ORIGINAL ARTICLE

# The Effect of Hank's Balanced Salt Solution Supplemented With L-Ascorbic Acid on the Gene Expression of Extracellular Matrix Markers (Type 1 Collagen and Vimentin) and Viability of Native Dental Pulp

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## ABSTRACT

**Introduction:** Tooth avulsion is the complete displacement of a tooth from its alveolar socket, presenting significant treatment challenges, particularly when immediate replantation is not feasible. Hence, a storage medium, Hank's Balanced Salt Solution (HBSS), is required to transport the avulsed tooth to the dental chairside for replantation. The supplementation of L-ascorbic acid (Vit. C) into HBSS may improve storage medium quality as it promotes fibroblasts' proliferation and maintains extracellular matrix (ECM) integrity. We aimed to determine the effect of HBSS supplemented with Vit. C on the gene expression of ECM markers (Type I collagen and Vimentin) and the viability of native dental pulp. **Materials and Methods:** The pulp tissues were extirpated from sound teeth collected at Klinik Bedah Mulut, Universiti Kebangsaan Malaysia. These pulps were exposed 24 hours to three types of media: HBSS (negative control/CTRL), HBSS with 350μg/ml Vit. C (experimental group), and KnockOut DMEM/F-12 (KO) with 10% fetal bovine serum (FBS) (positive control) respectively. Quantitative Reverse Transcriptase Polymerase Chain Reaction and Alamar Blue Proliferation Assay were used to determine expression of ECM markers and viability of native pulp respectively. **Results:** The expression of ECM markers and viability of pulp tissue after storage with HBSS supplemented with Vit. C were comparable to the positive control group (KO + 10% FBS). **Conclusion:** Hank's Balanced Salt Solution supplemented with L-ascorbic acid can potentially be served as a medium to store and transport the avulsed tooth before replantation.

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**Keywords:** Hank's balanced salt solution, L-ascorbic acid, Type I Collagen, Dental pulp, Vimentin

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## INTRODUCTION

Traumatic dental injuries (TDIs) are one of the most significant oral health challenges worldwide as they occur most frequently among the young population especially during childhood and adolescence, comprising 5% of all injuries. Based on International Association of Dental Traumatology (IADT) guidelines, one in four school-aged children experience dental trauma, while one in three adults have sustained injuries to their permanent teeth—most of which occur before the age of 19 (1). A global systematic review revealed that millions of people, aged 7 to 65 have experienced traumatic injuries to their secondary dentition and millions of children worldwide were expected to suffer primary tooth-related TDIs (2). Various studies have shown that children and young adults are more vulnerable to TDIs. The most common causes of TDIs include falls, sports, motor-vehicle-accidents (MVA), assaults and abuse (3). It can lead to consequences which can be seen immediately or later. Dental trauma can result in immediate effects such as pain, bleeding, swelling, mobility, fracture of bone and tooth, soft tissue injuries, and tooth avulsion. The consequences are tooth discolouration, dental/periodontal abscess, loss of periodontal attachment, periapical pathology, ankylosed tooth, delayed permanent tooth eruption and impaction (3). However, TDIs do not only lead to excruciating and upsetting experiences. Patients attending the clinic with TDIs may have shown the clinical presentation of fractured, displaced or lost teeth; without immediate and proper management, it could have caused significant impacts on mastication, aesthetic, phonetic and psychosocial aspects which further threaten the quality of life of an individual (4-5). Misdiagnosis and inappropriate treatment of dental injuries can further damage the traumatized teeth. Hence, accurate examination, diagnosis, clinical case management, and review are essential to achieve a positive outcome.

Permanent tooth avulsion is among the most severe dental injuries (6), occurring in 0.5% to 16% (7-8). Avulsion refers to total removal or loss of a tooth from its socket in alveolar bone with damage to the periodontium such as cementum, periodontal ligament (PDL), alveolar bone and gingival tissues (9). Its prevalence in primary dentition is about 7% - 13% while permanent dentition is 1% - 16% (10-11). An avulsed tooth's survival rate heavily depends on the immediate management at the injury site (12). Replantation is one of the treatment choices to manually reinsert the displaced tooth back into its socket. It is believed to offer a higher likelihood of tooth survival by immediate replantation at the injury site. However, managing tooth avulsion is often challenging due to several factors causing replantation not to be done immediately. Hence, it is suggested that the tooth be temporarily kept in a storage medium to maintain and preserve the viability of PDL cells (13). There are also some circumstances in which replantation of avulsed tooth is contraindicated which are excessive loss of tooth structures or extensive caries, tooth with periodontal disease, patient loss of consciousness, non-compliant patient or other complicated medical issues such as suppressed immune system and severe heart conditions or cardiovascular disease. Although replantation is aimed at saving the avulsed tooth, it is also crucial to identify teeth with poor long-term prognosis, which may require extraction in a later stage (12).

Tooth avulsion may involve severe injuries in pulp and periodontal tissues (14). Pulp necrosis and root resorption are common complications after avulsions. Necrosis occurs when there is presence of infection caused by bacteria invasion through the apex or cavity on the crown into pulp during avulsion, and consecutive infection can cause root resorption and ultimately lead to tooth loss (15-16). The pulpal vitality status is a key factor in determining the periodontal healing (17). Pulp necrosis in mature teeth is indicated for endodontic treatment; however, in immature teeth, there is a possibility of revitalizing the tooth by revascularization. Revascularization depends on factors such as the presence of infection (18–21), the diameter of the apical foramen, the length of roots and conditions under which the tooth was stored outside of the mouth. The success rate is approximately 60% if the tooth is replanted within 1 minute after avulsion but it drops to 10-30% if the teeth are stored in saliva, saline, or dry conditions for 5 minutes before replantation (22).

The golden hour of replantation is about 20-30 minutes. The tooth should be stored in a medium to maintain the viability of PDL cells during the transportation to the chairside for reimplantation (9). The American Association of Endodontics strongly recommends Hank's Balanced Salt Solution (HBSS) as a storage medium (23). Recent studies show that with its optimum pH and ideal osmolality, HBSS is biocompatible to maintain the viability of PDL fibroblasts and renew degenerated periodontal cells for at least 24 hours, which are comprised primarily of type I, III and V (24-25) when compared to milk, ViaSpan and Eagle's medium (26). Hence, it is regarded as a better transport and storage medium than other mediums (25). In relation to HBSS to pulpal tissue, HBSS consists of sufficient nutrients, an important factor influencing the viability of human dental pulp cells (27). Teeth with open apical foramen with short extraoral dry time have a higher chance of pulp revascularization (28). A case study has shown that the pulp revascularization rate reduced rapidly when the avulsed teeth were kept in an inadequate medium, even though the storage time is very short as it is detrimental to cell viability (29). Hence, maintaining cell viability in the storage medium should be helpful for revascularization especially in immature teeth (17).

Ascorbic acid (Vit. C) is water soluble and has antioxidant properties involved in the biosynthesis of collagen in fibrous tissue, connective tissues, teeth and bones. It can promote the growth of fibroblasts and enhances the survival and differentiation of osteoblasts (30). Other than that, it can also promote the proliferation of type I collagen production and increase the expression of specific markers (13). It has been proven that ascorbic acid aids in wound healing by facilitating matrix deposition, promoting neo-vascularization, and stimulating the release of inflammatory mediators (31). In case of pulp tissue exposure, ascorbic acid supports wound healing by inducing reparative dentin formation. This process is facilitated by sodium-dependent vitamin C transporter 2 and glucose transporter 1 in the pulp tissue, which helps transport ascorbic acid. Additionally, ascorbic acid plays an important role in collagen synthesis and differentiation of odontoblast-like cells during the healing process (32). Most studies have shown that HBSS is the recommended storage medium as it helps to maintain the cell viability during transportation (23-26). With the supplementation of Vit. C, the quality of storage medium can be further enhanced as Vit. C promotes type I collagen and vimentin expression, enhancing cell growth which is crucial for maintaining extracellular matrix integrity in the dental pulp tissues of an avulsed tooth.

Dental pulp tissue is a loose connective tissue, primarily consists of fibres and ground substances that form extracellular matrix and cellular components, mainly fibroblast and undifferentiated mesenchymal cells. Type I collagen is produced by pulp fibroblasts and odontoblasts, while type III collagen can also be produced by pulp fibroblasts (33). Traumatic injury induces inflammation in the pulp, thus promoting dentin synthesis through biomineralization. Type I collagen is essential in the biomineralization process and makes up 90% of the total protein in the organic matrix of bone and dentin (34). Collagen imparts viscoelastic properties of the skeletal and dentinal structures and facilitates the process of mineral deposition. Type I collagen promotes hydroxyapatite crystals' formation and initiates reparative dentine deposition, playing a key role in the regeneration of pulp and dentine (35).

Besides type I collagen, another protein known as Vimentin also plays a role in pulp regeneration and cell function. Vimentin was identified as a type III intermediate filament protein in pulp stem cells through the qualitative and quantitative protein expression patterns (36). Vimentin messenger RNA expression in pulp tissue was the highest among various human tissues. Thus, this study has suggested that high Vimentin expression and its migration effect in pulp stem cells make it a key marker for pulp regeneration and cell function (36).

In this present study, the objectives were to determine the effect of HBSS supplemented with L-ascorbic acid on 1. the gene expression of extracellular matrix (ECM) markers, which were type I collagen and Vimentin; and 2. cell viability of native dental pulp.

## MATERIALS AND METHODS

### Sample collection

All the healthy, sound and vital teeth (N=9) were extracted from healthy individuals and collected from Klinik Bedah Mulut, Faculty of Dentistry, Universiti Kebangsaan Malaysia (UKM). These sound and vital teeth were collected from patients who came for orthodontic purposes. The teeth were extracted by dental students under the supervision of oral surgeons from fit and healthy patients. Patients were given verbal and written informed consent with information sheets before tooth collection. After rinsing with 0.2% chlorhexidine for 60s, topical anaesthetic gel was applied and 2% Scandonest (Mepivacaine) with epinephrine 1/80000 was used as local anaesthesia.

### Pulp tissue extirpation

After extracting the teeth, they were immersed into sterile Phosphate Buffer Saline (PBS) 1x (Solarbio, China) and immediately sent for pulp extirpation. After the tooth surface was cleaned and disinfected with 0.2% chlorhexidine, a straight-line access cavity was done using a high-speed pear-shaped bur and Endo-Z bur with copious amount of water supply mounted on a high-speed handpiece to obtain the pulp tissues under a sterile condition. The intact pulp tissues were removed from the root canals using barbed broach instruments, inserted into petri dishes with sterile PBS, and then sent to the tissue culture lab for sample processing.

### Sample processing

After washing with sterile PBS 1x for three times, these tissues were exposed to three types of media: HBSS (Sigma, USA), HBSS with 350µg/ml Vit. C (Sigma, USA) and KO medium with 10% FBS (Invitrogen, USA). The negative control was HBSS while the positive control was KO + 10% FBS. HBSS + Vit. C was used as an experimental group in this study. The pulp tissues were inserted into 96 well plates with 220µL of media in each of the well. After that, incubation was done in a 37◦C incubator with an atmosphere of 5% $CO_2$ for 24 hours, respectively.

### Alamar Blue Proliferation Assay

In order to determine the cell viability of native dental pulp, Alamar blue assay was used. After incubation for 24 hours, 20 µL of the medium was gently withdrawn and replaced with 20 µL of Alamar Blue reagent. (Invitrogen, USA), respectively. The tissues were harvested after being incubated in an atmosphere of 37 ◦C and 5% CO2 for 3 hours. The remaining medium was separated into two wells with 100µL, respectively. Then, the optical density was measured at 570nm and 600nm wavelengths using microplate reader. The calculation of the percentage of reduction value of Alamar blue was based on the manufacturer's protocol while the percentage of cell proliferation was obtained using the following equation:

% Cell Viability=((% Reduction Value test )/(% Reduction Value control))x100%

### Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

The harvested tissue was washed with sterile PBS at the end of incubation time. It was minced into smaller pieces and lysed with 1mL TRIzol reagent (Invitrogen, USA). The ribonucleic acid (RNA) from native dental pulp was preserved using TRIzol reagent. To extract total RNA, 1 mL of the cell lysate was mixed with 200 µL of chloroform (Sigma, USA). The mixture was then centrifuged at 12,000 rpm for 15 minutes to separate the aqueous and organic phases. RNA remained in the aqueous phase and was precipitated by adding 500 µL of absolute isopropanol (Sigma, USA) and 5 µL of polyacrylic carrier. The RNA was then pelleted by centrifuging at 12,000 rpm for 8 minutes. The precipitate was washed with 1 mL of 75% ethanol (Sigma, USA) and centrifuged for 5 minutes. After removing the ethanol, the RNA pellet was air-dried and resuspended in 10 µL of RNase and DNase-free distilled water (Invitrogen, USA).

RNA concentration and purity were assessed using a Nanodrop ND-100 spectrophotometer. Complementary DNA (cDNA) was synthesized from 100 ng of total RNA using the SensiFAST cDNA Synthesis Kit (Bioline, UK), following the manufacturer's protocol. The reverse transcription process involved primer annealing at 25°C for 10 minutes, reverse transcription at 42°C for 15 minutes, and reaction termination at 85°C for 5 minutes. For data normalization, the housekeeping gene was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) while the two target genes were type I collagen (Col. I) and Vimentin. Table I shows 2 target genes (Col. I and Vimentin) and houkeeping gene (GAPDH) with their primer sequences (37-38). The Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) was performed using Luna® Universal qPCR master mix (New England Biolabs, USA) in Bio-Rad iCycler. The protocol condition was initiated with the activation of Taq DNA polymerase at 95◦C for 3 minutes, followed by 40 cycles of PCR amplification at 95◦C for 10 seconds and 60◦C for 30 seconds and then melt curve analysis. To analyze the date, the mRNA levels of the target gene were relatively quantifies against the hosekeeping gene using the following equation:

Relative Expression Ratio= $2^{-(\Delta CT\ \text{target gene} - \Delta CTGAPDH)}$

ΔCT target gene=threshold cycle value of target gene
ΔCTGAPDH=threshold cycle value of housekeeping gene

**Table I: Forward and reverse primer sequences of ECM markers and housekeeping genes.**

| Gene | GenBank Accession Number | Primer Sequence |
|---|---|---|
| GAPDH | NM_002046.5 | F: caatgacccccttcattgacc |
| | | R: ttgattttggagggatctcg |
| Col 1 | NM_000088.3 | F: gtgctaaaggtgccaatggt |
| | | R: accaggttcaccgctgttac |
| VIMENTIN | NM_003380.5 | F: TACGAGGAGGAGATGCGGGA |
| | | R: CCTCCTGCAATTTCTCCCGG |

## Statistical analysis

Statistical analysis was conducted using descriptive statistics with the Statistical Package for the Social Sciences (SPSS) version 29.0. Parametric tests, including one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) tests, were employed. One-way ANOVA was used to assess differences between group means, while post hoc Tukey HSD tests were applied to evaluate the statistical significance of differences among sample means. A p-value of $< 0.05$ was considered statistically significant, and $p > 0.05$ was deemed not significant.

# RESULTS

## Alamar blue cell proliferation assay

The cell viability of native dental pulp treated with media with HBSS, HBSS + 350µg/ml Vit. C and KO + 10% FBS were evaluated using the Alamar Blue Cell Proliferation Assay. Figure 1 showed HBSS supplemented with 350µg/ml Vit. C had the highest cell viability of native dental pulp. Medium with HBSS (CTRL) was only used as a negative control to compare other media's effectiveness on the dental pulp's cell viability. The cell viability of native dental pulp of HBSS + Vit. C (175±17%) when compared to KO + FBS (116±7%) show significant differences where p = 0.01 and when compared to CTRL (100%) where p = 0.002. One-way ANOVA revealed significant differences in the cell viability means of dental pulp across the three groups, $F(2,9) = 13.260$, $p = 0.002$.

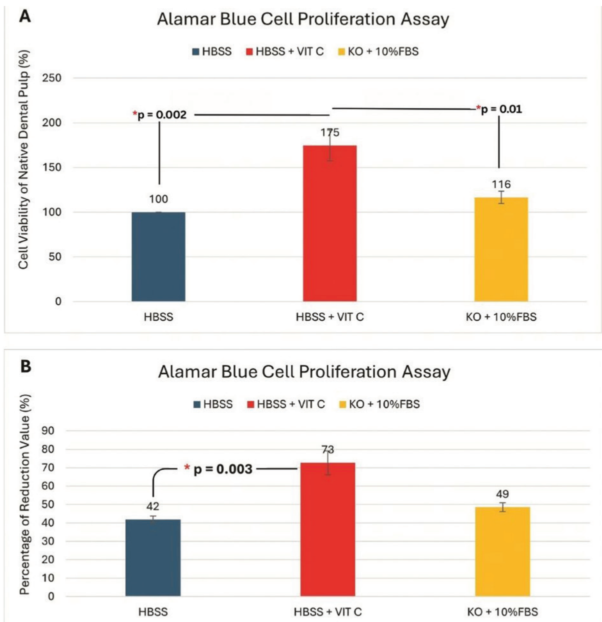


**Figure 1: The percentage of cell viability and reduction value of native dental pulp in 3 types of media. (A) The percentage of cell viability of native dental pulp in HBSS + 350µg/ml Vit. C and KO + 10% FBS were calculated versus the medium with HBSS only/ CTRL which was normalized as 100%. (B) The percentage of reduction value for each media was calculated. All the data were representative of three independent tests with n=4 by groups and means ± SEM. *p < 0.05 denoted the statistical significance of HBSS + Vit. C when compared to CTRL.**

**Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)**

One-way ANOVA was performed to compare the relative gene expression of Col. I and Vimentin in three different media HBSS only/ CTRL, HBSS supplemented with 350µg/ml Vit. C and KO + FBS (Figure 2). There are statistically significant differences between these three groups. The relative gene expression of HBSS supplemented with Vit. C (3.49030E-04±1.13597E-04) showed upregulating trends and highest Col. I gene expression when compared to CTRL (6.3207E-05±4.51323E-06) and KO + 10% FBS (1.40E-05±5.92189E-06). HBSS supplemented Vit. C (1.05E-01±9.20E-03) showed the highest upregulated expression in Vimentin, and there are statistically significant difference when compared to CTRL (2.64E-02±1.00E-02) and KO + 10% FBS (1.72E-02±2.89E-03). One-way ANOVA showed that all three groups resulted in different means of Col. I ($F(2,6) = 7.575$, $p = 0.023$) and Vimentin ($F(2,6) = 36.123$, $p = <0.001$) relative mRNA expression.

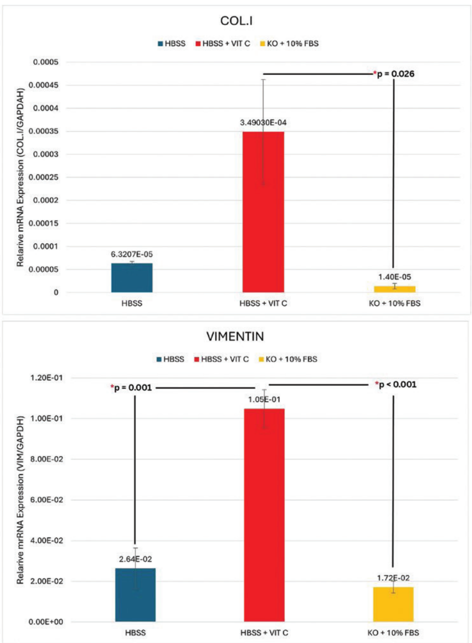


**Figure 2: Relative mRNA expression of ECM markers (a) Col. I; (b) Vimentin among medium with HBSS only/ CTRL, HBSS + 350µg/ml Vit. C and KO + 10% FBS. All the data were representative of three independent tests with n=3 by groups and means ± SEM. p<0.05 denoted the statistical significance of HBSS + Vit. C when compared to CTRL and KO + 10% FBS.**

## DISCUSSION

Numerous studies have reviewed and compared different media to store and transfer teeth in case of tooth avulsion. Maintenance of periodontal and pulp cell viability are the most crucial factors determining the success rate of avulsed tooth replantation. Previous studies were carried out to compare different storage media to assess effectiveness in preserving periodontal ligament fibroblast cells' viability and promoting cell growth. According to Adnan et al., 2018 it has been shown that HBSS can maintain the viability of periodontal cells and demonstrated clonogenic and mitogenic capacity of periodontal ligament fibroblast cells (39). To enhance the long-term prognosis and survival of avulsed teeth, management protocols should incorporate the care of both the pulp and periodontal ligament cells (40). This study focuses on maintaining dental pulp viability to determine the success rate of avulsed tooth replantation. Different storage media were used to determine the optimal storage media that supports native dental pulp cells' viability in transporting the tooth before tooth replantation can be done.

According to the Brazilian Dental Journal, the pursuit of an optimal storage medium for avulsed tooth includes the capability to preserve periodontal ligament and maintain the viability of pulp cells, promote clonogenic potential, possess antioxidant properties, minimize contamination by microorganisms, maintain compatible osmolality and physiological pH and cost-effective. Moreover, it is also stated that HBSS ensures cell maintenance, allows better conservation of tissues for an extended time, and has ideal osmolality and pH to preserve cell viability (41). The International Association of Dental Traumatology recommends using HBSS to store avulsed teeth (42). Hwang et al. and Pillegi et al. observed 90% to 94% cell viability after cultured human periodontal cells were kept in the medium of Hank's Balanced Salt Solution (41).

HBSS was compared to the other two storage media in this study, such as HBSS supplemented with Vit. C and KO medium with FBS to determine the most effective storage media to maintain cell viability of native dental pulp. Only sound teeth with healthy pulp were included in this study to ensure no unfavourable and irrelevant outcomes due to other factors, such as pulpal infection due to caries and periodontitis. In this study, Alamar Blue Proliferation Assay and Reverse Transcriptase Polymerase Chain Reaction were carried out to assess the effectiveness of various storage media on the native dental pulp cells viability and analyze the expression of gene of type I collagen and Vimentin, respectively.

The viability of native dental pulp cells after being stored in the test group of HBSS supplemented with Vit. C shows the highest trend, followed by KO medium with FBS and the lowest trend of native dental pulp cell viability is seen in HBSS alone. There are significant differences between HBSS supplemented with Vit. C and both KO medium with FBS and HBSS alone. In comparison of KO medium with FBS and HBSS alone, although there is an insignificant difference between these storage media, KO medium with FBS shows an upregulated trend in promoting cell viability compared to HBSS alone. However, according to Casaroto et al., a culture medium supplemented with 10% FBS is not suitable for clinical use as a storage medium for avulsed teeth, as it requires fresh preparation. Moreover, the pH also tends to increase when there is more release of carbon dioxide in this medium. Hence, it is crucial to monitor the pH of this medium continuously. Thus, storing an avulsed tooth in KO + 10% FBS is not feasible in a clinical scenario, which is transporting the avulsed tooth to the chairside (42). Moreover, there is a significant difference between HBSS supplemented with Vit. C and HBSS alone, which indicates that HBSS supplemented with Vit. C was more effective than HBSS alone. This is because of the presence of Vit. C, which promotes the fibroblasts' growth and enhances osteoblasts' survival and differentiation (30). Hence, HBSS supplemented with Vit. C is the most effective storage medium, which preserves cell viability before replanting avulsed teeth into the tooth socket.

Several studies indicated that type I collagen is an ideal scaffold material for dental pulp regeneration as it enhances human dental pulp stem cells (hDPSCs) proliferation and significantly increases gene expression associated with odontogenesis (43). In this study, HBSS supplemented with Vit. C shows the highest type I collagen gene expression and there is a statistically significant difference between HBSS supplemented with Vit. C and KO medium with FBS. Moreover, both KO mediums with FBS and HBSS alone show a low type I collagen gene expression trend compared to the test group of HBSS supplemented with Vit. C and HBSS alone, although there are distinct differences in the trend of type I collagen gene expression, there was an insignificant difference statistically between these two groups, due to the small sample size. However, according to Ohkura et al., the supplementation of Vit. C into HBSS is important in the biosynthesis and secretion of collagen (32).

Vimentin is an intermediate filament protein found in normal mesenchymal cells, where it helps maintain cellular integrity and provides resistance to mechanical stress. Murakami et al., 2012 (36) concluded that vimentin expression is the highest in pulpal tissue. The migratory role of Vimentin in pulp stem cells indicates that it serves as an essential marker for pulp regeneration and cellular function (36). Vimentin gene expression was the highest in HBSS supplemented with Vit. C. There was a significant difference between HBSS supplemented with Vit. C and KO medium with FBS, in which KO medium with FBS shows the lowest trend in vimentin gene expression. HBSS alone showed a lower trend than HBSS supplemented with Vit. C in vimentin expression, but these two groups show insignificant differences. In a study by Lan et al., 2020 adding vitamin C to stem cells increases gene expression of Vimentin (44).

In this study, some limitations are encountered, such as infection control during pulp extirpation to ensure minimal bacterial contamination on the native dental pulp to achieve maximum results. Another limitation is the requirement to investigate further the long-term effect of KO + 10% FBS on gene expression. Future studies should include more samples and time points to determine the maintenance of the viability of pulp cells in different storage media to improve and achieve more favourable outcomes. Determination of optimal volume and duration of treatment with HBSS supplemented with Vit. C should be performed to maintain cell viability, gene expression, and enhancement of pulpal cell growth and regeneration.

## CONCLUSION

In summary, we concluded that HBSS supplemented with Vit. C is the most effective among the positive control group (KO + 10% FBS) and negative group (HBSS) in promoting the Col. I gene expression and maintaining the cell viability of native dental pulp. Hank's Balanced Salt Solution, enriched with L-ascorbic acid, may be a viable storage medium for an avulsed tooth before replantation. The prognosis of an avulsed tooth largely depends on its immediate management at the site of the incident. Proper handling can improve long-term outcomes by enhancing the chances of revascularization in immature teeth and increasing the survival rate of reimplanted avulsed teeth.

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## COMPETING INTERESTS

The authors declare that they have no competing interests.

## ETHICAL CLEARANCE

This study was approved by the Research Ethics Committee, Faculty of Medicine, Universiti Kebangsaan Malaysia (Ethics reference no.: JEP-2023-407).

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