

CHAPTER III

MATERIALS AND METHODS

3.1 Subjects

The use of human subjects in this study was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University.

Teeth obtained from the subjects were divided into four groups: normal permanent teeth, inflamed permanent teeth, normal primary teeth, and inflamed primary teeth. The normal primary and permanent teeth served as negative control groups, while the inflamed permanent teeth served as positive controls.

Normal permanent teeth

Normal permanent teeth were obtained from subjects whose teeth were intact and were intended for extraction for orthodontic purposes (n = 6-18).

Inflamed permanent teeth

Inflamed permanent teeth were obtained from subjects whose teeth were diagnosed with irreversible pulpitis and required extraction due to inability to restore or because of financial problems (n = 5-7).

Normal primary teeth

Normal primary teeth were obtained from subjects, aged at least five years, whose primary teeth were intact, over-retained, and required extraction for

orthodontic purposes or to provide access for their succedaneous teeth. The degree of root resorption of these teeth was not more than 1/3 of their root length (n = 5-7).

Inflamed primary teeth

Inflamed primary teeth were obtained from subjects, aged at least five years, whose primary teeth had deep caries, were diagnosed with reversible or irreversible pulpitis, and required extraction due to inability to restore or because of financial problems (n = 9-16).

The subjects were excluded if they were unwilling to attend, or had a history of neuropathic disease, or received long-term analgesic drugs, steroids, or any drugs that alter pain perception.

The experimental procedures were explained to all the subjects satisfying eligibility criteria before the informed consent for participation in the study was obtained from subjects or the accompanying guardians, in cases of subjects under the age of 20.

3.2 Dental pain assessment

Before extraction, the subjects or guardians were interviewed to find out the following information: demographic data, medical condition, chief complaint, and history of pain. The clinical appearance, radiographic findings, diagnosis, and treatment indicated for the specific tooth to be studied were also recorded on the pain assessment form. The subjects were then asked to assess the maximum pain

experienced in association with the tooth to be treated on that day using VAS and WBFPS.

3.3 Pulpal tissue collection

Immediately after extraction, teeth were rinsed with normal saline solution to remove debris. Teeth were then flash-frozen in liquid nitrogen to prevent further protein degradation by proteases until pulpal tissue collection. To collect pulpal tissue, a vertical groove almost to the depth of the pulp chamber was cut along the buccal surface of each tooth from the incisal edge to the apex in anterior teeth, and to the furcation in molar teeth, using a round-end tapered diamond bur (D8). Then, the teeth were split open along the groove using an elevator. Pulpal tissues were carefully removed and stored in 1.5 ml Eppendorf tubes in liquid nitrogen, before being transferred to a freezer and stored at -80°C for further investigation.

3.4 Sample preparation

To prepare loading samples, frozen pulpal tissues were weighed on a digital scale. The pulpal tissues were then crushed with a plastic pestle in loading buffer at a ratio of 1 mg of pulpal tissue to 10 μl of buffer. One milliliter of loading buffer contained 900 μl of 2X sample buffer (stock 100 ml contained 2 ml of glycerol, 6 g of sodium dodecyl sulfate (SDS), and 1.4 g of Tris), 100 μl of 10% 20-mercaptoethanol, and 5 μl of 8% bromophenol blue in ethanol. The protein in the samples was denatured by boiling the mixture at 95°C for 5 minutes. These samples were kept in a -80°C freezer for further investigation.

3.5 Western blot analysis

Preparation of 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE)

The SDS-PAGE gels consisted of two layers: separating gel (10% acrylamide/bisacrylamide gel) and stacking gel (4% acrylamide/bisacrylamide gel). The separating gel contained a mixture of 2.5 ml of purified water, 2 ml of 30% acrylamide, 1.5 ml of 1.5M Tris-HCl pH8.8, 30 μ l of 10% ammonium persulfate, and 7.5 μ l of tetramethylethylenediamine (TEMED). The mixture of separating gel was loaded into a glass plates within the casting stand and was left for complete polymerization for about 30 minutes. Later, the stacking gel was prepared and was loaded on top of the separating gel. The stacking gel contained a mixture of 1.75 ml of purified water, 0.5 ml of 30% acrylamide, 750 μ l of 0.5M Tris-HCl pH6.8, 25 μ l of ammonium persulfate, and 2.5 μ l of TEMED. The well-forming comb was then quickly inserted between glass plates and the stacking gel was left for complete polymerization for about 15 minutes.

Electrophoresis

The SDS-PAGE gels were submerged in a tank of running buffer. One litre of running buffer contained 3.03 g of Tris, 14.42 g of glycine, and 1 g of SDS diluted in 1 L of purified water. The well-forming comb was gently pulled off under the running buffer and the excess gel remaining in wells were gently flushed off using a 5 ml syringe and 20G needle. Ten microliters of each loading sample, equal to 1,000 μ g of proteins, were carefully loaded into each well within the gel using the special gel loading tips. Five microliters of the molecular weight marker (Spectra™ Multicolor Broad Range Protein Ladder #SM1841, Fermentas Inc, Glen Burnie, MD,

USA) was loaded into the first well on the left side. After loading, the electric current was applied to the tank at 90 volts for 10 minutes. Later, the voltage was increased to 120 volts for 90 minutes.

Transfer of proteins

In order to make the proteins accessible to antibody detection, the proteins were removed from the gels onto polyvinylidene fluoride (PVDF) membranes. First of all, the gels were gently taken out of the glass plates. Then, they were placed within the solid supporter in the order as follows: sponge, blotting paper, gel, membrane, blotting paper, sponge. Ensuring that no air bubbles formed between gel and membrane, the solid supporter was submerged in the tank of transfer buffer at 4°C, to which an electric current of 90 volts was applied for 90 minutes. One litre of transfer buffer contained 3.03 g of Tris and 14.42 g of glycine diluted in 200 ml of methanol and 800 ml of purified water. After transfer was complete, the transferred proteins in PVDF membranes were checked by incubating the membranes in Ponceau S staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) for up to an hour with gentle agitation. At this stage, the proteins were seen as red bands on the membranes. The membranes were then cut into pieces before being rinsed in purified water until the background was clean. Each piece of membrane contains protein bands that correspond with the molecular weight of Nav1.8, Nav1.9, MMP-9, β -actin, and PGP9.5, which are 217, 201, 88, 43 and 26 kDa respectively.

Blocking the membrane

Non-specific background binding of the primary and secondary antibodies to the membranes was blocked by incubating the membranes in 5% non-fat dry milk in 0.1% Tris-buffered saline and Tween 20 (TBST) on the orbital shaker for 1 hour. One litre of 0.1% TBST contained 2.42 g of Tris and 8 g of sodium chloride added to 1L of purified water and 1 ml of Tween 20.

Incubation with the primary antibody

The milk was thrown away before the primary antibodies were added to the membranes without washing. The concentration of antibodies used in this study was as follows: 1:200 of rabbit polyclonal anti-sodium channel Na_v1.8 (S2071, Sigma Aldrich Inc, St. Louis, MD, USA) in 10% non-fat dry milk in 0.1% TBST, 1:200 of rabbit polyclonal anti-sodium channel Na_v1.9 (S2196, Sigma Aldrich Inc) in 5% bovine serum albumin in 0.1% TBST, 1:200 of rabbit polyclonal antibody to MMP-9 (ab38904, Biomed Diagnostics, Cambridge, MA, USA) in 1% non-fat dry milk in 0.1% TBST, 1:400 of rabbit polyclonal antibody to PGP9.5 (ab10404, Biomed Diagnostics) in 10% non-fat dry milk in 0.1% TBST, and 1:1,000 of rabbit monoclonal antibody to β -actin (sc-130657, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.1% non-fat dry milk in 0.1% TBST. The membranes were incubated in primary antibodies at 4°C overnight, followed by washing in 0.1% TBST 6x5 minutes.

Incubation with the secondary antibody

After washing, the membranes were incubated with a secondary goat-anti rabbit antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA), in a concentration of 1:5,000 with 0.1% TBST. The membranes were finally washed in 0.1% TBST 6x5 minutes.

Development method

The protein bands were visualized on Amersham hyperfilm ECL Western blotting detection reagent systems (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) by immersing the membranes in enhanced chemiluminescent (ECL) reagent mixed 1:1. The exposure time was adjusted according to the signal strength of protein bands and the specificity of primary antibodies. Band intensity was quantified by the Scion Image Program (Scion Corporation, Frederick, MD, USA) and was normalized with β -actin to control the amount of protein loading and transfer.

3.6 Data analysis

The relative values for each protein of each sample in the same group was pooled and presented as mean \pm SE. The statistically significant difference in relative protein quantity between normal and inflamed pulp of both primary and permanent teeth was tested using the Mann-Whitney rank sum test. The Spearman rank correlation test was used to determine the correlation between VAS or WBFPS score and the relative amount of protein expression. The correlation of pain score between VAS and

WBFPS was analysed using the Pearson's correlation test. A level of $p < 0.05$ was taken to be the threshold of statistical significance.



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