

COMPARATIVE ANALYSIS OF TWO FIXATING AND TWO DECALCIFYING SOLUTIONS FOR PROCESSING OF HUMAN PRIMARY TEETH WITH INACTIVE DENTIN CARIOUS LESION

ANÁLISE COMPARATIVA DE DUAS SOLUÇÕES FIXADORAS E DESCALCIFICADORAS PARA O PROCESSAMENTO DE DENTES DECÍDUOS HUMANOS COM LESÃO CARIOSA INATIVA EM DENTINA

Mattuella, Letícia Grando*
Bento, Letícia Westphalen**
Vier-Pelisser, Fabiana Vieira***
Araujo, Fernando Borba****
Fossati, Anna Christina Medeiros*****

RESUMO

O objetivo deste estudo foi analisar qualitativamente a preservação das características teciduais pulpares de dentes decíduos ântero-superiores humanos com lesão cariosa de natureza inativa em dentina, comparando-se duas soluções fixadoras (paraformaldeído a 4% e formalina a 10%, ambos com tampão fosfato 0,1 M) e descalcificadoras [ácido fórmico/citrato de sódio – solução de Ana Morse e EDTA (ácido etileno diamino tetracético) a 10%]. Oito dentes foram subdivididos em 4 grupos (n = 2), variando-se o agente fixador e descalcificador. Grupo 1: paraformaldeído a 4% e solução de Ana Morse; Grupo 2: formalina a 10% e solução de Ana Morse; Grupo 3: paraformaldeído a 4% e EDTA a 10%; Grupo 4: formalina a 10% e EDTA a 10%. Os dentes foram fixados e posteriormente descalcificados até que uma consistência borrachóide fosse obtida. Cortes histológicos de 6µm de espessura foram corados com hematoxilina e eosina (H&E). A fixação com ambas as soluções demonstrou boa conservação tecidual, enquanto que a descalcificação com a solução de Ana Morse pareceu mais adequada, por requerer um menor tempo de processamento das amostras e promover uma melhor preservação dos componentes celulares e da matriz extracelular do tecido pulpar. O EDTA, além de exigir um tempo mais longo de processamento das peças, alterou as características morfológicas da polpa. A combinação formol a 10% – solução de Ana Morse pareceu ser favorável para a metodologia deste estudo.

UNITERMOS: fixação de tecidos; descalcificação; dente decíduo; cárie dentária; matriz extracelular.

SUMMARY

The aim of this study was to analyze qualitatively the pulp tissue characteristics of maxillary anterior human primary teeth with inactive dentin carious lesion. Two buffered fixating solutions (4% paraformaldehyde and 10% formalin) and two decalcifying solutions [formic acid plus sodium citrate – Ana Morse solution and 10% ethylene-diaminetetraacetic

* DDS, MSc, Substitute Professor of Pediatric Dentistry, Department of Pediatric Dentistry, Faculty of Dentistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS.

** DDS, Postgraduate student of the Master's degree program in Pediatric Dentistry, Faculty of Dentistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS.

*** DDS, MSc, in Endodontics [Lutheran University of Brazil (ULBRA), Canoas, RS, Brazil] and PhD in Clinical Stomatology [Faculty of Dentistry of the Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil].

**** DDS, MSc, PhD, Coordinator of the Specialization Course in Pediatric Dentistry and Adjunct Professor of Pediatric Dentistry of the Faculty of Dentistry of the Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

***** DDS, MSc, PhD, Professor of the Department of Morphological Sciences, Institute of Basic Health Sciences, Faculty of Dentistry of the Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

acid(EDTA)] were evaluated. Eight teeth were assigned to 4 groups (n = 2), according to the fixating and decalcifying agents used. Group 1: 4% paraformaldehyde and Ana Morse solution; Group 2: 10% formalin and Ana Morse solution; Group 3: 4% paraformaldehyde and 10% EDTA; Group 4: 10% formalin and 10% EDTA. The teeth were fixed and thereafter decalcified until a rubber-like consistency was obtained. After histological processing, serial 6- μ m-thick sections were obtained and stained with hematoxylin and eosin (H&E). Both fixating solutions provided good preservation of the pulp tissue, showing the normal tissue topography. However, the use of 10% EDTA caused tissue damage, which was evidenced by the cell aspect and the altered extracellular matrix (ECM) characteristics. Decalcification with Ana Morse solution seemed to produce better preservation of cells and ECM morphology. The association of Ana Morse solution and 10% formalin was more favorable for the pulp tissue. In conclusion, fixation with either solution yielded good tissue preservation, while decalcification with Ana Morse solution required a shorter time for specimen processing and promoted better preservation of cell components and pulpal ECM. EDTA modified the pulpal morphological properties and required a longer processing time. The combination of 10% formalin and Ana Morse solution seemed to be the most favorable for the proposed methodology.

UNITERMS: tissue fixation, decalcification, primary teeth, caries, extracellular matrix.

INTRODUCTION AND REVIEW OF LITERATURE

The first step of the histologic technique is tissue fixation after specimen acquisition. Its main goal is to avoid cell destruction by its own enzymes (autolysis) or bacteria, and to reinforce the tissues, making them able to withstand the subsequent phases of the technique.¹⁰ The anatomic characteristics and the chemical properties of different tissues are therefore preserved.¹³

If specimen decalcification is not required, the next step is dehydration in ethanol solutions of increasing concentrations for removal of tissue water. Subsequently, the specimen is cleared with xylol, soaked in liquid paraffin and embedded in solid paraffin, thus providing blocks that will be cut into thin slices using a microtome. The histologic sections are placed in microscope glass slides and submitted to a staining technique.¹¹

A good fixating agent should have a high penetration capacity into the tissue to ensure proper fixation of superficial and deep cells.⁶ Several fixating agents with excellent properties are currently available. There is a consensus, however, on the use of 10% formalin because of the long experience with its use and the quality of specimen fixation with this solution.^{15,20} The addition of 0.1 M phosphate buffer to 10% formalin has been suggested and is currently practiced for better preservation of tissue and cell constituents.³

The 4% paraformaldehyde has lower formaldehyde concentration compared to 10% formalin and is buffered in the same way. It has been

considered a good tissue fixating solution because of its capacity of preservation, depending on the material evaluated and the technique used.⁹ The fixation time relies on the quality of the fixating agent, nature of specimen and temperature. If any of these requirements is not correctly fulfilled, the results may be compromised by alterations in normal tissue morphology.⁵

When the specimen has mineralized tissues, decalcification shall be performed immediately after fixation. The goal of decalcification is to remove calcium salts from the mineralized tissues, making them adequate for further cutting of histologic sections. Any acid, even if properly buffered, affects tissue stability. These effects depend on the solution's acidity and duration of the decalcification process. In addition, the faster the action of the decalcifying agent, the greater the damages and untoward effects to the staining technique performed in a subsequent step. The factors influencing the speed of decalcification include decalcifying solution concentration, temperature, stirring and tissue suspension.¹⁹

Decalcification is performed by chemical solutions, which may be acids or chelants. From a chemical standpoint, the decalcifying agents should have an acidic action in order to remove and turn the calcium salts, which are insoluble in the tissues, into soluble salts. Therefore, it should be emphasized that fixating agents that contain acid in their composition, such as formalin (that contains formic acid), are also able to act as decalcifying agents if the acid component is not neutralized.¹⁹

Acids may be divided into strong and weak acids and the Ana Morse solution is a weak acid. The addition of sodium citrate to its composition was suggested by Evans and Krajian⁸ (1930) apud Morse (1945)¹⁷ in order to neutralize the trend of formic acid to increase specimen volume during the decalcification process.

EDTA is the most widely used decalcifying agent and represents the group of chelants. It acts capturing metallic ions, mainly calcium, which is the only ion that is removed when binds to the chelating solution. This means that only the calcium ions from the external layer of the apatite crystal will be removed. When all calcium ions from the outer layer of the apatite crystal are removed, they will be replaced by ions from deeper layers. In this way, the crystal size decreases gradually, producing an excellent preservation of tissue components. A tissue is considered as completely decalcified when it presents a soft consistency to probing. Therefore, the material should be tested daily.¹⁹

Human teeth, as well as alveolar bone, must be decalcified during processing for histologic analysis because of its structure. Rapid fixation of all dental elements is difficult to obtain because penetration of the fixating agent through such structures as enamel, dentin and bone is a slow process. In such cases, the tissues in the center of the specimen may undergo some alterations before fixation is completed. The most seriously affected tissue is perhaps the pulp tissue.¹⁷

The pulp is a specialized loose connective tissue that contains cells, fibers, ground substance, blood vessels and nerve terminations. It is surrounded by rigid dentin walls and forms with the latter the embryologic and functional entity called the pulpodentinal complex.^{2,4} The histological analysis of the pulp reveals four different zones: the odontoblastic layer on pulp periphery; an acellular layer under the odontoblasts; a cell-rich layer; and the pulp core.^{1,2,4} The most predominant cell type in the pulp are the fibroblasts, but other cells are also observed, including odontoblasts, blood cells, Schwann cells, endothelial cells and undifferentiated mesenchymal cells.⁴

Collagen and reticular fibers are part of the pulpal ECM and have therefore an important role in the maintenance of this tissue. Type I collagen is the main fiber component, but type III collagen also consists of a significant part.¹² The cellular and fibrillar elements of the pulp are surrounded by a gelatinous ground substance with high water content and composed of different glycosamino-

glycans, glycoproteins and proteoglycans. This gelatinous substance is the vehicle for carrying metabolites into the cells and acts as an antibacterial barrier, consisting of an unspecific defense element.²

The components of pulpal ECM are mainly responsible for the physiological properties of this tissue. Several important clinical processes occur within an extracellular environment, such as inflammatory reactions and calcified tissue formation.¹²

The purpose of this study was to perform a qualitative analysis of pulp tissue preservation in maxillary anterior human primary teeth with inactive dentin carious lesions. The analysis involved two buffered fixating solutions (4% paraformaldehyde and 10% formalin) and two decalcifying solutions (Ana Morse solution and 10% EDTA). In addition, the total time required for specimen decalcification was also recorded.

MATERIALS AND METHODS

This study was approved by the Ethics in Research Committee of the Federal University of Rio Grande do Sul, according to the Brazilian National Health Council Resolution 196/96.

To be included in this study, maxillary anterior primary teeth (central incisors, lateral incisors, canines) should present on visual inspection a minimum of 2/3 of intact root, inactive carious lesion in dentin and absence of clinical (fistula, edema, mobility non-compatible with physiological root resorption) and radiographic (absence of pathologic alterations) pulp involvement. The teeth were retrieved from the Discipline of Pediatric Dentistry of the Faculty of Dentistry of the Federal University of Rio Grande do Sul (Brazil) and had been extracted for different reasons according to the treatment plan established for each child at the time of attendance.

Eight teeth were obtained and assigned to 4 groups (n = 2), as follows: Group 1: fixation with buffered 4% paraformaldehyde and decalcification with Ana Morse solution; Group 2: fixation with buffered 10% formalin and decalcification with Ana Morse solution; Group 3: fixation with buffered 4% paraformaldehyde and decalcification with 10% EDTA; Group 4: fixation with buffered 10% formalin and decalcification with 10% EDTA.

The teeth were immersed in the buffered fixating solutions [4% paraformaldehyde (4°C) or 10% formalin (room temperature)] immediately

after being collected and were fixed for 24 hours. After fixation, the teeth were decalcified in Ana Morse solution or 10% EDTA until present a soft consistency (rubber-like aspect) to cutting. Table 1 shows the decalcification times required for the specimens in each group. The specimens immersed in EDTA were stirred daily for approximately 12 hours using a magnetic stirrer. Both solutions were renewed twice a week.

After the decalcification process, the teeth were rinsed in running water for 12 hours, thereafter dehydrated in an increasing ethanol series, cleared with xylol, soaked in liquid paraffin and embedded in solid paraffin. The paraffin-embedded pieces were serially sectioned in a rotary microtome. Serial 6- μ m-thick sections were obtained and stained with H&E. Cell morphology as well as crown and root ECM were qualitatively assessed

by a single examiner blinded to the groups to which the histological sections belonged.

RESULTS

Both fixating solutions provided good preservation of the pulp tissue, showing the normal tissue topography in a satisfactory manner. However, the use of 10% EDTA as a decalcifying solution caused tissue damage, which was evidenced by the aspect of the cells and altered ECM characteristics (Figs. 1 and 2).

Decalcification with Ana Morse solution seemed to produce better preservation of cells and ECM morphology. The association of Ana Morse solution and 10% formalin was more favorable for the pulp tissue (Figs. 3 and 4).

TABLE 1 – Total decalcification time of the specimens in each group.

Group/Specimen	Fixating agent	Decalcifying Agent	Time (months)
Group 1 – Specimen 1	4% paraformaldehyde	Ana Morse solution	4
Group 1 – Specimen 2	4% paraformaldehyde	Ana Morse solution	4 ½
Group 2 – Specimen 1	10% formalin	Ana Morse solution	4 ½
Group 2 – Specimen 2	10% formalin	Ana Morse solution	5 ½
Group 3 – Specimen 1	4% paraformaldehyde	10% EDTA	11
Group 3 – Specimen 2	4% paraformaldehyde	10% EDTA	11 ½
Group 4 – Specimen 1	10% formalin	10% EDTA	8 ½
Group 4 – Specimen 2	10% formalin	10% EDTA	12

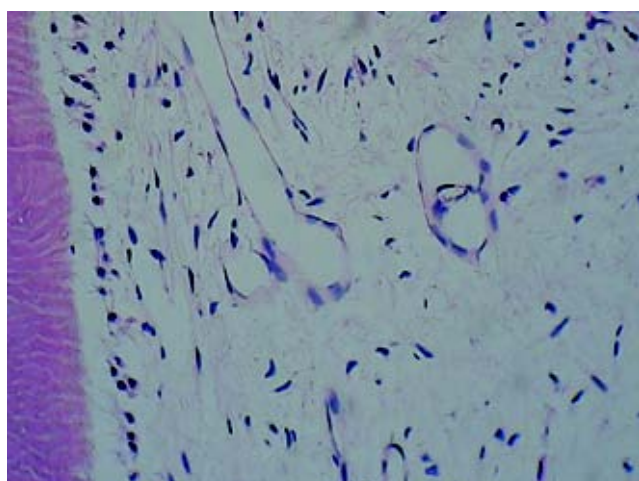


Figure 1 – Photomicrograph of the pulp of a primary tooth with inactive dentin carious lesion fixed with 10% formalin and decalcified with 10% EDTA. Notice the degradation of cell structures and extracellular matrix.

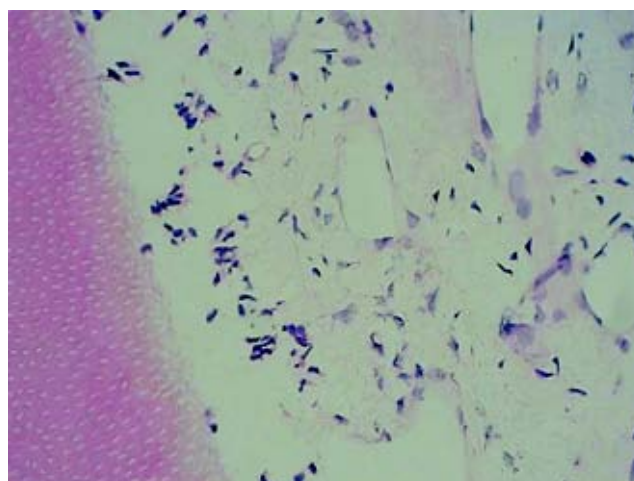


Figure 2 – Photomicrograph of the pulp of a primary tooth with inactive dentin carious lesion fixed with 4% paraformaldehyde and decalcified with 10% EDTA. Notice the disintegration of the odontoblastic layer and of the extracellular matrix structures.

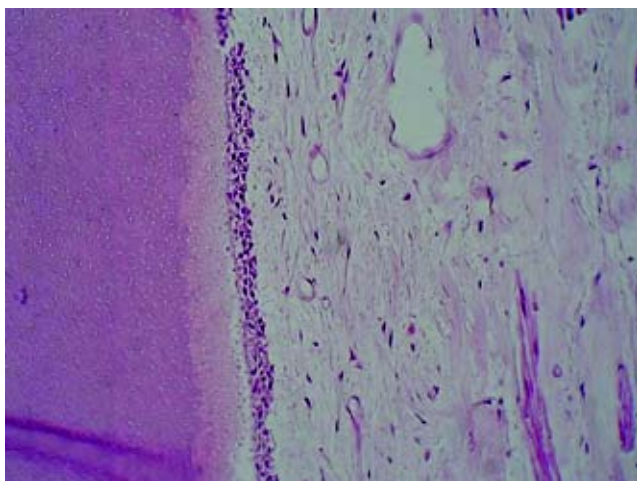


Figure 3 – Photomicrograph of the pulp of a primary tooth with inactive dentin carious lesion fixed with 10% formalin and decalcified with Ana Morse solution. Notice the integrity of the odontoblastic layer and cell structures.

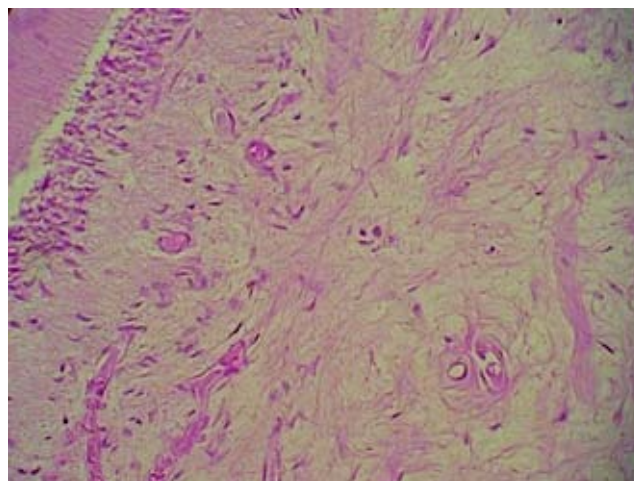


Figure 4 – Photomicrograph of the pulp of a primary tooth with inactive dentin carious lesion fixed with 4% paraformaldehyde and decalcified with Ana Morse solution. Notice the preservation of cell constituents and of the extracellular matrix morphology.

DISCUSSION

The nature of carious lesion is a good indicator of pulpal histologic status and the best prognoses are obtained with inactive carious lesions.⁷ Identification of active or inactive carious lesions in a patient is based on extremely scrupulous diagnostic criteria.¹⁸ Visual inspection and radiographic examination are among the most widely employed caries diagnosis methods.²³

In the present study, all teeth were clinically and radiographically examined to warrant that none of the specimens had pulp involvement. However, it is well known that, regardless their nature, irritating factors may affect the pulpal response and the predominant cellular structures in the pulp tissue.²²

Fixation with either 4% paraformaldehyde or 10% formalin seems to preserve the pulp tissue and maintain favorable conditions for examination and microscopic analysis of its cell components. Nevertheless, buffered 10% formalin is more commonly used because it is more readily available and may be stored for longer periods.¹⁶ On the other hand, 4% paraformaldehyde solution is short-lived and has technical requirements for preparation, such as heating and fine pH adjustment.⁹

For better penetration of the fixating agent, it is advisable to cut off the tooth apex.²¹ In this study, this was not required because we used primary teeth with physiological root resorption and therefore the specimens had wide foramen openings.

The tooth pulp is probably the most affected tissue by decalcification processes.¹⁷ Therefore, the goal of this study was to assess qualitatively the preservation of pulp components after decalcification with two decalcifying solutions, by analyzing histologic sections stained with H&E.

Although the effect of stirring during the decalcification process is a subject of great controversy in the literature, it is believed that this procedure accelerates fluid exchange within and around the tissues.¹⁹ However, this procedure was not effective in speeding up decalcification of dental tissue in the specimens examined in the present study (Table 1).

When an acidic solution is used for decalcification, it is advisable to test it on a daily basis. This can be performed using chemical, radiographic or physical tests, the latter including probing, cutting and palpation. Although, none of these tests is satisfactory and some of them are even destructive, palpation may be a useful guide for skilled hands and, in some cases, might avoid unnecessary tests in the initial phases of decalcification.¹⁹

Moreira (2001)¹⁶ investigated the histologic aspect and innervation of the pulpodentinal complex of human primary teeth at different stages of physiological root resorption, using sections stained with H&E and immunohistochemical technique. 10% EDTA was used for specimen decalcification and the solution was renewed every two days. The specimens took 4 to 6 weeks to be ready for embedding in paraffin. These results do

not agree with those obtained of the present study, in which a longer time was required for complete decalcification of the specimens. This may be explained by the greater mineral contents of inactive carious lesions. During the remineralization process after a cariogenic challenge, calcium and phosphate ions coming from saliva are deposited in the dental structure affected by caries disease, making it more resistant to future acid attack.¹⁴

In the present study, it was observed that, regardless of the employed fixating solution, ECM and pulp cell alterations were observed when 10% EDTA solution was used as a decalcifying agent. The long time required for complete decalcification of the pieces probably produced these alterations.¹⁹

The combination of buffered 10% formalin and Ana Morse solution seemed to be the most advised for analysis of structures stained with H&E technique. The specimens in this group presented a greater accuracy of anatomical details and better preservation of tissue matrix and cells. These findings are consistent with those of previous studies.²¹

A small number of specimens were used in this study because of the difficulty in obtaining primary teeth that fulfilled all inclusion criteria in addition to the time required for processing the pieces. Further research is required to corroborate or confront the results obtained with the solutions employed in this study.

CONCLUSIONS

The analysis of pulp components from maxillary anterior primary teeth with inactive dentin carious lesion showed that:

1. both 4% paraformaldehyde and 10% formalin fixating solutions buffered with 0.1 M phosphate buffer yielded tissue preservation;
2. both Ana Morse and 10% EDTA solutions were effective if decalcifying the specimens. However, the 10% EDTA solution required a longer time for decalcification and caused alteration of cells and pulpal ECM characteristics;
3. the association of 10% formalin and Ana Morse solution seemed to be the most favorable for the purpose of this study.

REFERENCES

1. Arana V, Katchburian E. Complexo dentina-polpa. In: Arana V, Katchburian, editors. *Histologia e embriologia oral: texto – atlas – correlações clínicas*. São Paulo: Panamericana; 1999. p. 181-236.
2. Berkowitz BKB, Holland GR, Moxham BJ. Polpa dentária. In: Berkowitz BKB, Holland GR, Moxham BJ, editors. *Anatomia, embriologia e histologia bucal*. 3rd ed. Porto Alegre: Artmed; 2004. p. 149-67.
3. Brasileiro Filho G, Barbosa AJA, Miranda D. Métodos de estudo em patologia. In: Brasileiro Filho G, Barbosa AJA, Miranda D, editors. *Bogliolo Patologia*. 5th ed. Rio de Janeiro: Guanabara Koogan; 1994. p. 6-19.
4. Chiego Júnior DJ. Histology of pulp. In: Avery JK, editor. *Oral development and histology*. 2nd ed. Stuttgart: Thieme; 1994. p. 262-81.
5. Costacurta L. Métodos histológicos. In: Costacurta L, editor. *Histologia*. São Paulo: Artes Médicas; 1969. p. 15-23.
6. Di Fiore MSH. Técnica histológica. In: Di Fiore MSH, editor. *Diagnóstico histológico: reconhecimento de los órganos por su estructura microscópica – Tomo I*. 5th ed. Buenos Aires: El Ateneo; 1963. p. 53-76.
7. Di Nicolo R, Guedes-Pinto AC, Carvalho YR. Histopathology of the pulp of primary molars with active and arrested dentinal caries. *J Clin Pediatr Dent*. 2000;25(1):47-9.
8. Evans N, Krajian A. New method of decalcification. *Arch Pathol*. 1930;10(3):447-51.
9. Fossati ACM. Integrinas e proteínas da matriz extracelular durante o desenvolvimento da glândula submandibular de ratos. São Paulo, 2000. [Tese de Doutorado – Instituto de Ciências Biomédicas Universidade de São Paulo].
10. Ham AW. Como estudar histologia. In: Ham AW. *Histologia*. Rio de Janeiro: Guanabara Koogan; 1956. p. 10-9.
11. Junqueira LC, Carneiro J. Histologia e seus métodos de estudo. In: Junqueira LC, Carneiro J. *Histologia Básica – Texto/Atlas*. 10th ed. Rio de Janeiro: Guanabara Koogan; 2004. p. 1-22.
12. Linde A. The extracellular matrix of the dental pulp and dentin. *J Dent Res*. 1985;64(5):523-29.
13. Kiernan JA. Fixation. In: Kiernan JA, editor. *Histological & histochemical methods: theory & practice*. 3rd ed. Oxford: Butterworth Heinemann; 1999. p. 11-40.
14. Maltz M, Parolo CCF, Jardim J. Cariologia clínica. In: Toledo AO. *Odontopediatria – Fundamentos para a prática clínica*. 3th ed. São Paulo: Editorial Premier; 2005. p. 103-50.
15. Miller RT, Swanson PE, Wick MR. Fixation and epitope retrieval in diagnostic immunohistochemistry: a concise review with practical considerations. *Appl Immunohistochem Mol Morphol*. 2000;8(3):228-35.
16. Moreira A. Histologia e inervação do complexo dentino-pulpar de dentes decíduos humanos em diferentes fases de rizólise. Belo Horizonte, 2001. [Dissertação de Mestrado – Instituto de Ciências Biológicas Universidade de Minas Gerais].
17. Morse A. Formic acid-sodium citrate decalcification and butyl alcohol dehydration of teeth and bones for sectioning in paraffin. *J Dent Res*. 1945; 24(3): 143-53.

18. Nyvad B, Machiulskiene V, Baelum V. Reliability of a new caries diagnostic system differentiating between active and inactive caries lesions. *Caries Res.* 1999;33(4):252-60.
19. Page K, Stevens A, Lowe J, Bancroft JD. Bone. In: Bancroft JD, Stevens A, editors. *Theory and practice of histological techniques*. 4th ed. New York: Churchill Livingstone; 1996. p. 309-39.
20. Sari, S, Aras, S, Gunhan, O. The effect of physiological root resorption on the histological structure of primary tooth pulp. *J Clin Pediatr Dent.* 1999;23(3):221-5.
21. Silva AF, Tarquinio SBC, Demarco FF, Piva E, Rivero ERC. The influence of haemostatic agents on healing of healthy human dental pulp tissue capped with calcium hydroxide. *Int Endod J.* 2006;39(4):309-16.
22. Soares IJ, Goldberg F. O cenário. In: Soares IJ, Goldberg F, editor. *Endodontia: técnica e fundamentos*. Porto Alegre: Artmed; 2001. p. 23-6.
23. Wenzel A. Digital radiographic and caries diagnosis. *Dentomaxillofac Radiol.* 1998;27(1):3-11.

Recebido para publicação em: 03/11/2006; aceito em: 07/03/2007.

Corresponding address:

LETÍCIA GRANDO MATTUELLA
Rua Roque Calage, 566/201
CEP 91350-090, Porto Alegre, RS, Brasil
Phone: (+55-51) 8402-7750
E-mail: lmattueLLa@gmail.com