The presence of bacteria on periodontal ligament cells in different storage environments for teeth after displacement from the alveolar bone: preliminary in vitro research results

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Abstract

Introduction: Teeth displaced from their alveolar bone can be replanted if properly handled and stored in suitable environments. To help minimize the failure of reimplantation, teeth should be preserved in a storage environment that is initially free of bacteria. **Research objective:** Evaluating the morphology of bacteria on the periodontal ligament cells in different storage environments at various time points after the teeth have been removed from the alveolar bone using Gram staining. **Materials and Methods:** An in vitro study without a control group was conducted using 48 premolars and third molars after extraction, which were stored in four environments: Dulbecco's modified Eagle's medium (DMEM), fresh whole milk (unsweetened), saline solution, and electrolyte-replenishing drink. The presence of bacteria was assessed at four time points: 30 minutes, 1 hour, 2 hours, and 24 hours. **Results:** In DMEM and fresh whole milk, Gram-positive cocci, Gram-positive bacilli, and Gram-negative bacilli were presented at 30 minutes, 1 hour, and 2 hours; by 24 hours, Gram-negative cocci were also presented. In the saline solution, Gram-positive cocci and bacilli were also presented at all time points; Gram-negative bacilli appeared at 2 and 24 hours. In the electrolyte-replenishing drink, Gram-positive cocci were the only bacteria found at all time points. **Conclusion:** Bacteria were presented on the periodontal ligament cells of the root surface in all four environments and at all four-time points of storage. However, at different time points, the number of bacterial groups found in DMEM and fresh whole milk was the highest and nearly equivalent, followed by the saline solution and the lowest count in the electrolyte-replenishing drink.

Keywords: *bacteria, periodontal ligament cells, alveolar bone.*

1. INTRODUCTION

Teeth that are displaced from the alveolar bone account for approximately 0.5 - 16% of all dental trauma cases [1]. This severe dental injury causes damage to the periodontal ligament, severs the neurovascular bundle at the root apex, and may lead to pulp necrosis. Immediate reimplantation of the tooth into the alveolar bone is considered ideal and is recommended only for permanent teeth; however, it may not always be feasible [2]. Teeth can be replanted into the alveolar bone if properly handled and stored in a suitable environment. This helps prevent drying, reducing surface root resorption and increasing the chances of survival for periodontal ligament cells [3]. Two of the most critical factors affecting the prognosis of a tooth displaced from the alveolar bone after reimplantation are the duration of external drying and the storage environment of the tooth [4]. Preserving the tooth in a suitable storage environment that can maintain the viability of the remaining periodontal ligament cells on the root surface for as long as possible is key to the successful reimplantation of the tooth into the alveolar bone [5].

Recent studies have diversified the options for the best storage environment; however, no single storage environment has all the necessary characteristics for tooth preservation. The ideal medium must be capable of maintaining the viability of periodontal ligament and pulp cells, have physiological osmolarity and pH, possess antioxidant properties, contain minimal or no bacterial contamination, and be readily available in locations where accidents occur, such as playgrounds, sports fields, homes, schools, and hospitals, all while being cost-effective [6].

The storage environments mentioned include Hank's Balanced Salt Solution (HBSS), Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), milk, coconut water, saline solution, Save-A-Tooth, propolis, egg white, green tea, aloe vera, saliva, soy milk, probiotics, royal jelly, rice water, electrolyte-replenishing drinks, oresol solution, and Emdogain [7-11]. Several national

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and international studies have been conducted on various tooth preservation media; however, there are currently limited studies evaluating the presence of bacteria on periodontal ligament cells of teeth displaced from the alveolar bone in these storage environments. Therefore, we conducted this preliminary study to evaluate the morphology of present bacteria on the periodontal ligament cells in different storage environments at various time points after the teeth have been removed from the alveolar bone using Gram staining.

2. MATERIALS AND METHODS

2.1. Research subjects: The study was conducted on 48 premolars and third molars scheduled for extraction due to orthodontic treatment at different dental clinics in Hue City.

2.1.1. Selection criteria

- Permanent teeth with fully formed apices and intact roots.

2.1.2. Exclusion criteria

- Teeth with a history of trauma or fractures.
- Teeth with severe caries, periodontal disease, pulp pathology, or periapical pathology.
- Patients with a history of systemic diseases or those currently on medication.
- Patients who are uncooperative or do not consent to participate in the study.

2.1.3. Duration and location of the research

- Duration: From 01/2023 to 12/2023
- Location: Skill lab of the Faculty of Odonto-Stomatology and the Department of Microbiology, Hue University of Medicine and Pharmacy.

2.2. Research methods

2.2.1. Study design: In vitro experimental study without a control group.

2.2.2. Sample selection method:

48 premolars and third molars that met the study criteria were selected and randomly assigned to four storage environment groups:

- Group 1 (n = 12): DMEM
- Group 2 (n = 12): Fresh whole milk (unsweetened)
- Group 3 (n = 12): Saline solution
- Group 4 (n = 12): Electrolyte-replenishing drink

Within each group, the 12 teeth were further divided into four subgroups, each containing three teeth, to evaluate the presence of bacteria at different storage time points (30 minutes, 1 hour, 2 hours, and 24 hours).

2.2.3. Research methods

2.2.3.1. Preparation of teeth and placement in storage environment

- The tooth, freshly extracted from the patient's mouth, was grasped using a forceps at the crown area. A #12 scalpel blade was used to carefully remove 3 mm of the periodontal ligament from the root's surface at the cervical region to eliminate damaged cells caused by the extraction process. Subsequently, the tooth was placed immediately into a Falcon tube (15 ml capacity) containing 10 ml of storage environment. The time from when the tooth was extracted from the alveolar bone through the scraping process and placement in the storage environment must not exceed 45 seconds to ensure consistency among samples and minimize errors.

- The storage environment had to submerge the tooth throughout the study entirely. All Falcon tubes containing the tooth samples in the storage environment were kept at room temperature (approximately 25°C) for the entire testing period. After the designated time had elapsed, the tooth was carefully retrieved using forceps at the crown area. The root and crown's surface were cleaned using a plastic pipette to irrigate the tooth with saline solution twice, ensuring the storage environment was thoroughly removed from the tooth's surface.

2.2.3.2. Assessment of bacterial presence on periodontal ligament cells of teeth after displacement from the alveolar bone

After cleaning, the tooth was grasped with forceps at the crown area. A #12 scalpel blade was used to scrape the apical two-thirds of the root surface to collect periodontal ligament cells. This process ensured that a sufficient quantity of cells was obtained for subsequent analysis. Then, the cell debris collected solution was added to a centrifuge tube (1 ml capacity) with 500 µl of PBS solution and mixed thoroughly. After that, this mixture was used for Gram staining.

The Gram staining procedure followed the standard protocol of the Department of Microbiology at Hue University of Medicine and Pharmacy. Firstly, a slide was made from the debris solution collected from the Eppendorf tube. Then, it was fixed by passing over the flame 2 or 3 times. The slide needed to be cooled down completely before processing the staining steps. A few drops of gentian violet solution were added to the surface of the slide and allowed to sit for 01 minute. After removing the gentian violet, the slide was fixed with Lugol's solution (iodine) for 1 minute. The slide was continued gently, rinsing under tap water to wash out the previous reagents thoroughly. The slide was decolonized using 96% ethanol within 30 seconds before being cleaned with tap water. The final step was to apply safranin counterstain for 01 minutes, washing and drying the

slide, which could be ready for observation under the microscope. Different Gram-staining bacteria were reported at 1000X magnification under a light microscope. The characteristic properties of those bacteria were also examined and recorded.

2.3. Research variables and evaluation methods

The results from the debris solution collected from storage media at different time points were checked and reported for:

- Determine the presence of different Gram bacteria throughout the entire field of view **(**Gram-negative bacteria appeared in pink color, Gram-positive bacteria in purple color) [12].

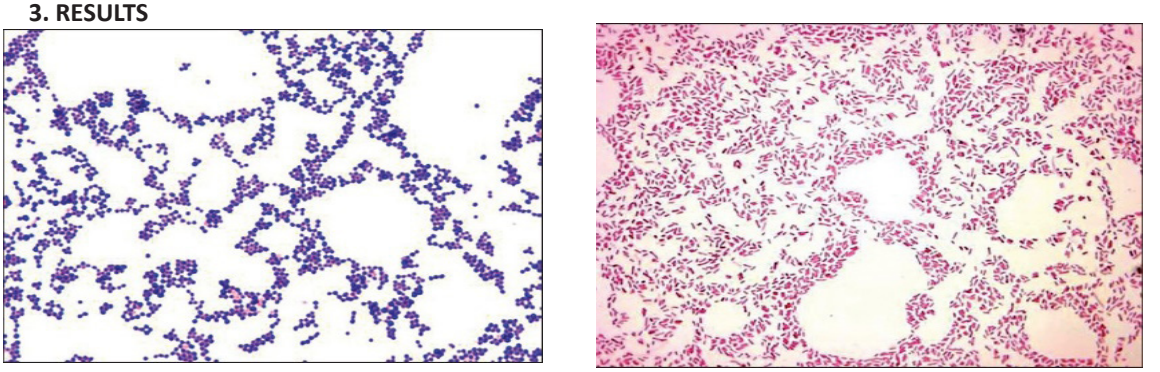
- Describe the bacterial morphology, which is cocci or bacilli bacteria.

statistical analysis

2.4. Statistical analysis

Use Excel 2021 software to enter collected data. Use SPSS 20 software to manage and process data

3. RESULTS



A **B**

Figure 1. The image of Gram staining from the Gram-Stained Bacterial Images [13]
A. Image of Gram-positive cocci. B. Image of Gram-negative bacilli.

Table 1. The presence and morphology of bacteria on periodontal ligament cells on the root surface stored in DMEM at different time points.

Storage media	Time points	Presence of bacteria	Bacterial morphology
DMEM	30 minutes	Yes	(1) Gram-positive cocci (2) Gram-positive bacilli (3) Gram-negative bacilli
	1 hour	Yes	(1) Gram-positive cocci (2) Gram-positive bacilli (3) Gram-negative bacilli
	2 hours	Yes	(1) Gram-positive cocci (2) Gram-positive bacilli (3) Gram-negative bacilli
	24 hours	Yes	(1) Gram-positive cocci (2) Gram- negative cocci (3) Gram-positive bacilli (4) Gram-negative bacilli

When stored in DMEM, bacteria were presented on the periodontal ligament cells on the root surface at all 4 time points.

- At 30 minutes, 1 hour, and 2 hours, there was the presence of Gram-positive cocci, Gram-positive bacilli, and Gram-negative bacilli.

- At 24 hours, there was an additional presence of Gram-negative cocci.

Table 2. The presence and morphology of bacteria on periodontal ligament cells on the root surface stored in fresh whole milk (unsweetened) at different time points.

Storage media	Time points	Presence of bacteria	Bacterial morphology
Fresh whole milk (unsweetened)	30 minutes	Yes	(1) Gram-positive cocci (2) Gram- negative cocci
	1 hour	Yes	(1) Gram-positive cocci (2) Gram-positive bacilli (3) Gram-negative bacilli
	2 hours	Yes	(1) Gram-positive cocci (2) Gram-positive bacilli (3) Gram-negative bacilli
	24 hours	Yes	(1) Gram-positive cocci (2) Gram- negative cocci (3) Gram-positive bacilli (4) Gram-negative bacilli

When stored in fresh whole milk (unsweetened), bacteria were also present on the periodontal ligament cells on the root surface at all 4 time points.

- At 30 minutes, Gram-positive cocci and Gram-negative bacilli were present.
- At 1 hour and 2 hours, both Gram-positive cocci and Gram-positive bacilli were observed, along with Gram-negative bacilli.
- At 24 hours, there was an additional presence of Gram-negative cocci.

Table 3. The presence and morphology of bacteria on periodontal ligament cells on the root surface are stored in a Saline solution at different time points

Storage media	Time points	Presence of bacteria	Bacterial morphology
Saline solution	30 minutes	Yes	(1) Gram-positive cocci (2) Gram-positive bacilli
	1 hour	Yes	(1) Gram-positive cocci (2) Gram-positive bacilli
	2 hours	Yes	(1) Gram-positive cocci (2) Gram-positive bacilli (3) Gram-negative bacilli
	24 hours	Yes	(1) Gram-positive cocci (2) Gram-positive bacilli (3) Gram-negative bacilli

When stored in a saline solution, bacteria were present on the periodontal ligament cells on the root surface at all four time points.

- Gram-positive cocci and Gram-positive bacilli were presented at all time points in saline solution storage. In addition, at 2 hours and 24 hours, Gram-negative bacilli bacteria were present.

Table 4. The presence and morphology of bacteria on periodontal ligament cells on the root surface stored in an electrolyte-replenishing drink at different time points.

Storage media	Time points	Presence of bacteria	Bacterial morphology
Electrolyte-replenishing drink	30 minutes	Yes	(1) Gram-positive cocci
	1 hour	Yes	(1) Gram-positive cocci
	2 hours	Yes	(1) Gram-positive cocci
	24 hours	Yes	(1) Gram-positive cocci

When stored in an electrolyte-replenishing drink, bacteria were present on the periodontal ligament cells on the root surface at all 4-time points. However, only Gram-positive cocci were observed.

4. DISCUSSION

DMEM is an improved version of Eagle's Minimum Essential Medium (EMEM), supplemented with additional nutrients. It is used as a cell culture medium, helping to maintain cell survival and promote superior cell proliferation.

Sterilized milk**:** Milk is significantly better than other solutions in terms of its physiological properties, including pH (6.5 - 7.2) and osmolarity (270 mOsm/kg), which are compatible with cells. It also contains essential amino acids, carbohydrates, and vitamins. Milk is readily available, bacteria-free, and contains epidermal growth factors that stimulate cell proliferation and regeneration.

Saline solution: This solution contains 0.9% NaCl with an osmolarity of 280 mOsm/kg. Although it is compatible with cells (since cell survival and growth occur at an osmolarity range of 230 - 400 mOsm/kg and a pH of 6.6 - 7.8), it lacks essential nutrients necessary for cellular metabolism, such as magnesium, calcium, and glucose. The saline solution appears suitable for the short-term preservation of avulsed teeth for under 2 hours.

Electrolyte-replenishing drinks are a potential storage medium commonly found at sporting events. They should only be used as a storage medium if no better option is available.

From the results of Tables 1 to 4, it is evident that all four storage environments exhibited the presence of bacteria on the periodontal ligament cells on the root surface at four different time points. This can be explained by the growth and dispersion of bacteria on the tooth's surface into the storage medium, which subsequently adheres to the periodontal ligament cells on the root surface. Our study collected periodontal cell samples from only the apical two-thirds of the root surface; thus, the bacteria found in our research are directly related to those suspended in the storage medium.

Tables 1 and 2 indicate that both DMEM and fresh whole milk (unsweetened) exhibited the presence of multiple bacterial groups on the periodontal ligament cells on the root surface. Even at 30 minutes**,** 1 hour, and 2 hours, three bacterial groups were presented, and later on, by 24 hours**,** four groups of bacteria were identified. This can be explained by the fact that DMEM and fresh whole milk are nutrient-rich environments, providing ideal conditions that support the survival of human cells as well as promoting bacterial growth, particularly oral bacteria. These bacteria proliferate rapidly in these media, leading to a correspondingly high number of bacteria adhering to the root surface.

Table 3 shows that the saline solution maintained a consistent presence of two bacterial groups at all four storage time points. Gram-negative bacilli could also be present at 2 hours and 24 hours. While there is currently a lack of research on the virulence of these Gram-negative bacilli and other bacterial groups contributing to increased complications such as root resorption following tooth re-implantation, some studies indicate a correlation between certain bacterial groups and chronic periodontal inflammation, similar to those found in periodontal diseases. This highlights the potential risks associated with bacterial presence in the context of dental re-implantation [14].

Table 4 indicates that only one bacterial group - Gram-positive cocci - was detected in the electrolyte solution at all four storage time points. The pH plays a crucial role in the homeostasis of microorganisms, as many bacteria require a specific pH for growth. Additionally, the redox potential is a key physicochemical parameter characterizing the growth state of microorganisms, and continuous fluctuations can favor the development of various bacterial groups. Changes in pH and concentrations of reactive gases (O_2, H_2, and H_2S) are considered primary factors influencing microbial development [15]. Thus, aside from the nutrient richness of DMEM and milk that allows for rapid bacterial proliferation and the more limited growth observed in saline, the low pH, redox potential, and CO_2 concentration in the electrolyte solution effectively inhibit bacterial growth compared to the other three environments.

Different environmental factors, such as temperature, pH, redox potential, ion strength, and osmotic pressure, significantly influence the growth and metabolism of microorganisms [15]. Thus, when the storage environment has optimal factors for maintaining the viability of periodontal ligament cells, it also creates opportunities for the growth of various bacterial species and the rapid multiplication of their numbers. It is important to note that the primary purpose of the tooth storage medium is to preserve the viability of periodontal ligament cells, and bacterial contamination is a secondary concern that needs to be considered [16]. However, when re-implanting a tooth into the socket, if it is not adequately cleaned and disinfected, it may carry many pathogens due to various microorganisms adhering to it, leading to root resorption and other infectious diseases, particularly tetanus. During the re-implantation process, systemic or local antibiotics can effectively prevent and eliminate bacteria. According to the International Association of Dental

Traumatology, broad-spectrum antibiotics (e.g., tetracycline) to soak and clean the tooth before re-implantation can eliminate most bacterial species, minimizing clinical complications related to root resorption and inflammation and replacement resorption [17].

This is only a preliminary study on the morphology and presence of bacteria assessed by Gram staining. Bacterial identification will provide a more detailed view of the type of bacteria in the storage environment, thereby providing a more accurate assessment of actual pathogenic bacteria or common bacteria in the oral cavity.

5. CONCLUSION

- Different bacteria were reported on the periodontal ligament cells of the root surface in all four environments and at all four-time points of tooth storage.

- Within four-time points, the number of bacterial groups found in the DMEM and fresh whole milk was the highest and nearly equivalent, followed by a lower count in the saline solution. However, the electrolyte-replenishing drink was only presented with one group of positive cocci.

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