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Effects of ethanol washing and storage duration on primary culture of stem cells from human exfoliated deciduous teeth

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ABSTRACT

Purpose: Since the oral environment harbors various microorganisms, the removal of contaminants during the primary culture process of stem cells from human exfoliated deciduous teeth (SHEDs) is very important. We investigated optimal methods for primary culture of SHEDs with minimal contamination rates.

Materials and methods: Three different storage conditions for deciduous teeth were utilized:1) storing teeth in Hank's Balanced Salt Solution (HBSS) with 3% penicillin and streptomycin (P/S), 2) storing teeth in HBSS with 3% antibiotics and antimycotics (A-A), and 3) storing teeth in HBSS with A-A, and additional washing with 70% ethanol just before primary culture of dental pulp. In addition, the storage time from the extraction of teeth to the primary culture was measured.

Results: The contamination rates were about 70% for HBSS with P/S, 40% for HBSS with A-A, and less than 10% for HBSS with A-A and additional washing with 70% ethanol. When the primary culture was conducted within 12 h after teeth extraction, the contamination rate was the lowest in all conditions. Furthermore, when the teeth were delivered in HBSS with A-A and an additional 70% ethanol washing was performed, the contamination rate was 0% until 48 h after teeth extraction. Ethanol washing had little effect on the cellular characteristics and stemness of SHEDs, including their morphology, growth rate, expression of surface markers, and differentiation potential.

Conclusions: We suggested that both delivering teeth in HBSS with A-A and additional 70% ethanol washing are critical considerations for the successful culture of SHEDs without contamination.

1. Introduction

Deciduous teeth had been considered only as clinical waste. However, since the existence of stem cells was reported,[1] deciduous teeth have become an important source of stem cell therapeutics. Dental pulp stem cells (DPSCs) reside in the dental pulp tissues and are involved in pulp regeneration. DPSCs can be obtained from the permanent teeth of adults or the deciduous teeth of young children.[1,2] Moreover, it is relatively easy to culture stem cells from human exfoliated deciduous teeth (SHEDs) since they are obtained from naturally exfoliated deciduous

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teeth without invasive and painful extraction processes. SHEDs have MSC-like characteristics and regenerative potential.[3] Many preclinical reports of SHEDs have demonstrated the potential therapeutic effects of SHEDs for various human diseases, including stroke, spinal cord injury, and cardiovascular disease.[4–8]

The experimental techniques to isolate and culture SHEDs have been upgraded.[1,9,10] However, in the oral microenvironment, more than 700 different bacteria, viruses, fungi, protozoa, and archaea exist, which could result in exogenous infection and contamination during the primary isolation and culture of SHEDs.[11–13] Therefore, for the clinical application of SHEDs as cell therapeutics, it is important to develop an inexpensive, simple, and efficient method to minimize contamination without changing the key characteristics of SHEDs. In this study, we investigated optimal conditions to isolate SHEDs without causing contamination or loss of stemness. We tested three conditions for storing deciduous teeth; Hank's Balanced Salt Solution (HBSS) with penicillin/streptomycin (P/S), HBSS with antibiotics-antimycotics (A-A), and HBSS with A-A and additional washing with 70% ethanol before extracting dental pulp to reduce the contamination rate of the primary culture of SHEDs. In addition, the effects of the time from teeth extraction to dental pulp isolation on the contamination rate were analyzed. To determine the effects of the three conditions on the stemness of SHEDs, we compared the cell surface markers and differentiation potential of SHEDs.

2. Materials and methods

2.1. In vitro primary culture of SHEDs

To acquire deciduous teeth, informed written consent was obtained from the patients according to the guidelines approved by the Institutional Review Board of Sungkyunkwan University (Suwon, South Korea) (IRB File No. 2021-11-016). Deciduous teeth were delivered and processed under three different conditions, 1) storing teeth in Hank's Balanced Salt Solution (HBSS)(Gibco, Grand Island, NY, USA) with 3% Penicillin/Streptomycin (P/S)(Gibco; penicillin 10000 units/mL, streptomycin 10000 μg/mL) at 4 °C for 1–120 h (h) (n = 64), 2) storing teeth in HBSS with 3% antibiotics-antimycotics (A-A)(Gibco; penicillin 10,000 units/mL, streptomycin 10,000 μg/mL, amphotericin B 25 μg/mL) at 4 °C for 1–120 h (n = 29); 3) storing teeth in HBSS and A-A at 4 °C for 1–120 h, sinking them in 1 mL of 70% ethanol for 3 s (sec) at room temperature (RT), and then drying in air for 30 s at RT just before extracting the dental pulp tissue (n = 172). The time from teeth extraction to primary culture was recorded for each sample. Teeth were placed in 1 mg/mL of collagenase type I (Serva, Heidelberg, Germany) diluted in DMEM/F12 (Gibco). Red Dental pulp tissues were identified by eyes, and then extracted using fine forceps. When they could not be captured by forceps, they were harvested by multiple pipetting with 1 ml pipettes. Dental pulp tissues were minced mechanically by fine scissors until there were no clump-like structures. The process took up to 5 min (min). Minced dental pulps were incubated in the collagenase solution at 37 °C for 1 h. After enzyme was inactivated by adding 1 mL complete media consisting of α-modified Eagle's medium (αMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 5 μg/mL gentamicin (Gibco). They were incubated at RT for 3 min, and then filtered through a 70-μm strainer (Falcon, NY, USA). Live cells were counted using a 0.4% trypan blue solution (Thermo Fisher Scientific, Waltham, MA, USA), and then seeded in 12-well plates (1×10^3–1×10^4 cells/well) (Thermo Fisher Scientific) in complete media. Half of the medium was replaced with complete medium every 3 days. Subculture was performed at > 80% confluence using TrypLE (Gibco). The number of cells was counted, and 4×10^3 cells/cm^2 were seeded in cell culture flasks (Thermo Fisher Scientific). Cell growth at each passage was determined by cumulation of population doubling length (CPDL) using (logB–logA)/log2, where B = harvested number and A = seeding number. Contamination was determined by observation under a microscope. Cells were photographed at every passage until passage nine.

2.2. In vitro adipogenic and osteogenic differentiation

To determine differentiation potential, SHEDs at *in vitro* passage 6 (P6) were utilized. For each condition, 3 lines of SHEDs derived from 3 different donors were analyzed (total n = 9). The SHEDs were dissociated and cultured within 48 h after tooth extraction. The cells (2×10^4 cells/well) were seeded in 12-well cell culture plates (Thermo Fisher Scientific). At more than 90% confluency, the medium was changed to adipogenic or osteogenic differentiation medium. StemPro™ Adipogenesis Differentiation Kit (Gibco) and StemPro™ Osteogenesis Differentiation Kit (Gibco) were utilized. The differentiation media was replaced every three days. After 21 days in differentiation media, the cells were fixed with 10% formalin (Biosesang, Gyeonggi, South, Korea) for 10 min at RT and washed with distilled water (DW). Then the cells were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) for 30 min or Alizarin Red S (Sigma-Aldrich) for 5 min at RT in dark. After washing three times with DW, the cells were photographed. The red colors in the images were separated using QuPath (Queen's University Belfast, Northern Ireland, UK) image analysis software.[14] The percentage of Oil Red O or Alizarin Red-positive pixels were calculated and compared.

2.3. Flow cytometric analysis

To verify the expression surface markers, SHEDs at *in vitro* passage 6 (P6) were utilized. For each condition, 3 lines of SHEDs derived from 3 different donors were analyzed (total n = 9). The SHEDs were dissociated and cultured within 48 h after tooth extraction. Cells were suspended at FACS buffer consisting of Dulbecco's modified phosphate buffered saline (DPBS) (Welgene, Daegu, South Korea) containing 2% FBS (2×10^5 cells/100 μL). Cells were incubated with the following primary antibodies according to the suggested dilution factor for 20 min at RT; anti-human CD11b, CD14, CD19, CD34, CD44, CD45, CD73, CD90, CD105, CD166, or HLA-DR antibody (BD Biosciences, NJ, USA). After washing by FACS buffer three times, the fluorescence intensity was measured using a FACS Calibur (BD Biosciences). FLOWJO software (Tree Star Inc., Ashland, OR, USA) was used for data analysis. Positive cells were determined by comparing with the appropriate isotype controls (BD Biosciences).

2.4. Statistical analysis

GraphPad Prism 9.5.1 (GraphPad Software, Boston, MA, USA) was used for statical analysis. Data are presented as mean ± standard deviation (SD). P-values of less than 0.05 ($P < 0.05$) were considered significant.

3. Results

3.1. Primary culture of SHEDs in three conditions

Deciduous teeth are usually stored in HBSS with antibiotics because oral bacteria are expected to contaminate the primary culture of SHEDs. To determine the optimal primary culture method of SHEDs with minimal contamination rate, we tested three conditions: 1) storing deciduous teeth in HBSS with 3% Penicillin/Streptomycin (P/S) (n = 64, condition 1, C#1), 2) storing deciduous teeth in HBSS with 3% antibiotics-antimycotics (A-A) (n = 29, condition 2, C#2), and 3) storing deciduous teeth in HBSS with A-A and then washing them with 70% ethanol just before extracting the dental pulp (n = 172, condition 3, C#3). Primary cultured SHEDs were maintained until *in vitro* passage 9 (P9).

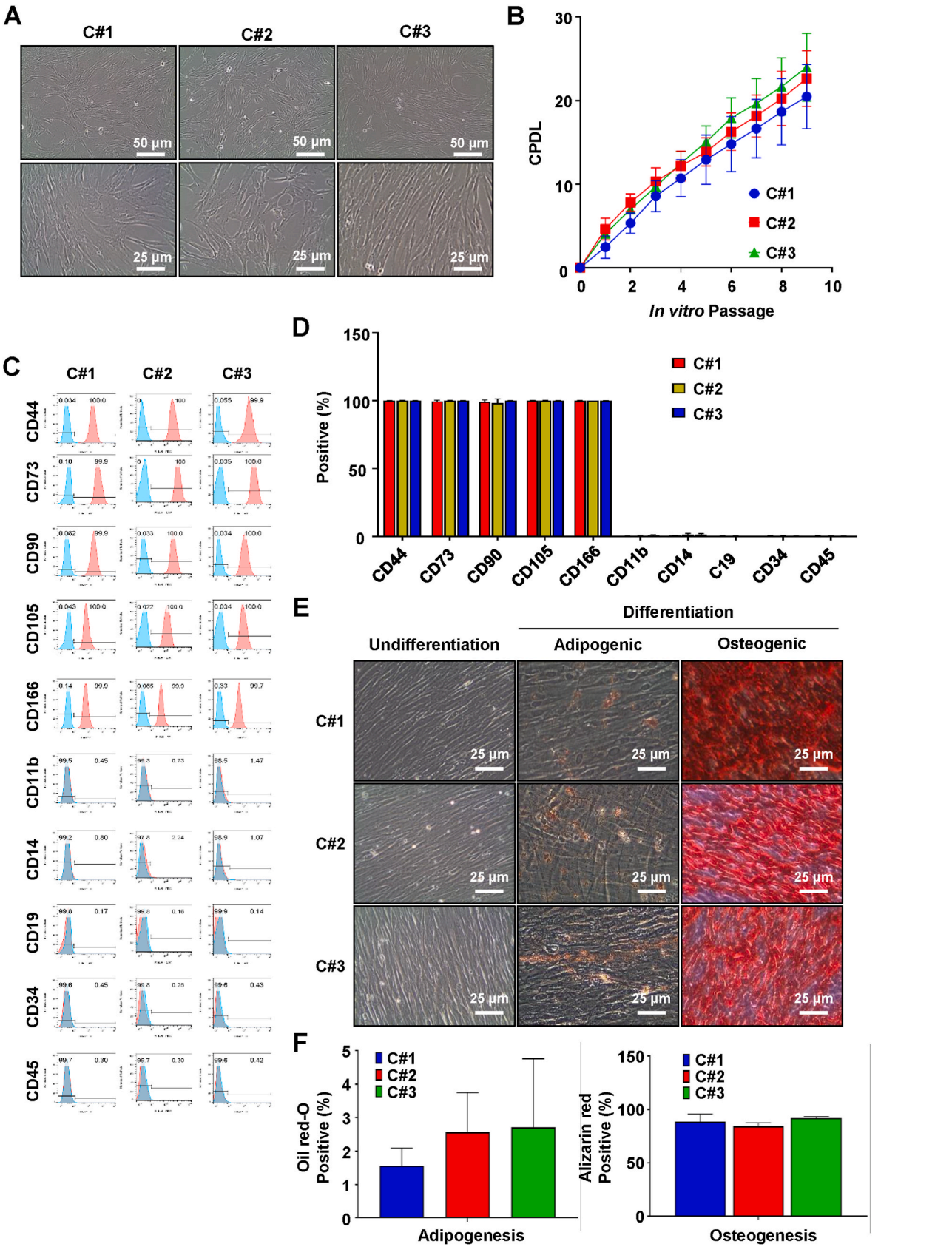

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Fig. 1. Characterization of SHEDs.
SHEDs were isolated from the dental pulp of deciduous teeth and cultured until *in vitro* passage 9 (P9). Primary culture of SHEDs was conducted in three conditions; Condition#1 (C#1) stored in HBSS with P/S (n = 64), Condition#2 (C#2) stored in HBSS with A-A (n = 29), or Condition#3 (C#3) stored in HBSS with A-A and washed by 70% ethanol (70% EtOH) (n = 172). (A) SHEDs at P6 showed typical MSC-like morphology irrespective of conditions. These images were one of representatives of SHEDs at each condition. (B) Growth curves were illustrated by average ± standard deviation calculated from three lines of SHEDs of each condition. There were no significant differences ($P > 0.05$, two-way ANOVA with repeated measures) in growth among the three conditions until P9. (C) To characterize immunophenotypes of SHEDs at P6, FACS analysis was performed (n = 3 for each condition). Results of one of three representatives of SHEDs for each condition were illustrated. SHEDs were positive for MSC-specific markers (CD44, CD73, CD90, CD105, and CD166), but negative for hematopoietic or endothelial cell markers (CD11b, CD14, CD19, CD34, CD45, and HLA-DR). (D) There were no differences in positive-cell percentages among the three conditions ($P > 0.05$, one-way ANOVA). (E) To confirm differentiation potential of SHEDs, adipogenic or osteogenic differentiation were performed at P6 (n = 3 for each condition). Lipid vacuoles and calcium deposits were stained with Oli red O and Alizarin Red, respectively. Undifferentiated SHEDs and Wharton's Jelly-derived MSCs (data not shown) were used as negative and positive controls, respectively. Results of one of three representatives of SHEDs for each condition were illustrated. (F) When the red signals were quantitatively analyzed, there were no differences among the three conditions ($P > 0.05$, one-way ANOVA).

3.2. Effects of culture conditions on characteristics of SHEDs

In all conditions, SHEDs showed typical fibroblast-like morphologies after adhering to the culture plates (Fig. 1A). During the primary culture, the growth of the three lines of SHEDs of each condition was stable until P9, and there was no significant difference among the three conditions (n = 3 for each condition, $P > 0.05$, two-way ANOVA with repeated measures, Fig. 1B). These data suggest that the three conditions do not affect cellular characteristics such as the morphologies and growth rates of SHEDs.

The expression of MSC-specific cell surface markers was determined using flow cytometry. The analysis was performed on *in vitro* passage 6 (P6). SHEDs derived from each condition (n = 3 for each condition) expressed MSC-specific cell surface markers CD44, CD73, CD90, CD105, and CD166 but did not express hematopoietic or endothelial cell markers; CD11b, CD14, CD19, CD34, CD45, and HLA-DR (Fig. 1C). There were no differences in positive-cell percentages among the three conditions ($P > 0.05$, one-way ANOVA, Fig. 1D). To determine *the in vitro* differentiation potential, SHEDs derived from each condition (n = 3 for each condition) were cultured in adipogenic or osteogenic induction medium for 21 days. Calcium deposits were stained with alizarin red after osteogenic induction, and lipid vacuoles were stained with Oil red O after adipogenic induction (Fig. 1E). When the red signals were quantitatively analyzed, there were no differences among the three conditions ($P > 0.05$, one-way ANOVA, Fig. 1F). These data suggested that the stemness of SHEDs was not altered by the three conditions.

3.3. Optimal condition for primary culture of SHEDs minimizing contamination

Contamination rates of primary cultures of SHEDs were evaluated according to the three conditions and storage duration. When contamination by microorganisms was visually observed in the culture flasks it was considered contamination. To avoid contamination, the flasks were immediately discarded. When SHEDs were more than 80% confluent without contamination, subculture was performed. The contamination rate was calculated to be approximately 70% (Fig. 2A) and 40% (Fig. 2B) for teeth stored in HBSS with P/S (C#1) and in HBSS with A-A (C#2), respectively. However, when the teeth were stored in HBSS with A-A and an additional 70% ethanol washing (C#3) was done, the contamination rate decreased dramatically to less than 10% (Fig. 2C). In the statistical analysis, the condition with additional 70% ethanol washing (C#3) showed significantly lower contamination rates than the other two conditions ($P < 0.0001$, Fisher's exact test, Fig. 2D).

Moreover, we tested the effects of storage time from tooth extraction to primary culture on contamination rate. The contamination rate was 0% when the SHEDs were isolated within 12 h of tooth extraction under storage conditions using HBSS with 3% A-A. Furthermore, no contamination was observed until 48 h in the condition of storing teeth in HBSS with A-A and an additional 70% ethanol washing before extracting dental pulp. The difference in contamination rate between before and after 48 h in the condition 3 (C#3) was statistically significant ($P = 0.0002$, Fisher's exact test, Fig. 2E). These results suggest that the optimal conditions for primary culture of SHEDs are 1) storing teeth in HBSS with A-A and additional 70% ethanol washing before extracting dental pulp and 2) performing dental pulp extraction within 48 h after teeth extraction.

4. Discussion

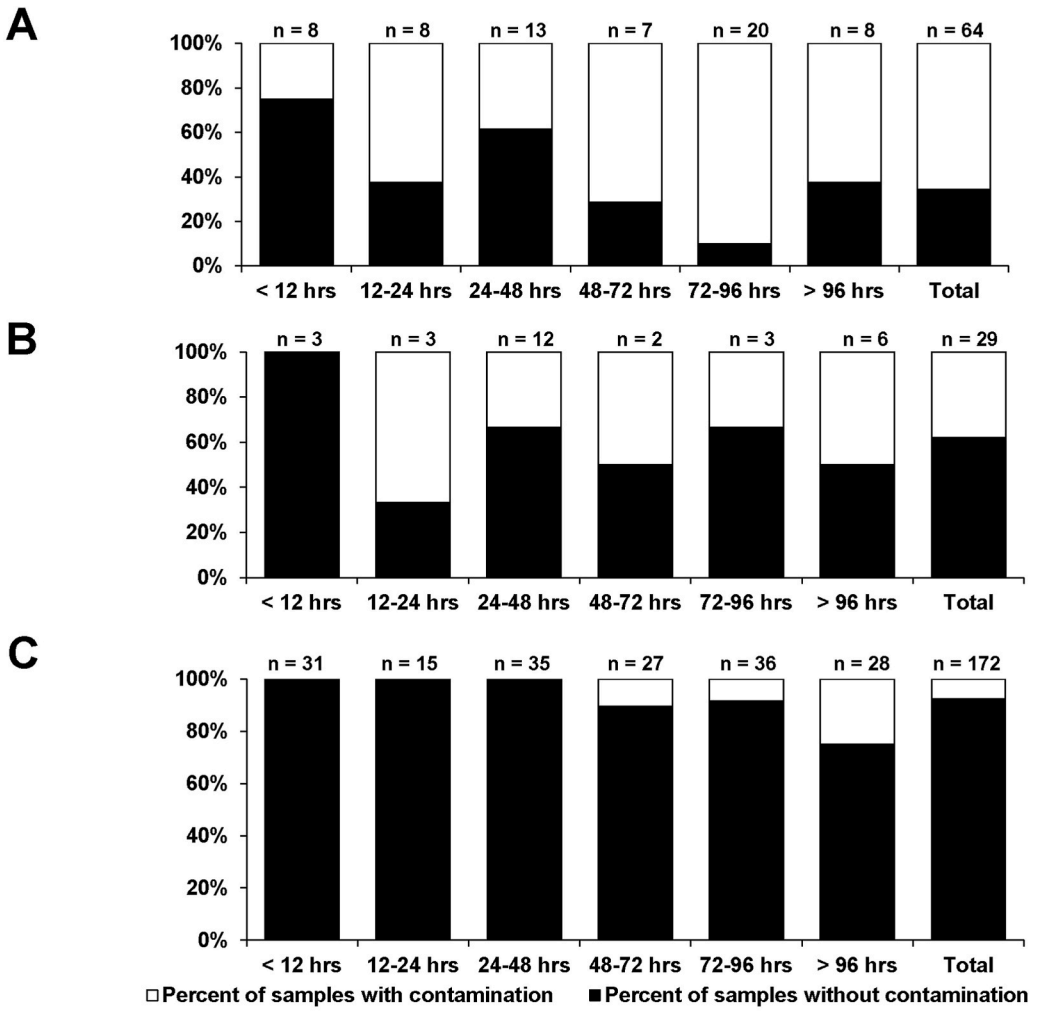
In this study, we introduced a method to reduce the contamination rate during primary culture of SHEDs by comparing several storage and washing methods, and the time from teeth extraction to primary culture of dental pulp. Antibiotics were the first consideration, because some bacteria and fungi in the oral microenvironment can resist penicillin and/or streptomycin.[15] A-A is a commonly used and commercially available product that prevents cell cultures from bacterial and fungal contamination. It consists of penicillin, streptomycin, and amphotericin B, which can prevent the contamination of fungi and bacteria.[16] The extracted teeth were immediately stored in a solution consisting of HBSS with P/S or A-A. The contamination rate was lower in HBSS with A-A than with P/S. However, the results were not sufficient to reduce the contamination rate to <10%. Accordingly, we tried empirical disinfection methods, such as additional washing of teeth with 70% ethanol just before extracting the dental pulp. It is well known that 70% ethanol immediately kills most microorganisms.[17] Consequently, the contamination rate decreased by less than 10%.

The addition of an anti-fungal agent, amphotericin B, reduced the contamination of SHEDs in this study. The results indicated that not only bacteria but also fungus could be the source of contamination. Many kinds of fungus have been reported to exist in the oral cavities of ordinary healthy people.[18] In addition, growth of yeast-like microorganisms was observed in several primary culture cases of SHEDs in this study.

We also evaluated the contamination rate according to storage time from tooth extraction to primary culture. The contamination rate was the lowest when the primary culture was conducted within 12 h after teeth extraction under all conditions. Furthermore, when the teeth were delivered in HBSS with A-A and an additional 70% ethanol washing was performed before extracting the dental pulp, the contamination rate was 0% until 48 h after teeth extraction (Fig. 2C). This result suggests that it is also important to reduce storing time.

There are numerous factors that could influence the contamination rate of primary culture of SHEDs including proficiency of dentists and technicians, environments of hospitals and laboratories, and general hygiene status of regions and nations. Even exfoliated deciduous teeth might be collected by non-medical personnels without any disinfection procedures. Therefore, contamination rate might be various among institutions. Easily applicable experimental protocols such as additional 70% ethanol washing and storage less than 48 h in this study would be helpful to reduce the variability and apply SHEDs to regenerative medicine in various situations.

In this study, cases of deciduous teeth with cavities or stainless-steel crowns resulted in contamination, regardless of the conditions (data not shown). Stainless-steel crown was introduced by Rocky Mountain



D

	C#1	C#3	Total
No contamination	23	161	184
Contamination	44	13	57
Total	67	174	241

Odds ratio (95% CI) = 0.04221 (0.01995 – 0.08907)
P < 0.0001 (Fisher's exact test)

	C#2	C#3	Total
No contamination	18	161	184
Contamination	11	13	24
Total	29	174	203

Odds ratio (95% CI) = 0.1321 (0.05199 – 0.3460)
P < 0.0001 (Fisher's exact test)

E

	C#3 Within 48 h	C#3 After 48 h	Total
No contamination	81	80	161
Contamination	0	13	13
Total	81	93	174

Odds ratio (95% CI) = +infinity (3.253 – +infinity)
P = 0.0002 (Fisher's exact test)

Fig. 2. Contamination rate of primary culture of SHEDs.
Contamination rates were analyzed according to the three conditions and storage time from extracting teeth to primary culture. Contamination by oral microorganisms was visually determined. (A) Condition #1 (C#1). (B) Condition #2 (C#2). (C) condition 3 (C#3). (D) Contamination rates of C#1 and C#2 were compared with that of C#3. P values were calculated by Fisher's exact test. (E) When primary culture was performed within 48 h after tooth extraction in C#3 (n = 81), contamination rate was significantly lower than that of 93 C#3 cases in which primary culture were done after 48 h after tooth extraction (P = 0.0002, Fisher's exact test).

Company in 1947 and has been the best practice to manage multi-surface carious lesions in decayed primary teeth.[19] However, *Streptococcus* bacterial adhesion to stainless-steel crowns was significantly higher than that to naive crowns.[20,21] Because teeth with stainless-steel crowns or cavities might cause much more contamination, it is not recommended to use these teeth for primary isolation and culture of SHEDs.

Our results showed that SHEDs isolated from the dental pulp of deciduous teeth have MSC-like characteristics, including expression of surface markers, including CD44, CD73, CD90, CD105, and CD166, and *in vitro* differentiation potential into osteoblast- and adipocyte-like cells, which is in accordance with previous reports.[22] However, the SHEDs in this study showed preferential differentiation into osteoblast-like cells compared to adipocyte-like cells. This indicated that SHEDs in this study might have preferential differentiation potential, which is focused on skeletal regeneration. This suggests the potential therapeutic indications of SHEDs. Since deciduous teeth are naturally exfoliated and replaced with permanent teeth in childhood,[23–27] it is possible to bank SHEDs at this age for future use. Banking SHEDs is one of autologous cell sources used to treat various diseases. Moreover, autologous cell therapy is better than allogenic cell therapy[28] because low immune responses or rejection could enhance the survival of transplanted cells and therapeutic efficacy in damaged organs.

5. Conclusion

We have provided a simple and cheap primary culture method for SHEDs to minimize contamination, which could maintain the MSC-like characteristics of SHEDs. We expect that primary isolation of SHEDs within 48 h after tooth extraction and washing extracted teeth with 70% ethanol just before isolation of SHEDs will contribute to optimizing the primary culture method of SHEDs for the clinical use and development of teeth banking.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgments

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