Study Protocol with Statistical Analysis Plan

Title:

Evaluation of IL-2, IL-5, and IL-17 Levels in the Saliva of Patients Treated with Stainless Steel Crowns

NCT Number: NCT06439095

Date: May 1,2025

This prospective before-and-after clinical study was approved by the Local Ethics Committee of Van Yüzüncü Yıl University in accordance with the Declaration of Helsinki (Approval No: 2024/01-03). The study protocol was registered on ClinicalTrials.gov (NCT06439095). All parents/guardians were informed about the procedures and potential outcomes and were asked to sign a written informed consent form prior to participation. Children whose parents declined consent were excluded from the study.

Selection of Participants

Participants were selected among 30 patients who applied to the Pediatric Dentistry Clinic of Van Yüzüncü Yıl University Faculty of Dentistry. The study was conducted on 20 observations from 10 pediatric patients undergoing general anesthesia. Data were collected at two time points: baseline (before treatment) and seven days after treatment. All treatment plans followed routine pediatric dental protocols, and no modifications were made for study purposes. Therefore, the study posed no harm to the patients.

Inclusion criteria:

- Children aged 3–10 years
- Voluntary participation
- Clinical indication for stainless steel crowns (SSC) without need for additional crowns in the next month
- Adequate unstimulated saliva volume in the morning
- No need for space maintainers in the following month
- Candidates for treatment under general anesthesia

Exclusion criteria:

- History of previous dental treatment
- History of systemic disease or syndromes
- History of allergic reactions
- History of using imitation jewelry
- Presence of metal restorations or orthodontic appliances
- Patients not undergoing general anesthesia

This was a pilot/exploratory study, and no formal sample size calculation was performed. As no previous studies were available evaluating IL-2, IL-5, and IL-17 gene expression after SSC placement, the primary aim was to obtain preliminary data and provide a basis for future large-scale research.

Study Design

The study was performed under general anesthesia in children with material loss requiring SSC treatment. Patients were selected among those needing at least four posterior SSCs. To avoid delays and ensure no new SSC needs arose during the study period, all treatments were completed in a single session following standard pediatric protocols. Of 30 children evaluated, 12 met inclusion criteria and were enrolled.

Only patients receiving SSC or SSC combined with composite restorations were included. Those receiving other metal restorations, such as amalgam or space maintainers, were excluded.

Dental Treatments

The proximal surfaces of the teeth planned for SSCs were contoured with a #69L round bur on a high-speed handpiece. Occlusal surfaces were reduced by ~1 mm. Caries removal was performed using a round bur on a low-speed handpiece. In case of pulp exposure, an access cavity was prepared with an 008 fissure bur, and appropriate pulp therapy was conducted. SSCs (Kids Crown, Shinhung, Korea) were selected and cemented with nickel- and chromium-free glass ionomer cement (Meron, Voco, Germany).

Saliva Sampling

Saliva samples were collected at two time points: before treatment (control) and seven days after treatment. Parents were advised to follow a low-nickel diet and avoid nickel-containing materials until saliva collection was completed. The diet excluded canned foods, acidic foods cooked in stainless steel, herring, oysters, asparagus, beans, mushrooms, onions, corn, spinach, tomatoes, peas, whole grain flour, pears, rhubarb, tea, cocoa, chocolate, and baking powder. The use of fluoride-containing mouth rinses was also prohibited. Approximately 5 mL of unstimulated saliva was collected into polyethylene tubes free of nickel/chromium, washed with acetone and distilled water. Saliva was collected in the morning before surgery and at follow-up, without prior rinsing.

Clinical Examination

At follow-up, a pediatric dentist examined the oral mucosa and soft tissues for signs of allergic reactions, inflammation, or tissue changes using a dental mirror and light. Parents and children were asked about any discomfort or changes over the previous seven days.

RNA Isolation

Tissue samples were carefully collected with a sterile spatula, weighed at 25 mg, and placed in RNase-free tubes with 700 μL TRIzol reagent. Samples were homogenized, incubated at room temperature for 5 minutes, mixed with chloroform, and centrifuged at 12,000 × g, 4°C for 15 minutes. The upper phase was transferred, mixed with isopropanol, incubated, and centrifuged. The RNA pellet was washed with 75% ethanol, centrifuged, and air-dried. Pellets were dissolved in RNAse-free water or buffer.

cDNA Synthesis

cDNA synthesis was performed on a cold plate at +4°C using the All-in-One cDNA synthesis kit (Solver). 1 μL of RNA (10 ng/μL) was used in a 20 μL reaction, incubated at 42°C for 60 minutes and 80°C for 10 minutes.

Gene Expression Analysis

cDNA products were analyzed using RotorGene Q. β-actin was used as the reference gene, and primers were designed for IL-2, IL-5, and IL-17. The PCR mixture included 12 μL SYBR Green qPCR Master Mix, 2 μL forward primer, 2 μL reverse primer, 5 μL water, and 4 μL cDNA

(total volume 25 µL). PCR cycling conditions were: 95°C for 15 min, followed by 45 cycles of 95°C for 30 s and 60°C for 30 s.

Statistical Analysis Plan

CT values obtained from Real-Time PCR were normalized using the Bio-Q online bioinformatics tool. Gene expression levels were calculated using the 2−ΔΔCT method. Group differences were analyzed using one-way ANOVA, and the Tukey HSD post-hoc test was used to identify significant differences. ΔCT values were calculated by subtracting housekeeping gene CT values from target gene CT values, and expression differences between groups were visualized using bar graphs.

Key Results Summary

Patients included were aged 3–6 years. Of the 12 enrolled, two were excluded due to loss to follow-up, leaving six girls and four boys. IL-2, IL-5, and IL-17 gene expression changes were assessed. Control group expression levels were low and homogeneous; follow-up samples showed marked increases, particularly in IL-5 and IL-17. IL-2 expression increased slightly without statistical significance. IL-5 increased moderately and reached statistical significance ($p < 0.05$), while IL-17 showed a sixfold increase and the highest variability, also reaching statistical significance. Heatmap and Venn diagram analyses illustrated expression changes and potential interactions among the genes.

Informed Consent Form for Participation in a Research Study

Dear participant,

Van Yüzüncü Yıl University, Department of Pediatric Dentistry, is conducting a research study titled "Evaluation of IL-2, IL-5, and IL-17 Levels in the Saliva of Patients Treated with Stainless Steel Crowns." This study aims to evaluate the release of nickel ions from pediatric stainless steel crowns on days 1 and 7, assess pH levels, and compare cytokine levels in saliva before and after the procedure to understand the body's reaction.

We invite you to participate in this research. Please note that your participation is entirely voluntary. Before making your decision, we will inform you about the study. After reading and understanding this information, if you agree to participate, please sign this form.

The purpose of this research is to determine the nickel and chromium levels in the saliva of patients, considering the possibility that components of stainless steel crowns interact with saliva and may release heavy metals. We will also assess the body's response using cytokine levels. This study will be conducted in collaboration with the Departments of Pediatric Dentistry and Oral Diagnosis and Radiology at Van Yüzüncü Yıl University, Faculty of Dentistry. Your participation is important for the success of the research.

If you agree to participate, you or your child will be examined by the research team, and findings will be recorded. If eligible, approximately 10 mL of saliva will be collected before the procedure. Additional saliva samples may be collected during follow-up. Saliva samples will be analyzed for metal ion release and cytokine levels such as IL-2, IL-5, and IL-17.

There is no risk from saliva collection. Participation is free of charge, and no additional payment will be provided. All medical information will be kept confidential and may only be reviewed by study monitors, ethics committees, or official authorities if necessary. Participation is voluntary, and refusal will not affect medical care. You have the right to withdraw consent at any time.

I have been informed about the study and have been invited to participate. I understand that confidentiality will be maintained, and that the research results may be used for educational and scientific purposes without revealing personal identity.

I understand that I can withdraw from the study at any time without providing a reason. I understand the researcher may exclude me from the study if necessary. I will not bear any financial responsibility for expenses related to the study and will not receive payment.

I have been assured that any health issues arising from the study will be addressed without financial burden to me. I understand that I am under no obligation to participate and have not been coerced. Refusal to participate will not affect my medical care or relationship with my healthcare providers.

I have read (or heard) and understood the above information. I voluntarily consent to participate (or allow my child to participate) in this study and consent to the use, presentation, and publication of the collected information in accordance with confidentiality rules. A signed copy of this form will be provided to me.

I want to receive information about the results related to me in this study. ( )
I do not want to receive information about the results related to me in this study. ( )

Participant Name Surname Date of Birth Address/Phone Signature

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Parent/Guardian Name Surname Date of Birth Address/Phone Signature

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Witness (if any) Name Surname Date of Birth Address/Phone Signature

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Dentist:

Title, Name Surname:

Address/Phone:
Signature: