Pesquisa Brasileira em Odontopediatria e Clínica Integrada 2025; 25:e230209
https://doi.org/10.1590/pboci.2025.026
ISSN 1519-0501 / eISSN 1983-4632

ORIGINAL ARTICLE

Cross-Sectional Microhardness and Chemical Composition of Primary Teeth with Green Discoloration due to Hyperbilirubinemia

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Academic Editor: Alessandro leite Cavalcanti

Received: November 05, 2023 / **Review:** May 05, 2024 / **Accepted:** May 14, 2024

How to cite: Macedo AF, Diniz MB, Azevedo RA, Fujita RR. Cross-sectional microhardness and chemical composition of primary teeth with green discoloration due to hyperbilirubinemia. Pesqui Bras Odontopediatria Clín Integr. 2025; 25:e230209. https://doi.org/10.1590/pboci.2025.026

ABSTRACT

Objective: To evaluate the chemical components and cross-sectional microhardness of primary teeth with hyperbilirubinemia-induced green pigmentation. **Material and Methods:** The sample consisted of two anterior and two posterior green primary teeth discolored by bilirubin and regular primary teeth, paired according to child age and tooth type. Scanning energy dispersive spectroscopy was used to investigate the mass percentage of calcium, phosphorus, and carbon, and a microhardness tester was used to assess the cross-sectional microhardness of enamel and dentin. The collected data were analyzed using the Student's t-test and Mann-Whitney ($p<0.05$). **Results:** There was a significant decrease in calcium in the dentin of the green discoloration group compared with the control group. Although the differences in cross-sectional microhardness were not significant, all data showed lower microhardness in both enamel and dentin in the green discoloration group. **Conclusion:** The calcium content in the dentin of green-discolored primary teeth is lower than in regular primary teeth, but the microhardness of enamel and dentin is not affected.

Keywords: Tooth; Child; Hyperbilirubinemia; Liver Diseases; Hardness Tests.

Introduction

The liver is composed of parenchymatous tissue in the upper part of the abdominal cavity with an ample blood supply. It has vital functions such as energy production and control, metabolism of carbohydrates, proteins, and lipids, metabolism and excretion of bile and steroids, maintenance of water balance, participation in immune defense, and a blood reservoir [1]. When these functions are disrupted, chronic liver disease (CLD) results and liver transplantation is often necessary [1,2]. Liver transplantation, along with the administration of immunosuppressants, has become the main therapeutic intervention in children with liver disease, especially in children with biliary atresia, a condition characterized by sclerosis of the extrahepatic bile ducts and abnormal branching of the intrahepatic ducts [3].

Several systemic complications of CLD are primarily related to liver dysfunction, such as cholestasis, portal hypertension, ascites, variceal bleeding, impaired protein synthesis, coagulopathy, hepatic encephalopathy, and hepatorenal and hepatopulmonary syndromes [2]. Several oral manifestations can occur, including enamel developmental defects, impaired salivary secretion, delayed tooth eruption, oral mucosal lesion, and green staining of teeth and soft tissues [1,2,4,5].

Intrinsic green pigmentation of the teeth is an unusual condition first described in 1912 that can affect both the primary and permanent dentitions [6,7]. This pigmentation is a consequence of the high concentration of bilirubin in the dentinal tubules [1]. Bilirubin is a breakdown product of hemoglobin. The non-conjugated bilirubin is converted to conjugated bilirubin in the liver and eventually excreted in the urine. Any disturbance in this pathway can lead to high blood bilirubin levels and hyperbilirubinemia, which causes dental pigmentation, especially if the serum bilirubin level is above 30 mg/dL [8].

A dental pigmentation can be classified according to its origin: intrinsic or extrinsic. Green tooth pigmentation is inherent and caused by high serum bilirubin concentration during periods of dentinogenesis, resulting mainly from biliary atresia due to liver disease. During tooth development, odontoblasts synthesize and secrete the collagen-rich organic matrix, which mineralizes. In contrast, ameloblasts secrete a protein matrix in which about 25% of hydroxyapatite crystals are deposited by the end of the secretory phase of amelogenesis. Then, ameloblasts degrade almost all of the organic matrix, including the green pigment that enters the teeth, to allow the mineral content to increase during the maturation phase [8]. Thus, bilirubin is gradually deposited in the dentin during the development of the tooth germ, resulting in a greenish coloration of the tooth [9]. Although greenish pigmentation in soft tissues is reversible due to rapid cell renewal, bilirubin deposited in mineralized tissues of teeth is permanently incorporated during the formation period because metabolic activity ceases after tissue maturation [6-8,10,11].

There are no studies on the chemical composition and microhardness of greenish deciduous teeth. There are some reports of cases and studies of pigmented permanent teeth, but there are few studies of deciduous teeth because sample collection is complex due to the age of the children and the severity of the pathology, which can often lead to death [4,7,9]. Knowledge about these discolored teeth helps formulate preventive and specific dental treatments in children with dental pigmentation due to biliary atresia, whether transplanted or not. This contributes to correctly monitoring the oral condition and avoids aggravation of the general health condition due to oral infections.

Therefore, the aim of this pilot study was to evaluate the chemical components and cross-sectional microhardness of primary teeth with hyperbilirubinemia-induced green pigmentation. The hypothesis is that there are differences in dental tissues when comparing normal primary teeth and bilirubin-induced green primary teeth of children with biliary atresia.

Material and Methods

Ethical Statement

The Federal University of São Paulo's Human Ethics Committees, Brazil, approved this study under protocol #2.905.636. Written informed consent was obtained from the children's parents/guardians.

Sample Selection and Specimen Preparation

Primary teeth were obtained from normal children (NC) and children with biliary atresia aged 5 to 7 years of both sexes attending the Dental Clinic of the Cruzeiro do Sul University in São Paulo, São Paulo, Brazil, and the Pediatric Gastroenterology and Transplantation Department of the Federal University of São Paulo, respectively. The primary teeth analyzed in this pilot study were collected from patients who needed extraction due to prolonged retention. The green teeth were extracted from children before or after their liver transplant due to biliary atresia. Green staining can be classified as follows: score 0: no staining; score 1: mild green staining; score 2: severe green staining [11]. In our sample, teeth were classified as score 2.

The green teeth sample consisted of two anterior (one lower central incisor and one upper central incisor) and two posterior (one lower first molar and one upper first molar) primary teeth. The teeth of the control group were paired according to age and tooth type (Figure 1). Teeth were cleaned and stored refrigerated in a 0.1% to 4°C thymol solution. Then, they were cross-sectioned using a diamond disk attached to a water-cooled cutting machine (Isomet 1000, Buehler Ltda., Lake Bluff, II, EUA).

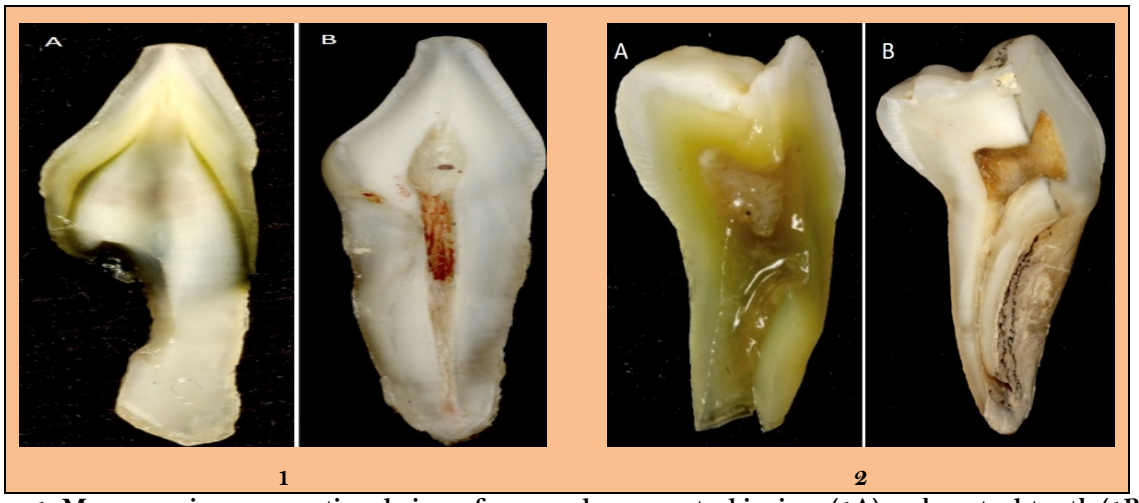

Figure 1. Macroscopic cross-sectional view of a green lower central incisor (1A) and control tooth (1B); Macroscopic cross-sectional view of a green primary molar (2A) and control tooth (2B).

Analysis with Scanning Energy Dispersive Spectroscopy

Prior to the analysis, specimens were cleaned with ultrasound and fixed in a metallic stub using double-sided carbon tape. Then, scanning energy dispersive spectroscopy (JEOL6460 LV, Tokyo, Japan) was performed at a voltage of 20 kV. The enamel and dentinal surfaces were analyzed to identify calcium, phosphorus, and carbon elements [12,13] (Figures 2 and 3).

Cross-Sectional Microhardness

All teeth were prepared based on a previous study [14]. For cross-sectional microhardness analysis, tooth halves were embedded in epoxy resin and then polished on a rotary electric polishing machine (Ecomet

250, Buehler Ltd., Lake Bluff, II, USA) with 220-, 600-, and 1,200-grit silicon carbide abrasive paper (Teclago, Vargem Grande Paulista, Sao Paulo, Brazil) under cooling. Each tooth specimen was tested using a microhardness tester (HMV-2T, Shimadzu, Kyoto, Japan) with a Knoop diamond under a load of 25 grams for 5 seconds. Three rows of indentations were made at 100 µm intervals at depths of 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160, 180, 200, and 220 µm for enamel and at depths of 10, 30, 50, 70, 90, 110, and 220 µm for dentin. The average depth was determined for each sample and group [15].

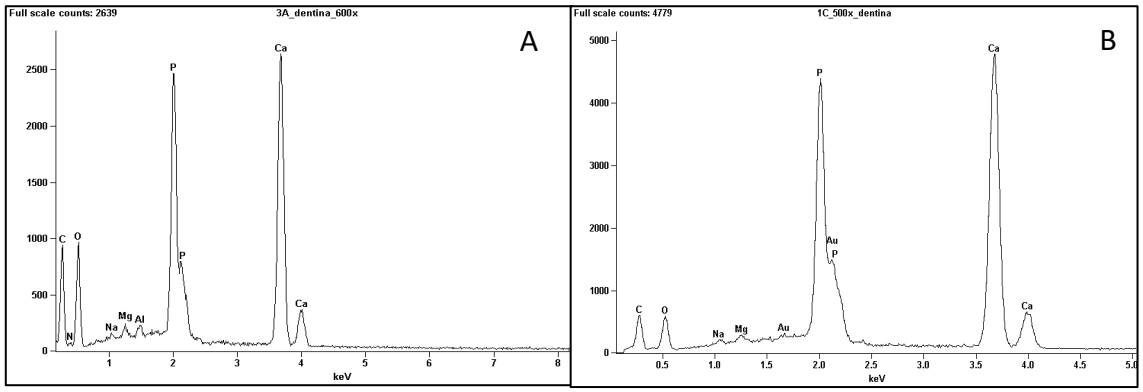

Figure 2. Ion percentages in the green pigmentation primary teeth group (A) and the control group (B).

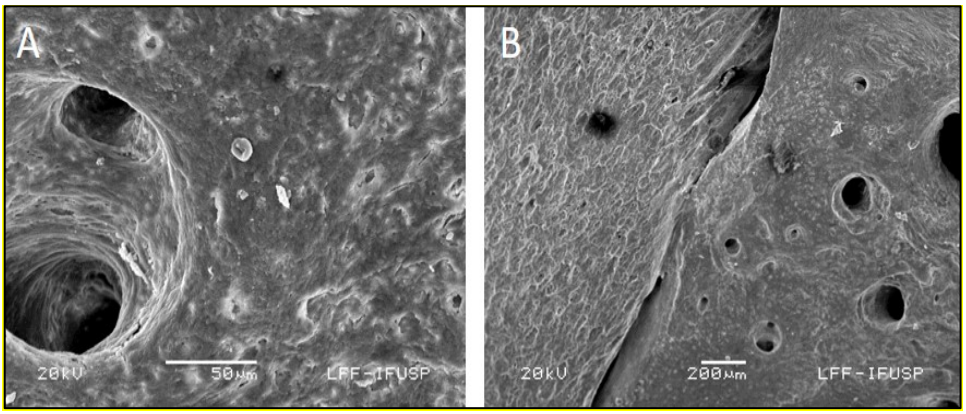

Figure 3. Scanning electron microscopy images showing the dentin structure of a green pigmentation tooth (A) and control tooth (B).

Statistical Analysis

Data were analyzed using SigmaStat software for Windows® (Systat Software, San Jose, CA, USA). Cross-sectional microhardness and chemical component analyses were evaluated as continuous data. The Kolmogorov-Smirnov test was used to verify data normality. Thus, the Student's t-test was used for comparisons between groups, and the Mann-Whitney test was used when the data was not normally distributed. The significance level was set at 5%.

Results

Spectroscopy analysis demonstrated changes in dentin, with the percentage of calcium being lower in the green pigmentation group compared to the control group ($p=0.033$). The percentage of phosphorus and carbon did not differ in enamel and dentin in both groups ($p>0.05$) (Table 1).

Table 1. Means and standard deviations of ion percentages of each chemical component in the two groups.

Dental Substrate	Chemical Components	Green Primary Teeth Pigmentation Group	Control Group	p-value	Normality Test
Enamel	Phosphorus	12.045 ± 2.164	11.910 ± 2.814	0.942[a]	0.588
	Calcium	24.993 ± 4.970	28.545 ± 4.779	0.343[a]	0.520
	Carbon	28.668 ± 6.784	23.195 ± 5.671	0.262[a]	0.755
Dentin	Phosphorus	9.478 ± 1.675	10,762 ± 2.784	0.343[b]	<0.05
	Calcium	19.865 ± 3.799	25.415 ± 1.295	0.033[c]	0.316
	Carbon	35.230 ± 3.391	28.545 ± 4.470	0.055[a]	0.118

[a]Student's t-test; [b]Mann-Whitney test; [c]Indicates difference in chemical component obtained by t-Student test ($p<0.05$).

The cross-sectional microhardness analysis of the enamel and dentin did not show statistically significant differences between the groups when individual depth layers were evaluated (Tables 2 and 3).

Table 2. Mean and standard error of the cross-sectional Knoop microhardness of the enamel structure in the two groups.

Cross-Sectional Microhardness Analysis	Green Primary Teeth Pigmentation Group	Control Group	p-value	Normality Test
10 µm	125.822 ± 21.861	147.478 ± 25.003	0.524[a]	0.266
20 µm	118.075 ± 8.782	131.125 ± 12,969	0.437[a]	0.448
30 µm	120.738 ± 19.075	130.438 ±19.176	0.725[a]	0.276
40 µm	118.883 ± 9.127	118.317 ± 11.231	0.970[a]	0.240
50 µm	125.900 ± 12.229	123.480 ± 18.810	0.917[a]	0.604
60 µm	128.100 ± 15.416	135.600 ± 14.045	0.728[a]	0.591
80 µm	116.283 ± 15.045	134.983 ± 16.709	0.425[a]	0.573
100 µm	124.100 ± 28.946	130.471 ± 13.475	0.845[a]	0.343
120 µm	139.480 ± 24.311	130.000 ± 21.335	0.777[a]	0.225
140 µm	133.457 ± 31.929	141.529 ± 20.017	0.834[a]	0.407
160 µm	146.614 ± 34.799	137.800 ± 24.147	0.839[a]	0.172
180 µm	143.500 ± 29.145	131.871 ± 22.548	0.758[a]	0.079
200 µm	135.750 ± 12.684	137.750 ± 12.605	0.915[a]	0.571
220 µm	131.000 ± 16.946	132.250 ± 16.770	0.960[a]	0.079
Total	129.124 ± 2.386	133.082 ± 1.789	0.193[a]	0.443

[a]Student's t-test; [b]Mann-Whitney test.

Table 3. Mean and standard error of the cross-sectional Knoop microhardness of the dentin structure in the two groups.

Cross-Sectional Microhardness Analysis	Green Primary Teeth Pigmentation Group	Control Group	p-value	Normality Test
10 µm	55.888 ± 7.831	57.637 ± 7.0,32	0.870 [a]	0.287
30 µm	45.186 ± 6.065	40.114 ± 5.597	0.550 [a]	0.501
50 µm	34.457 ± 4.518	46.200 ± 7.996	0.225 [a]	0.602
70 µm	33.229 ± 6.308	42.400 ± 7.134	0.354 [a]	0.304
90 µm	31,343 ± 4.431	38.057 ± 5.086	0.339 [a]	0.811
110 µm	28.600 ± 3.682	39.643 ± 5.897	0.156 [a]	0.782
220 µm	32.500 ± 10.260	39.386 ± 7.909	0.295 [b]	<0.05
Total	37.315 ± 3.673	43.348 ± 2.587	0.097 [b]	<0.05

[a]Student's t-test; [b]Mann-Whitney test.

Discussion

Biliary atresia is one of the most common pathologies in CLD, with liver transplantation being the final treatment option. Bilirubin is normally insoluble and is converted to a soluble conjugate in the liver for excretion

from the body. In the first few days of life, liver cells are immature and unable to successfully release the enzyme that conjugates bilirubin, causing unconjugated bilirubin levels to rise and pigmentation to develop [6].

In the oral cavity, hyperbilirubinemia leads to green-stained teeth, and the presence of this intrinsic pigmentation is a diagnostic factor of biliary atresia [6,7,9]. Tooth discoloration in children with liver disease is directly related to total serum bilirubin levels, indicating the duration and severity of CLD [5]. However, this condition is exceptional, and there are few studies on green discolored deciduous teeth, mainly because these teeth are rarely available for analysis [2,6,7,9,15]. It should be noted that the bilirubin level that leads to staining still needs to be well-defined in the literature [4].

To date, no studies have investigated the chemical composition and microhardness of enamel and dentin of greenish deciduous teeth of children with biliary atresia. Thus, the hypothesis of the present pilot study was confirmed because there were differences in dental tissue between normal deciduous teeth and green pigmentation deciduous teeth of children with bilirubin-related biliary atresia.

The degree of enamel and dentin mineralization is directly related to microhardness, which was determined in this study on cross-sectional tooth slices. The cross-sectional microhardness of the different depths of the enamel of the teeth with pigmentation showed no changes compared with the teeth of normal children. Since pigmentation occurs in the dentin due to hyperbilirubinemia and the enamel remains unchanged, its mineral structure didn't change according to the data obtained. The color change is visible because enamel translucence reveals the greenish coloration of the dentin, but the enamel composition and hardness aren't affected [6,9]. The reduction of enamel microhardness in children with rickets, who also have reduced calcium levels, was observed in a previous study [15], while the deciduous teeth of children with cerebral palsy didn't show differences in enamel microhardness compared to normal children [14]. It can be concluded that some systemic changes may affect the tooth structure and its mineralization.

Although there was no statistically significant difference in this study, the dentin of the greenish teeth had lower overall cross-sectional microhardness, probably due to the presence of bilirubin. Macroscopic examination of the sectioned greenish teeth showed that the outer third of the dentin was pigmented and surrounded the entire crown area. The greenish color of bilirubin may influence the density of the organic matrix during tooth development [9]. There are reports that the tubular structure and thickness of the peritubular dentin aren't altered in these teeth, but only an abrupt change in the direction of the dentinal tubules occurs [6,16]. However, when analyzed under the scanning electron microscope, hyperbilirubinemia was associated with a lower density of dentinal tubules and a lower thickness of the peritubular dentine of green deciduous teeth [10]. This morphological change of the dentin directly affects the adhesion of composite resin used for restorations [10].

The results of the present study show that the structural composition of the dentin layer changes with a decrease in calcium ions, consequently affecting microhardness. Since odontoblasts, the precursor cells of dentin, have postmitotic properties, proteolysis of collagen, the most abundant protein in dentin, has a negative effect and compromises the integrity of this tissue [12,17]. It should be noted that pigmentation of dentin occurs during dentin calcification and can affect both the primary and permanent dentition [7]. Calcium and phosphorus are hydroxyapatite's main inorganic elements, a dentin crystalline component [13]. The decrease of these inorganic elements may affect the mineralization process and increase the susceptibility to caries [10,18]. This dentin abnormality may be associated with a failure of the calcium mineralization process, and insufficient coalescence of these calcification globules results in an irregular calcification pattern [15].

In this pilot study, the data showed a reduction in calcium levels, similar to studies conducted in children with diabetes [13]. This inorganic element could be the main factor in tooth discoloration. In teeth with tetracycline pigmentation, the discoloration is due to the formation of the tetracycline-calcium orthophosphate complex, which is formed by the chelation of calcium with the hydroxyapatite of mineralizing dentin [19]. Bilirubin-induced greenish pigmentation is thought to follow a similar pattern by binding to calcium, thus affecting the amount of this element in dentin and, consequently, its mineralization process and reducing its hardness.

The reduction in calcium ions and decreased microhardness of pigmented deciduous teeth due to high serum bilirubin in CLD, especially in biliary atresia, during tooth development is relevant to managing these patients. This condition impairs mineralization and consequently increases the risk of caries lesions. Children with CLD have poor oral hygiene, often due to their poor general condition and especially to frequent meals to compensate for nutrient deficiencies [11,20]. In this context, the salivary pattern also changes, increasing the predisposition for developing dental caries. In a salivary study conducted on children with permanent dentition and liver transplantation, the average salivary sodium level was lower, and the potassium level was higher [21].

Some limitations of the present study must be mentioned. The deciduous teeth used were in the process of rhizolysis, i.e., physiological resorption with high mineral maturation [22]. In addition, the sample was small because it was difficult to obtain deciduous teeth without caries lesions or enamel development disorders. Another important factor is liver disease. Children with biliary atresia require a surgical procedure, Kasai portoenterostomy, and in most cases, later liver transplantation [23]. If portoenterostomy isn't performed, progression of the disease can lead to death by 3 years, i.e., a short survival time [24,25].

The results of this pioneering study clearly demonstrate that preventive measures are needed to reduce the likelihood of oral disease occurrence to maintain the integrity of the teeth and improve the quality of life of these children. New studies in this area should be encouraged to provide better knowledge and new perspectives on a preventive approach for this population of children.

Conclusion

Although there was no change in the microhardness of enamel and dentin, the calcium content in the dentin of primary teeth with green pigmentation caused by hyperbilirubinemia was lower than in deciduous teeth of normal children. This condition may increase susceptibility to caries lesions, and an oral health monitoring program is essential for these children.

Authors' Contributions

AFM	https://orcid.org/0000-0001-7639-8893	Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation and Writing - Original Draft.
MBD	https://orcid.org/0000-0002-0693-2162	Methodology, Formal Analysis, Investigation, Data Curation, Writing - Review and Editing and Visualization.
RAA	https://orcid.org/0000-0002-3725-9572	Conceptualization, Formal Analysis, Writing - Review and Editing and Visualization.
RRF	https://orcid.org/0000-0002-0222-3596	Formal Analysis, Data Curation, Writing - Review and Editing and Supervision.

All authors declare that they contributed to a critical review of intellectual content and approval of the final version to be published.

Financial Support

None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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