverse transcription polymerase chain reaction (RT-PCR) assays, were described. For the descriptive analysis, measures of central tendency, dispersion and proportions were used. Differences between the groups ($p<0.05$) were established by the Student t-test. Results: Mean age was 8.6 (SD=1.9) years, 51.1% were female; 54 children were diagnosed with fluorosis and 38 without fluorosis; prevalence of dental fluorosis was 58.7% (95% CI: 48.4% -68.9%). Gene expression of COL1A2 was statistically significantly lower ($p<0.05$) in the participants with dental fluorosis. Conclusion: There are differences in the expression levels of the COL1A2 gene among the population under study. Therefore, COL1A2 may be potentially involved in the development of dental fluorosis.

Keywords: Dental fluorosis; dental enamel; gene expression; collagen.

INTRODUCTION.

Dental fluorosis is characterized by white or opaque spots on the enamel, caused by the ingestion of high concentrations of fluoride during the process of tooth formation.[1] Over 60% prevalence of this condition has been reported in Cartagena (Colombia). The main risk factors associated with fluorosis are the consumption of fluorinated foods and the use of toothpastes.[2] Its causes has not been not completely clarified, but in recent years it has been suggested that genetic factors may be involved in its etiology.[3]

Collagen is a protein that strengthens and supports bones, as well as many other tissues, including cartilage, tendons and skin. Collagen genes type I alpha 1 (COL1A1) and alpha 2 (COL1A2) encode the necessary instructions to form the components of collagen. Specifically, the COL1A1 gene produces a type I collagen component, called the pro-alpha1 chain; while the COL1A2 gene produces the pro-alpha2 chain. These chains bind to form a type I procollagen molecule. Procollagen molecules organize themselves into long, thin fibrils that intertwine in the spaces around the cells, resulting in the formation of mature and strong Type I collagen fibers.[4] Due to its important role in bone formation and architecture, mutations in the COL1A2 gene have been associated with a broad spectrum of diseases of bone, cartilage and blood vessels. Likewise, the

influence of polymorphisms of the COL1A2 gene on the appearance of dental fluorosis has also been evaluated.[4-6] However, its role in the development of dental fluorosis is not completely clear, so that studies of gene expression analysis that may contribute to the understanding of the etiology of this condition are required.

As the role of the COL1A2 gene in the development of dental fluorosis is partially unclear, the aim of this study is to compare gene expression levels of COL1A2 in children with and without dental fluorosis.

MATERIALS AND METHODS.

Study design and participants

Cross-sectional study involving 92 children who voluntarily participated in “a health day session” organized by Universidad de Cartagena, Colombia, as part of its social action activities, carried out in the community of "El Pozón" in the city of Cartagena, Colombia. “El Pozón” is a neighborhood located in the southeast of the city, characterized by high rates of vulnerability and severe poverty. The study was approved by the ethics committee of Universidad de Cartagena and complied with all recommendations of the declaration of Helsinki.

Selection criteria

Children from 5 years of age onwards who had four fully-erupted teeth of permanent dentition, with or without a diagnosis of fluorosis, were included in the study. Those who reported a personal history of neoplastic diseases, immunodeficiency and autoimmune disorders were excluded.

Variables and instruments

An instrument to obtain information related to socio-demographic characteristics and to report clinical findings obtained in the dental examination was designed (1. Age; 2. Sex; 3. Socio-economic stratum; 4. Place of origin 5.Presence of fluorosis; 6. Severity of fluorosis). The instrument was tested in a pilot test that included five participants. It evaluated the comprehension of each one of the questions, the sufficiency of the categories, their extension and relevance.

This allowed to make the necessary modifications before its application, adapting it to the context. Two experts validated its structure and layout.

Procedures

During the “health day sessions”, attending parents were invited to participate in the study and its objectives, benefits and risks were explained. All participating children were authorized by their parent or guardian who signed a written informed consent. Participation consisted of filling out a questionnaire, going through a clinical dental examination, and donation of a blood sample. The questionnaire was completed immediately after signing each informed consent. Then the clinical dental examination was performed, and finally, peripheral blood samples (5mL) were collected from the anterior forearm in EDTA-coated Vacutainer tubes (BD, Biosciences) by a specialist in bacteriology. They were then stored on ice and transported to the Biochemistry Laboratory at Universidad de Cartagena for further analysis. The researcher in charge of collecting the information from the questionnaire had been previously trained and was not the same person who performed the clinical examination and molecular tests.

Dental clinical examination

Visual diagnosis was performed by a dentist (Kappa Cohen >0.80). The presence of dental fluorosis was determined using the Thylstrup-Fejerskov index, classifying the macroscopic appearance of the teeth using an ordinal scale from 0 to 9. Artificial light, oral mirror, triple dental syringe and a portable dental unit fitted in the facilities of the COOSALUD community service in the El Pozón neighborhood were used. Each participant was assigned to one of the two groups: with fluorosis and without fluorosis. In addition, all those who presented an oral disease apart from fluorosis also participated in prevention activities in the health day sessions, and a visit to a dental specialist was suggested to their parents.

Lymphocyte isolation

Lymphocyte isolation was performed through centrifugation and separation by density gradient using Lymphosep® (Biowest, Nuaillé, FR). The lymphocyte suspension was centrifuged at 800 x g, at 4ºC, for 10 minutes and the cell pellet obtained was washed twice in sterile phosphate-buffered saline (PBS, Invitrogen).

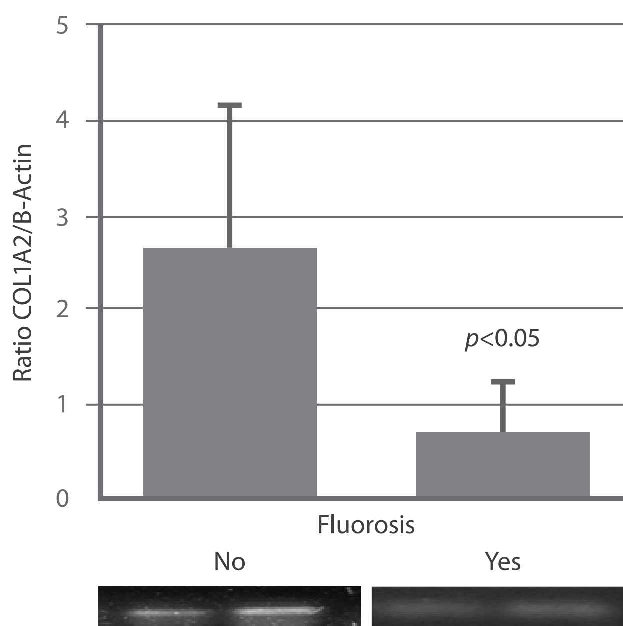
Gene expression assays

RNA was isolated from lymphocytes with QIAzol Lysis Reagent® (Qiagen, CA, USA). cDNA was obtained using the QuantiTect® Reverse Transcription kit (QIAGEN, CA, USA), following the manufacturer's instructions. Amplification of the genetic loci was performed under the following conditions: 5 minutes at 95ºC, followed by 35

Table 1. Distribution of degree of dental fluorosis and the Thylstrup-Fejerskov index.

Degree	n	Percentage (%)
Mild	51	55.4
Moderate	3	3.3
Severe	0	0.0
Does not apply	38	41.3
TF Index		
0	38	41.3
1	33	35.9
2	11	12.0
3	7	7.6
4	2	2.2
5	1	1.1
6	0	0
7	0	0
8	0	0
9	0	0
Total	**92**	**100**

Figure 1. Comparison of gene expression levels of COL1A2 in children with and without dental fluorosis.



cycles of 94ºC for 0.5-1 minute, 60-62ºC for 30 seconds, 72°C for 0.5-1 minute and final elongation at 72°C for 10 minutes. PCR products were separated by electrophoresis in a 1.0% agarose gel, and visualized using EZ Vision® (Amresco, OH, USA) with the ChemiDoc™ XRS+System (BIORAD, CA, USA). β Actin was used as the normalizing gene. Relative gene expression (COL1A2/β Actin) was analyzed by densitometry using the QuantityOne® one-dimensional image analysis software (BioRad, CA, USA).

The sequences of the primers used were COL1A2 (for-CTGGTAGTCGTGGTGCAAGT, rev AATGTT-GCCAGGCTCTCCTC), ACTIN (for-GTGGGGC-GCCCCAGGCACC, rev-CTCCTTA ATGTCACGCA-CGATT).

Statistical analysis

The assumption of normality of the data was evaluated using the Shapiro Wilk test. For the descriptive analysis the measures of central tendency, dispersion and proportions, were used. The Student t-test was used to establish differences between the groups. For all tests a cutoff for significance of 0.05 was assumed. The STATA® program (Stata Corp. LP, College Station, TX, USA) was used.

RESULTS.

The percentage of participants was 88.5% of 92 children. Mean age was 8.6 (SD=1.9) years. Minimum age was 5 years and the maximum age was 12 years. Female participants accounted for 51.1%; 95.6% belonged to stratum 1, that is, low socio-economic level, and 100% were from the city of Cartagena.

In relation to the dental clinical examination, 54 cases of dental fluorosis were diagnosed, with a prevalence of 58.7% (95% CI: 48.4%–68.9%). According to the severity of the condition, 94.4% (51/54) presented mild fluorosis, and 5.6% (3/54) moderate. Distribution of fluorosis according to the Thylstrup-Fejerskov index is shown in Table 1.

There were no significant differences ($p>0.05$) when comparing the expression levels of the β-Actin gene. Significant differences were found in the relative gene expression of COL1A2 between the groups ($p<0.05$), being lower in children with fluorosis (Figure 1).

DISCUSSION.

Fluorosis has been possibly related to the reduced expression of the COL1A2 gene, which plays a significant role in the remodeling of the extracellular matrix of the dental enamel.[7] This suggests that children with dental fluorosis exposed to high concentrations of fluorine may have alterations in the COL1A2 gene expression. Therefore, it could be suggested that this gene is involved, along with other genes, in the etiology of this condition.

In vitro studies in cells and osteoclasts of mice have reported significant differences, showing that a decrease

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in collagen formation is the result of the inhibition of the maturation of collagen fibers, and that an increase in its degradation is fluoride-dependent. Similarly, Dawson[5] and Huang *et al.*,[4] concluded that some polymorphisms of the COL1A2 gene are involved in the development of dental fluorosis. However, Escobar-García *et al.*,[6] and Pragya & Vandana[8] argue that there is no evidence of association between polymorphisms of the COL1A2 gene and dental fluorosis in populations exposed to high concentrations of fluoride. Therefore, other studies with larger samples and more sensitive techniques are necessary to corroborate this possible relationship.

Among the hypotheses that explain this relationship, it can be proposed that an effect of fluoride exposure is the delay in the elimination of the amelogenin protein secreted in the matrix during the development of enamel.[9] In dental fluorosis the activity of ameloblasts, osteoblasts, osteoclasts, osteocytes, matrix formation and calcium homeostasis are altered.[10] Osteoblasts contribute to bone formation and actively secrete type I collagen, growth factors and minerals in the matrix.[11] The mechanism of action of fluoride is directly related to the composition and structure of the bone matrix, acting specifically on collagen and non-collagen proteins, which play an important role in biomechanical integrity and mineralization.[11]

High doses of fluoride can cause structural changes in the collagen fibers and directly alter the quantity/quality of collagen in the connective tissues, even in the dentin-pulp complex. It can also induce a delay in the elimination of the amelogenin matrix protein and inhibit some proteinases that participate in the hydrolysis of amelogenin.[5] However, it is important to clarify that in the present study fluoride intake was not quantified; therefore, it would be appropriate to determine the levels of fluoride present in the blood or urine in this population, and to assess the total daily intake of fluoride, calculating the intake of fluoride from the diet, and from drinking water, among other sources .

Among the strengths of this study is the performance of standardized procedures and the use of validated instruments, which allow for reliable results regarding the gene expression of COL1A2 and its possible relationship with dental fluorosis. However, there are some limitations, such as the delays between the cause-effect variables under study, and the non-use of more sensitive molecular techniques, such as reverse-transcription quantitative PCR.

Because molecular processes involved in the development of dental fluorosis are not fully understood, the main applications of this study are related to the development of future research focused on determining the potential effect of fluoride on the collagen gene and other genes, related with the extracellular matrix and the formation of dental enamel.

CONCLUSION.

There are differences in the expression levels of the COL1A2 gene among the population under study. Therefore, COL1A2 may be potentially involved in the development of dental fluorosis. However, more studies are needed to corroborate this relationship.

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