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Original

Immunohistochemical localization of transient receptor potential vanilloid-type 5 in ameloblasts of Swiss mice

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ABSTRACT

Objective. To determine the localization of transient receptor potential vanilloid 5 (TRPV5) in the ameloblasts of Swiss mice. **Materials and methods.** In vitro experimental study where paraffin sections of 12 continuously growing incisors of 7-day-old male Swiss mice were analyzed. Developmental stages of dental enamel formation were identified by hematoxylin-eosin staining. Immunodetection of the transient receptor potential vanilloid 5 was performed using the primary polyclonal antibody anti-TRPV5. Observations were carried out using a Leica DM 500 microscope. **Results.** The different stages involved in the formation of dental enamel were identified, including the secretory and maturation stages. In them, it was evidenced that the ameloblasts were elongated cells with oval nuclei in a proximal position and with developed areas of medial and distal secretion. TRPV5 immunolabeling was visualized in the cell membrane and cytoplasm of the secretory and maturation ameloblasts of all mice tested. TRPV5s were also immunolocalized in odontoblasts, vascular endothelium, and pulp cells. **Conclusions.** The transient receptor potential vanilloid 5 is located on the secretory and maturing ameloblasts of Swiss mice. In particular, TRPV5s are immunodetected in odontoblasts, vascular endothelium, and dental pulp cells.

Keywords: Ameloblasts; TRPV cation channels; calcium channels; odontoblasts; sental enamel; dentin (*Source: MeSH*).

RESUMEN

Objetivo. Determinar la localización de los receptores de potencial transitorio de tipo vaniloide-5 (TRPV5) en los ameloblastos de ratones Swiss. **Materiales y métodos.** Estudio experimental *In vitro* donde fueron analizados cortes en parafina de 12 incisivos de crecimiento continuo de ratones machos Swiss salvajes de 7 días de nacidos. Las etapas del desarrollo de la formación del esmalte dental se identificaron por medio de la coloración hematoxilina-eosina. La inmunodetención de los receptores de potencial transitorio de tipo vaniloide-5 fue efectuada por medio del anticuerpo primario policlonal anti-TRPV5. Las observaciones se llevaron a cabo mediante un microscopio Leica

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DM 500. **Resultados.** Se identificaron las diferentes etapas implicadas en la formación del esmalte dental, entre ellas, la etapa de secreción y maduración. En ellas, se evidenció que los ameloblastos eran células alargadas con núcleos ovalados en posición proximal y con áreas manifiestamente desarrolladas de secreción medial y distal. Fue visualizado un inmunomarcaje de TRPV5 en la membrana plasmática y citoplasma de los ameloblastos de secreción y maduración de todos los ratones analizados. Los TRPV5 también fueron inmunolocalizado en los odontoblastos, endotelio vascular y células pulpares. **Conclusiones.** Los receptores de potencial transitorio de tipo vaniloide-5 se localizan en los ameloblastos de secreción y maduración de ratones Swiss. Particularmente, los TRPV5 son inmunodetectados en los odontoblastos, endotelio vascular y células de la pulpa dental.

Palabras clave: Ameloblastos; canales catiónicos TRPV; canales de calcio; odontoblastos; esmalte dental; dentina (*Fuente: DeCS*).

INTRODUCTION

Ameloblasts are cells responsible for protein secretion and maintenance of an extracellular environment that favors the formation of the most mineralized tissue produced by vertebrates, dental enamel. Ameloblasts have a particular dynamism, having three stages of differentiation: presecretory, secretory, and maturation. At each stage, ameloblasts have a specific activity. In the presecretory stage, enamel formation begins, which will be thin and with crystals integrated into the dentin to reinforce resistance to pressure and shock. In the secretory stage, the enamel is composed of a mixture of forming crystals and a variety of proteins and ions such as calcium. Finally, in the maturation stage, the enamel increases mineral density and the massive arrival of phosphate and calcium occurs (1).

The modifications of the physiological events in the differentiation stages of the ameloblasts can induce structural alterations in the disposition of the hydroxyapatite crystals that integrate dental enamel (2). These modifications could lead to loss or decrease in dental enamel hardness and chewing function. Therefore, it is interesting to elucidate the role of ameloblasts and the constitutional elements of enamel during its formation. One of them is calcium (Ca^{2+}), an element of the alkaline-earth metal family whose homeostasis is vital in all forms of life. This element is essential for dental enamel, the content of Ca^{2+} in this structure is significantly elevated, compared to organs such as the liver or muscle (3).

In recent years, it was considered that Ca^{2+} transport in ameloblasts occurred as a passive process. However, this concept has changed due to the identification of channels involved in its transport at the cellular level (4). It has been known that ameloblasts are capable of regulating the transport of ions and molecules, making it easier for crystals to grow and tolerate excess acidity through HCO_{3-} (a by-product of the formation of hydroxyapatite) (5). This physiological process occurs thanks to the presence of channels with high selectivity for Ca^{2+} over other cations. Among these, the TRPV5 epithelial calcium channels stand out, members of the subgroup ('V' for vanilloid) belonging to the family of transient receptor potential (TRP) channels. In addition to their specificity for Ca^{2+}, TRPV5s are located in the apical membrane of epithelial tissues and are responsive to 1.25-dihydroxyvitamin D3 (1.25[OH]2D3), facilitating the entry and transport of Ca^{2+} at an intestinal and renal level (6).

However, at the dental level, particularly in ameloblasts, the study of TRPV5s has been poorly addressed. Given this, it would be interesting to know the location of TRPV5s, an event that will help elucidate the role of these channels during the formation of dental enamel or amelogenesis. Therefore, the objective of this work was to determine the localization of the transient receptor potential vanilloid 5 (TRPV5) in the ameloblasts of wild-type mice using an immunohistochemical study.

MATERIALS AND METHODS

Tissue preparation. Twelve continuously growing incisors from 7-day-old male Swiss WT mice (Janvier, St Berthevin) were used. The incisors were obtained from the mandibles by microdissection with a Stereomicroscope (Leica MZ FLIII, Leica Microscopy Systems), after anesthesia and perfusion with 4% paraformaldehyde (PFA, Sigma-Aldrich Co.). Initially, the mandible was isolated from the

basal bone and then, the continuously erupting incisors were extracted using a Gracey curette (Hu-Friedy). The obtained teeth were carefully washed in phosphate-buffered saline (PBS 1X, Gibco™) and fixed by immersion in 4% paraformaldehyde (PFA, Sigma-Aldrich Co.) and 0.1% glutaraldehyde (Sigma-Aldrich Co.) for 24 hours. Then, the teeth were decalcified in 10% EDTA (ethylenediaminetetraacetic acid) for 4 weeks.

Subsequently, the samples were dehydrated and embedded in paraffin. Once the blocks were obtained, 6 µm sections were made using a Leica RM2125 RST microtome. Then, slides containing sections of ameloblasts in the secretory/maturation stage were identified and selected using hematoxylin-eosin staining, previously described by Simancas-Escorcia et al (7). This work took into account the ethical aspects contemplated in resolution 008430 of 1993 of the Republic of Colombia and was carried out following the international 3R principles and the guidelines found in the Guide for the care and use of laboratory Animals (8).

Hematoxylin-eosin staining. Once immersed in xylene (Leica Biosystems), the sections were rehydrated in decreasing concentrations of alcohol (100%, 95%, 80%, 60%, 30%) and then stained with Mayer's hematoxylin for 5 minutes and immediately washed in Water. Then, an Acid-Alcohol differentiation was carried out for 30 seconds followed by eosin staining for 3 minutes. Finally, the sections were dehydrated, washed in xylene, and mounted in an anhydrous mounting medium (DPX).

Immunohistochemistry. After selecting the incisor sections to be studied, they were deparaffinized and rehydrated in xylene and alcohol baths, respectively. Antigenic retrieval was performed by incubating the sections in a pH6 citrate buffer for 20 minutes at 97°C. Endogenous peroxidase blockade was performed for 10 minutes in 3% hydrogen peroxide. Then, the sections were immersed in a washing solution (Tris-buffered saline solution (TBS 1X, Gibco™) + polyoxyethylene monolaurate (20 sorbitan or Tween®20 at 0.05%)) (3x5 minutes) and, proceeded to perform blocking of specific sites with Normal horse serum (Vector Laboratories) for 20 minutes. Sections were then incubated with the primary antibody Anti-TRPV5 (Rabbit Polyclonal, 1:200 dilution, PA577320, Invitrogen) at 4°C overnight. The next day, after washing, the slides remained in contact with the anti-Rabbit biotinylated secondary antibody (Vector Laboratories) and with the R.T.U Vectatastain® ABC peroxidase system (Vector Laboratories) for 30 minutes each at room temperature. The reaction was visualized after incubating the sections with Diaminobenzidine (Impact DAB System, Vector Laboratories) for 4 minutes. The reaction was stopped by washing with distilled water. Finally, tissues were counterstained with Mayer's hematoxylin for 30 seconds, dehydrated in ethanol, followed by xylene, and mounted with DPX.

The TRPV5 reaction was considered positive when visualizing brown staining. The negative control was performed without the presence of the primary antibody, replaced by the blocking solution. All observations were made using a Leica DM 500 microscope coupled to a camera.

RESULTS

Hematoxylin-eosin staining allowed the identification of the structures that compose and surround the continuously growing incisors of the wild-type mice evaluated in this study (Figure 1, A). The different stages involved in the formation of dental enamel were recognized, including the secretory and maturation stages. There, the ameloblasts were elongated cells with oval nuclei in a proximal position and with a noticeably developed area of medial and distal secretion. In the superior extremity of the secretory ameloblasts, the stratum intermedium was observed, which disappeared in the maturation ameloblasts. An anatomical area composed of layers of flat cells with elongated central nuclei. Likewise, the formation of a well-defined mineralized enamel matrix adjacent to the apical area of the ameloblasts was identified. An intermediate zone between enamel and dentin was also identified in all the analyzed samples, as well as odontoblasts located in the periphery of the dental pulp (Figure 1, B).

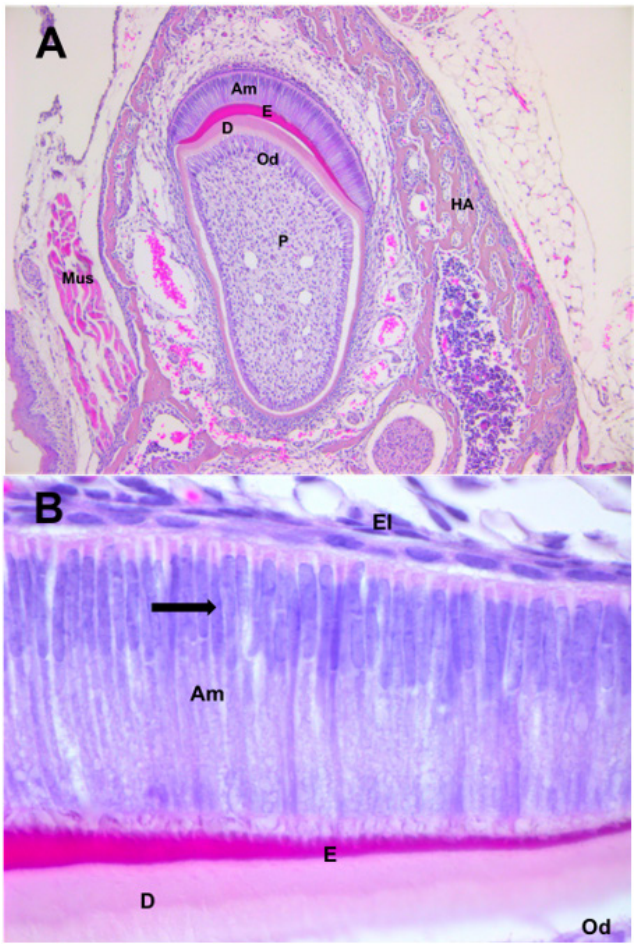

Figure 1. Histological sections of the continuously growing incisor of a 7-day-old Swiss WT mouse. **(A)** View of the incisor with adjacent structures (25x). **(B)** Anatomical appearance of ameloblasts surrounded by stratum intermedium (SI) and mineralized dental structures (200x). **Mus:** muscle; **AB:** alveolar bone; **Am:** ameloblasts; **E:** enamel; **Od:** odontoblasts; **D:** dentin; **P:** pulp. Hematoxylin-eosin staining.

Through the immunohistochemical technique, it was possible to verify the location of TRPV5 in the continuously growing incisors of 7-day-old Swiss WT mice. The distribution of these receptors was widely identified in dental structures, but also in muscle, periodontal ligament, and epithelial cell rests of Malassez (Figure 2, A). In all the dental incisors analyzed, the immunolocalization of TRPV5 was constant in the area of secretory and maturation ameloblasts. There, its location was in the cell membrane and the cytoplasm, with an important distribution in the proximal zone that is in contact with the enamel matrix and the distal area, limiting the stratum intermedium. In this last zone, TRPV5s were detected during the secretory stage in the cytoplasm of the elongated cells that compose it (Figure 2, B, C). No immunohistochemical staining was identified in the histological sections where the primary antibody anti-TRPV5 was replaced by the blocking solution (negative control) (Figure 2, D).

It was also possible to verify the immunolocalization of TRPV5s in the cell membrane and cytoplasm of odontoblasts. In this case, TRPV5s were distributed in the apical secretory pole, including the areas of odontoblastic extensions that were in contact with the mineralized matrix layer or predentine (Figure 3, A). TRPV5 immunodetection was notable in dental pulp cells, including Höehl's cells and pulp fibroblasts. In the cell-rich zone and the central zone of the dental pulp, particularly, in the endothelial cells that form the blood vessels (Figure 3, B-D).

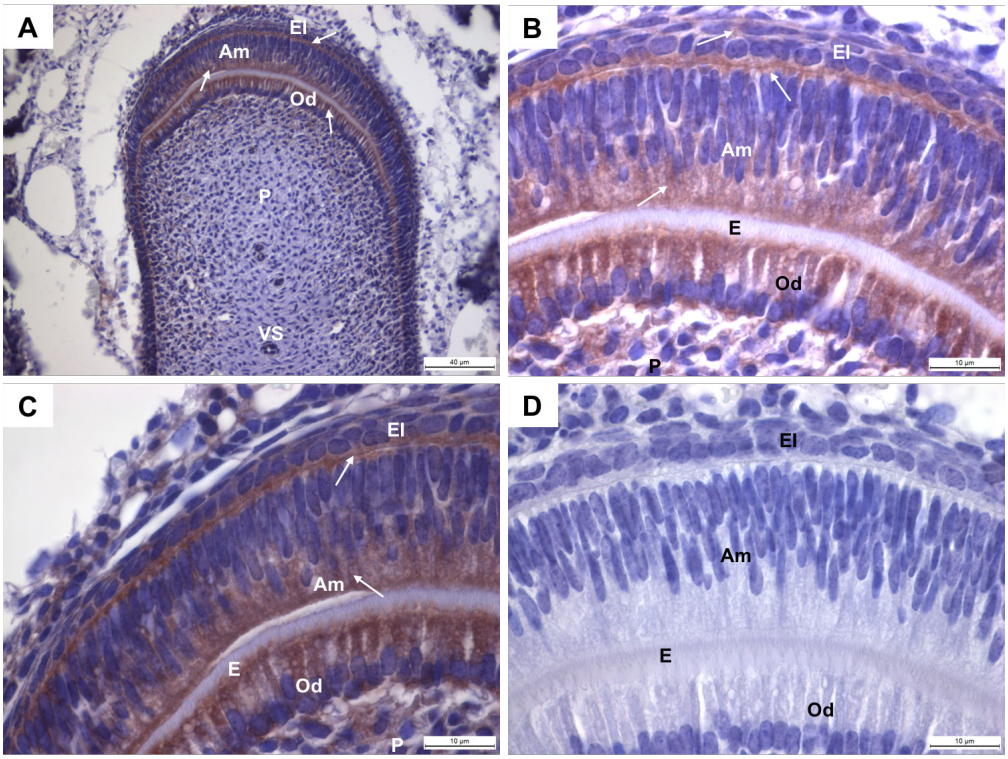

Figure 2. Immunohistochemical localization of TRPV5 in ameloblasts of Swiss WT mice **(A)** Photomicrograph of continuously growing incisor and surrounding structures. Note the brown staining of TRPV5s. **(B,C)** TRPV5 immunostaining in ameloblasts, stratum intermedium, and odontoblasts (arrows). **(D)** Negative control. **Am:** ameloblasts; **SI:** stratum intermedium; **E:** enamel; **Od:** odontoblasts; **P:** pulp; **BV:** blood vessels. Black bar: 40 µm (A); 10 µm (B,C,D).

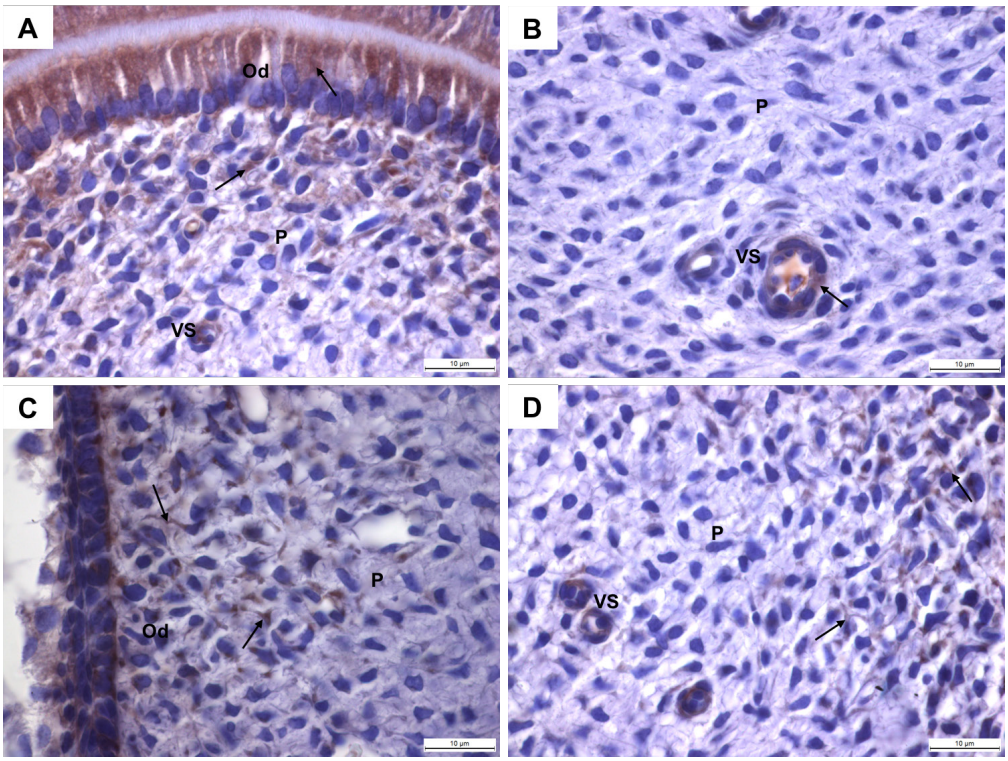

Figure 3. Immunodetection of TRPV5 in pulp cells of Swiss WT mice aged 7 days. **(A)** Distribution of TRPV5 immunolabeling in odontoblasts and adjacent cells. **(B,C,D)** TRPV5 immunostaining in blood vessel endothelial cells, Höehl cells, and pulp fibroblasts (arrows). **Od:** odontoblasts; **P**: pulp; **BV:** blood vessels. Black bar: 10 µm.

DISCUSSION

Answering fundamental questions about the mineralization process of dental enamel implies understanding the movements of calcium throughout the formation of this structure. The identification of the membrane proteins that form calcium channels and participate in the movements of this ion in the ameloblasts of wild mice, particularly TRPV5, was the object of this research work. The results obtained show the unequivocal expression of TRPV5 in the secretory and maturation ameloblasts of the continuously growing incisors of 7-day-old Swiss WT mice. This expression was particularly localized in the cytoplasm and cell membrane of the proximal and distal areas of the ameloblasts. These findings would indicate that TRPV5 could play an essential role in the mineralization processes of dental enamel.

The cytoplasmic location of TRPV5 in ameloblasts suggests their involvement in intracellular Ca^{2+} regulatory functions. Studies indicate that these proteins are stored inside cells waiting to supply the required Ca^{2+} needs (9). For example, in the distal convoluted tubules of the kidneys, TRPV5s are the main facilitators of Ca^{2+} uptake. TRPV5s have even been identified as a critical element for Ca^{2+} homeostasis and increased cell proliferation (10). In ameloblasts, the relationship between the localization and the physiological mechanism of TRPV5 has not been established. However, it is likely that TRPV5s not only facilitate calcium influx across the plasma membrane but also have a potential intermediary role in intracellular calcium uptake thanks to its storage in compartments such as endosomes. In both situations, TRPV5s could be accompanied by other Ca^{2+} facilitators, such as the Ca^{2+} ATPase pump (PMCA1) and Na+/Ca^{2+} exchangers (11).

The model of continuous growth of rodent incisors used in the present investigation has been widely used for understanding the processes related to the dental formation (12,13). Thanks to this model, it has been possible to determine the high rate of Ca^{2+} transport in secretory and maturation ameloblasts during amelogenesis (14). This physiological aspect of calcium in ameloblasts would justify the immunolocalization of TRPV5s in these dental structures, as was observed in the present investigation. We hypothesize that potential modifications in these channels could involve biological changes in calcium transport/storage and, consequently, affect the physiological formation of dental enamel. These events would be responsible for the presence of developmental and structural defects in dental enamel, including amelogenesis imperfecta (AI) (15).

Although at present a direct relationship between TRPV5s dysfunction and AI has not been established, recently, it has been pointed out that the extrusion of Ca^{2+} from the apical pole of ameloblasts can also be mediated by other transporters. A variable number of mutations in genes encoding for proteins involved in calcium transport and regulation are responsible for AI (7). To date, no pathogenic mutations of TRPV5s have been reported in humans, but nucleotide polymorphisms have. It is not clear to what extent these genetic variations would affect the function of the TRPV5 protein, but it is believed that they could decrease the stability of the calcium channels (16). Studies show that the chemical and specific modulators of TRPV5s have the potential to contribute to the treatment of calcium homeostasis disorders such as nephrolithiasis and hypercalcaemia (17). Precisely, calcium precipitation in the renal cortex or the medulla has been associated with syndromes where the dental phenotype is compatible with structural defects of dental enamel or hypoplastic amelogenesis imperfecta (18,19). Therefore, the implication of possible structural and functional defects of TRPV5s at the dental level is a question that remains unresolved.

Another important finding of the present study was the localization of TRPV5s in odontoblasts and dental pulp cells, including vascular endothelial cells. The identification of TRP markers (TRPV1-4) in odontoblasts (not specifically TRPV5), is related to the numerous ion channels and neuronal receptors present in odontoblasts and involved in the transduction of dental pain (20). Odontoblasts usually have mechanical sensors capable of perceiving changes in calcium concentration, these sensors are mechano-sensitive ion channels that facilitate the transduction of mechanical stimuli into electrical signals (21). Recent studies indicate that pulpal sensory axons adjacent to odontoblasts express glutamate receptors. Thus, suggesting that odontoblasts contain glutamate as a neuroactive substance necessary for the activation of pulpal axons (22). Therefore, it is not ruled out that TRPV5s have a role related to the signal transduction in odontoblasts and dental pulp cells. However, functional studies will be useful to clarify the role of these proteins in pulp cells.

On the other hand, the identification of TRPV5s in the cytoplasmic membrane and cytoplasm of odontoblasts is probably the result of an important flow of calcium in the formation of dentin, as has been reported in other investigations (23). The identification of TRPV5s in blood vessels was also an interesting finding. Although there are no reports of the role of these proteins in vascular beds, perhaps their location corresponds to calcium transport through them. But in the endothelial cells of the pulpal blood vessels, where they were identified, it is probably a vascular myogenic response resulting from depolarization of the smooth muscle cell membrane and calcium flux through the channels. Studies report that the activation of TRPV2 channels is responsible for the depolarization of the cell membrane and, this can also contribute to calcium influx and constriction of blood vessels. The physiological role of TRPV5s in dental pulp cells, including odontoblasts, has not been determined, but the immunolocalization described in this work constitutes a starting point for future studies that seek to elucidate the function of this protein in the aforementioned dental structures.

In conclusion, the immunohistochemical expression of TRPV5s was identified in the cell membrane and cytoplasm of secretory and maturing ameloblasts of 7-day-old Swiss mice. An important expression of TRPV5s was detected in odontoblasts, vascular endothelium, and dental pulp cells. This would indicate that TRPV5s could participate in calcium transport and homeostasis during the formation of mineralized dental structures. Future studies will be necessary to establish the physiological mechanisms that involve the transport and regulation of calcium homeostasis by TRPV5s in ameloblasts and odontoblasts, which would help to clarify the role of TRPV5s in the mineralization of enamel, dentin, and its participation in pathological processes during the formation of these structures.

Conflict of interests

None, declared by the authors.

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