Royal jelly promotes the viability and proliferation of periodontal ligament fibroblasts in an *In Vitro* tooth avulsion simulation

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Abstract

The best treatment for tooth avulsion is immediate replantation. If not possible, the tooth should be kept in a proper storage medium before seeking dental treatment. Hank's balanced salt solution (HBSS) is the recommended medium of choice for tooth storage; however, it is not always readily available at the scene of accidents. Until today, numerous storage media have been studied, but they rarely contain all the required properties for the avulsed tooth preservation. Royal jelly (RJ) is a natural bee product with several biological functions such as promoting cell longevity, and wound healing, as well as eliminating inflammation.

Objectives: This study aims to investigate the effect of RJ on the viability and function of periodontal ligament fibroblasts (PDLF) in an *in vitro* tooth avulsion model.

Material and Methods: PDLF separated and cultured from human sound tooth, were plated and subjected to tooth avulsion simulation. The storage media were then added including HBSS, RJ solutions and low fat milk. Cell viability and proliferation were assessed by MTT assays.

Results: The results show that RJ solutions dose-dependently maintain higher PDLF viability when compared to HBSS ($p<0.05$). However, when compared to low fat milk, PDLF survival was somewhat comparable in RJ solution at concentrations of 500 and 900µg/ml. In addition, royal jelly solutions also promote the proliferation of the survived PDLF when compared to HBSS.

Conclusions: Royal jelly demonstrates beneficial properties as a potential tooth storage medium.

Keywords: cell proliferation, cell viability, dental trauma, low fat milk, storage media, tooth storage

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Introduction

Avulsion of permanent teeth is among 0.5% to 3% of all dental injuries [1], characterized by complete displacement of the teeth from their bony sockets. It is mostly caused by accidents, violence or contacting sports involving the facial area. Consequently, the compromised blood supply to the teeth most likely affects the viability of cells in the pulp tissue, resulting in pulp necrosis [2]. In addition, the tear in periodontal ligament (PDL) tissue at the root surface affects PDL cells survival and their regenerative capabilities [3]. The best treatment option for the avulsed tooth is immediate replantation to its socket but it is rarely possible to do so. Alternatively, it is advised to place the avulsed tooth in a proper storage medium, to ensure the highest degree of cell viability, and have it replanted by a dentist as soon as possible. Better prognosis would be achieved if the tooth could be replanted to its socket within 30 minutes or stored in a proper storage medium in the first couple of hours after avulsion [4].

Studies have shown that the viability of PDL cells at the root surface is considered an essential factor for long-term success of the replanted teeth, since they are needed for new PDL tissue regeneration. The extra-alveolar duration, degree of bacterial contamination and storage condition of the tooth before replantation directly affect the survival of PDL cells [3, 5]. Diminished numbers of viable cells result in post-replantation healing complications such as surface resorption, inflammatory root resorption or replacement resorption [3, 6-7].

To date, studies are still ongoing in search of a suitable storage medium for avulsed teeth which should be easily accessible in emergency situations, effective in various conditions and temperatures, inexpensive, and should have a long shelf-life. In addition, to maintain the viability and function of PDL cells, a storage solution should have the same pH and osmolarity as body fluid, contain antimicrobial activity, not induce antigen-antibody reactions, and reduce the risk of post-replantation root resorptions [8].

So far, various types of tooth storage media have been studied and they could be roughly classified into groups according to their efficacy and accessibility. Firstly, the solutions with excellent effectiveness but limited availability include cell and tissue culture media e.g. Hanks's Balanced Salt Solution (HBSS), and Minimum Essential Medium; organ transporting media e.g. Viaspan® and Euro-Collins®; propolis, and green tea. Secondly, milk, coconut water, egg, and Ricetral are more commonly available and have shown good to excellent outcomes. Lastly, water, saliva, saline, Gatorade, and contact lens solution can be easily obtained; however, they demonstrated poor efficacy in maintaining PDL cells vitality [9].

Hank's Balanced Salt Solution (HBSS) is a widely accepted storage medium of choice for avulsed teeth since it is non-toxic, nutrients-enriched, pH-balanced and has a proper osmolarity for cell growth [1, 9]. Most importantly, it can maintain high levels of PDL cells viability when immersed for duration up to 24 hours [1, 10-14]. Moreover, it has been developed into a commercialized emergency tooth preservation kit (Save-a-tooth®, Pottstown, PA, USA) [15]; however, it is not always readily available at the scene of accidents and studies have shown that its efficacy is inferior to the original HBSS [11, 16]. Second to HBSS, milk has been recommended as an effective tooth storage medium from its physiological properties and ease of access [1, 9, 17-20]. It has been reported that soaking the tooth in milk for 2-6 hours is as effective as HBSS while at durations longer than 12 hours, the effectiveness of milk significantly drops [17]. However, the duration for effective PDL cells preservation in milk varies among different studies, possibly from the different temperatures, origin and types of milk used [10, 12, 16, 19-21].

Royal jelly (RJ) is a well-known natural bee product, essential for the growth of the queen honeybee. It is secreted from the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera*). Numerous studies have reported health-promoting activities of RJ for examples, antimicrobial activity, immuno-modulation, anti-inflammation, anti-oxidation, and anti-aging effects [22]. Moreover, RJ supports the wound healing process by promoting human dermal fibroblast migration [23] and enhances collagen production of skin fibroblast by the function of 10-hydroxy-2-decenoic acid (10H2DA), the active component of RJ which induces TGF- β1 [24]. Yanagita *et al*. have shown that RJ at the concentrations between 4 – 500 μg/ml were not toxic to PDL cells [25]. In addition, Dhanesuan *et al* demonstrated that RJ crude extract at the concentrations from 0.1-1mg/ml enhanced PDL cell proliferation while significant inhibition was observed with RJ at 5mg/ml [26]. To the best of our knowledge, RJ has never been studied as a potential media for tooth storage.

In the present study, we aimed to determine whether RJ solution could be a suitable storage medium for the avulsed tooth by assessing its efficacy in maintaining the viability and proliferative capability of human PDL fibroblasts (PDLF) when compared to the well-accepted storage media, HBSS and low fat milk.

Materials and Methods

Cell culture

This project was approved by the Ethics Committee of the Faculty of Dentistry, Srinakharinwirot University. Human PDL tissue was retrieved from healthy premolars, extracted for orthodontic purposes with informed consent. PDLF primary culture and cell strain were established according to the technique described by Dhanesuan *et al.* [26], maintained in Dulbecco Modified Eagle's Medium high glucose (DMEM/HIGH, HyClone™) (GE Healthcare, UT, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA, USA), 2mM L-Glutamine (Invitrogen, Carlsbad, CA, USA), 100IU/ml Penicillin, 100ug/ml Streptomycin and 5ug/ml Amphotericin B (Invitrogen, Carlsbad, CA, USA), and incubated in 5% CO_2 atmosphere at 37°C. PDLF with passage numbers less than 8 were used in this study.

Preparation of Royal Jelly solution

Freeze dried RJ capsules (Supa Farm, Chiang Mai, Thailand) were obtained and the powder was gently dissolved in HBSS (Invitrogen, Carlsbad, CA, USA) at the initial concentration of 10mg/ml at 4°C, overnight. The mixture was then centrifuged at 9,660 x g at 4°C for 20 minutes and the supernatant was collected and sterilized by 0.20μm syringe filtration (Minisart®, Sartorius stedim, Germany). Total protein concentration in the RJ solution was measured by Bradford assay.

Storage media

The storage media tested in this study include 1) HBSS (Invitrogen, Carlsbad, CA, USA), 2) RJ 100μg/ml 3) RJ 500μg/ml 4) RJ 900μg/ml and 5) low fat, Ultra-high Temperature processed (UHT) milk (Foremost, Bangkok, Thailand).

Cell Viability Assay

PDLF were trypsinized, counted and plated in 96-well tissue cultures plates at the number of 1.5×10^4 cells/well/100μl of DMEM/10% FBS. The plates were then incubated overnight at 37°C in 5% CO_2 to allow cell attachment. Subsequently, tooth avulsion was simulated, according to the methods by Gjertsen *et al.* [27] with some adjustments, by removing 80μl of medium from each well and placing the plates on the laboratory bench at room temperature for 30 minutes. Next, the remaining medium was

completely aspirated and followed by the addition of 100μl of the test storage media, in triplicate wells. The test conditions included 1) HBSS 2) RJ 100μg/ml 3) RJ 500μg/ml 4) RJ 900μg/ml and 5) low fat UHT milk. PDLF in the wells which DMEM/10% FBS was not removed were used as controls. The cells were immersed in the storage media at room temperature for 1 hour. The storage media were then completely removed from each well, which was further rinsed with 200μl/well of phosphate buffered saline (PBS) and subjected to the MTT assay to determine PDL cell viability. MTT powder (USB Corporation, Cleveland, OH, USA) was dissolved in PBS to the concentration of 5mg/ml and then mixed with serum-free DMEM at the ratio of 10:100 microliter. Next, 110μl of MTT/serum-free DMEM mixture was added to each well and the plates were placed in a 37°C incubator for 30 minutes. Following the incubation, the solution was gently removed by pipetting and the formazan crystals formed were dissolved by adding 100μl of DMSO into each well. The absorbance, representing the amount of viable cells, was then measured at the wavelength of 550nm on Asys UVM340 Microplate reader (Biochrom, Cambridge, UK) and calculated as a percentage relatively to the control.

Cell Proliferation Assay

PDLF were trypsinized, counted and plated in 96-well tissue cultures plates (one plate for each timepoint) at the number of 10^4 cells/well/100μl of DMEM/10% FBS. The plates were then incubated at 37°C in 5% CO_2 for overnight to allow cell attachment. Then, the cells were exposed to tooth avulsion simulation and the same storage media conditions, as stated in the Cell Viability Assay. After the immersion of PDLF in the storage media for 1 hour follow by the rinse with PBS, 100μl DMEM/10% FBS was added to each well and the plates were further incubated at 37°C. MTT assays were then performed to determine cell viability at 2 and 4 days after the tooth avulsion simulation and storage media incubations.

Statistical Analysis

Statistical differences were determined by Kruskal-Wallis test and Dunn's multiple comparisons post test. The data were presented as means ± S.D., and a *p* value less than 0.05 was considered significant.

Results

Cell Viability

Following tooth avulsion simulation and subsequent exposure of PDLF to the storage media for 1 hour, immediate MTT analyses (Figure 1) showed that there were significant differences in the percentages of cell survival among all the storage media conditions tested ($p < 0.001$). HBSS was able to maintain the viability of PDLF at the level of 56 %. When compared to HBSS, RJ solutions at the concentration of 100, 500 and 900 μg/ml dose-dependently supported higher PDLF survival at 63%, 68%, and 71% respectively. However, statistical significant differences were only observed between HBSS and RJ500 and RJ900 ($p < 0.05$). On the other hand, low fat milk demonstrated a slightly lower level of cell survival (86%) compared to the control. In addition, only PDLF immersed in HBSS and the RJ solution at 100 μg/ml showed significant lower levels of cell viability when compared to those in low fat milk ($p < 0.001$ and $p < 0.05$ respectively).

Cell Proliferation

To determine the effects of different storage media on the proliferative ability of the survived PDLF, we performed MTT assays at 2 and 4 days after subjecting the plated cells to the tooth avulsion simulation and the immersion

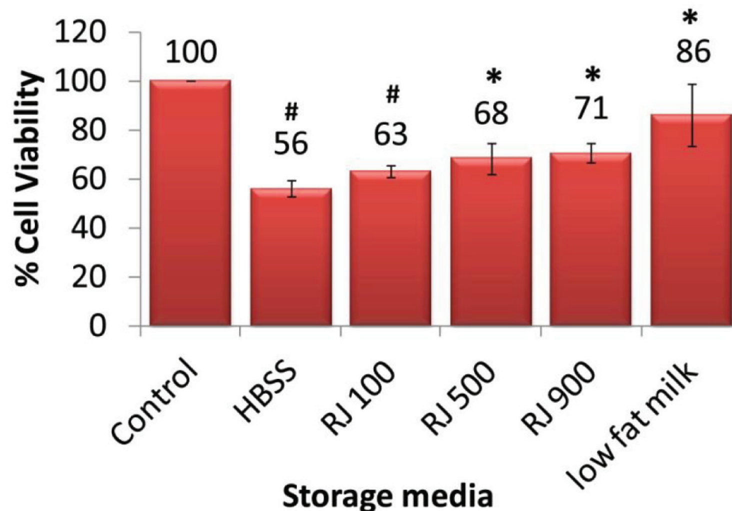

Figure 1 Peridontal ligament fibroblast viability. Following the culture media depletion for 30 minutes, PDLF were stored in various storage media, at 25°C, for 1 hour and then subjected to MTT assay. PDLF viability is shown as an average percentage relative to the control. Error bars, S.D. from 7 separate assays. *, significantly different from HBSS ($p<0.05$); #, significantly different from low fat milk ($p<0.05$). Control, PDLF in the wells which DMEM/10% FBS was not removed; HBSS, Hank's Balanced Salt Solution; RJ, Royal jelly.

of cells in various storage media for 1 hour. The rate of PDLF proliferation in each condition from day 2 to day 4 is shown by line graph plots in Figure 2A and their slope analyses in the bar graph plots in Figure 2B. Among the storage media tested, immersion of PDLF in low fat milk resulted in the fastest rate of cell proliferation, shown by the steepest slope value (0.280) which is comparable to those in the control (0.275). On the other hand, PDL cells previously exposed to RJ solutions showed slightly slower rate of cell proliferation (slope value 0.240, 0.224 and 0.219) comparing to those in the control and low fat milk, however clearly faster than those in HBSS (0.153). Repeated experiments using the same conditions showed different growth rates but essentially similar trend comparing among the storage media.

Discussion

One of the crucial prognostic factors for the success of the avulsed tooth replantation is the survival of PDL cells at the root surface. They are essential for normal PDL tissue repair which may prevent healing complications e.g. root resorptions following tooth replantation [7, 28-29]. Preservation of PDL cells viability relies mainly on shortening of the extra-alveolar duration, and more importantly, on the suitable type and condition of storage media used prior to replanting the tooth back into its socket [30-31].

Several studies have utilized the 30-minute dry time in their experimental design, even though some PDL cells are damaged, however; some viable cells are still available for investigation. Moreover, it closely resembles the real situation before which the avulsed

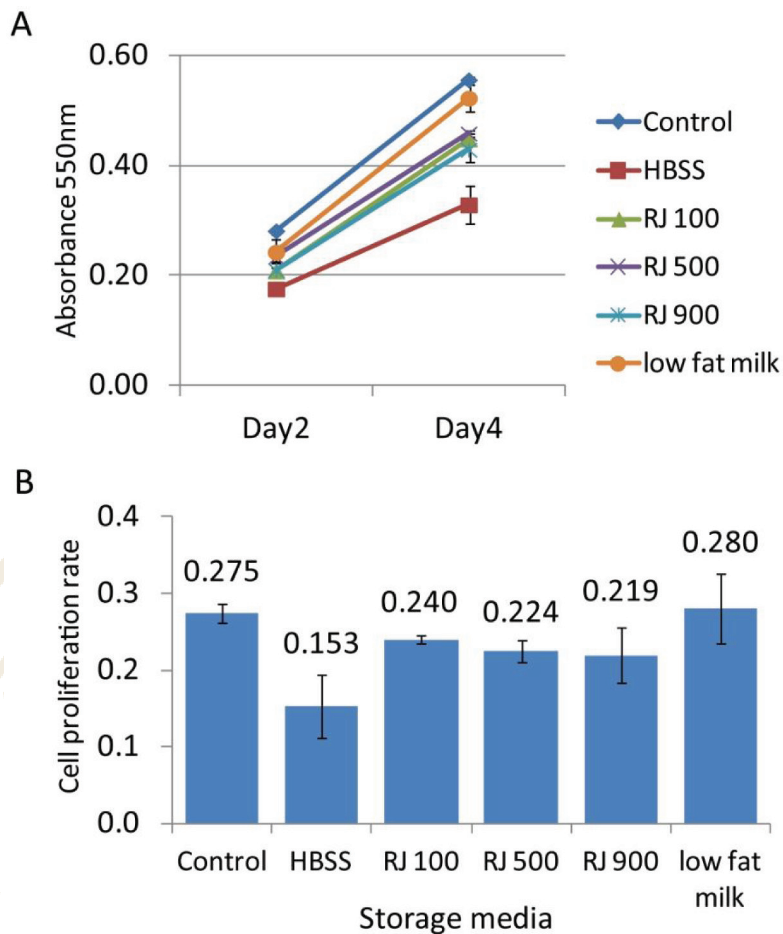

Figure 2 Periodontal ligament fibroblast proliferation rate. PDLF previously stored in various storage media for 1 hour were further cultured for 2 and 4 days and later subjected to MTT assays, to determine the proliferation rates of the cells in each condition.
A) Line graph plots demonstrate the proliferative potential of PDLF from day 2 to day 4 in each storage media condition.
B) Bar graph plots represent the rates of PDLF proliferation shown by the slope analyses.
Control, PDLF in the wells which DMEM/10% FBS was not removed; HBSS, Hank's Balanced Salt Solution; RJ, Royal jelly.

tooth could be placed in a proper storage medium [27, 30, 32-35]. In most of the studies, the extracted teeth were left dry for a specific duration, immersed in the test storage media, and subjected to enzymatic extraction and determination of viable PDL cells [30, 32-35]. On the contrary, most reports on the efficacy of various storage media on the viability of cultured PDL cells did not include the dry-time period in their methods. Following cellular attachment to the plate, the culture media was removed and immediately replaced with the test storage media[10-13, 16, 18-21, 36-40]. The outcome shown in these studies may reflect the effect of the storage media on PDL cells in almost normal condition, unlike PDL cells in the real

tooth avulsion situation. Using the PDL cell culture setting, Gjertsen *et al.*, has factored in the 30-minute dry time at room temperature after the 50% removal of culture medium from the wells [27]. In our study, however, we have made some adjustments to the reported method [27] by removing 80% of culture medium from the wells and placing the plates on the bench for 30 minutes to closer simulate the extra-alveolar duration and condition affecting the PDL cells on the root surface. Initially, 100% removal of culture medium followed by 30-minute dry time was evaluated, but no viable cells could be detected from any storage media tested.

Among all of the osmolarity-balanced media (e.g. HBSS, saline, and milk), milk is still the most recommended physiologic storage solution for the avulsed tooth due to the presence of essential nutrients and, most importantly, its ease of access at the scene of accidents [1]. Milk with lower fat content was shown to support better PDL cell viability[18]. In addition, the long shelf-life UHT milk, which does not require refrigeration, is more readily available and shows comparable preservation of PDL cells survival as regular pasteurized milk [19]. From our study, the low fat UHT milk showed the highest capability in promoting PDLF viability and proliferation. Interestingly, the higher efficacy of low fat milk over HBSS at room temperature, observed in our study, is in agreement with previous reports [12, 16, 37-38] but in contrast with some studies showing higher effectiveness of HBSS over milk[10, 13-14, 20]. The discrepancy among the studies may result from the differences in the origin and composition of milk, the experimental design, and the analysis methods employed in each study. Recently, Zhan *et al.* have studied the effect of storage media and time on the involvement of PDL cells in the root resorption process. They reported that PDL cells could induce the formation of multinucleated, osteoclast-like cells, when stored in HBSS, milk or α- MEM for 1 hour or more while the highest effect was observed with the PDL cells soaked in milk for 6 hours [52]. Therefore, the ability of storage media to suppress osteoclastogenesis may be another essential factor, aside from maintaining the viability of PDL cells, to predict the long term success of the replanted avulsed tooth.

Results from our study indicate that RJ solution supports higher PDLF survival and faster proliferation rate when compared to HBSS. However, when compared to low fat milk, RJ solutions at the concentration of 500 and 900µg/ml showed slightly lower PDLF survival and slightly slower or comparable cell proliferation rate. The result shown here is, somewhat, in agreement with a recent study from Dhanesuan *et al.*, which reported that RJ at a concentration up to 1mg/ml was able to enhance the proliferation of human PDLF, whereas the growth of human gingival fibroblasts gradually declined with the RJ concentration above 0.1mg/ml[26]. Unlike what we have observed, Yanagita *et al.* have shown that RJ did not induce significant mouse PDL cells proliferation [25]. The distinct proliferative potential, observed in PDL cells, may somewhat reflect the species-specific responses to RJ or result from the difference in the RJ preparations used in the studies [25-26].

In our study, the use of crude RJ extract, which would contain lesser amount of active components, may possibly result in the less dramatic positive effects of the RJ solution demonstrated. To date, numerous components in RJ have been identified and their biological functions reported. For instance, it was shown that 10-hydroxy-2-decenoic acid (10H2DA) promotes collagen production in skin fibroblasts [24] and also contains immunomodulating effects [47]. More importantly, about 50% of dried RJ is

comprised mainly of proteins, which may hold the keys to the health promoting benefits of RJ. The most abundant proteins in RJ belong to the major royal jelly proteins (MRJPs) family [48], and proteins in this family have shown cell proliferation functions in various cell types [49-51]. Therefore, it is likely that the positive effect of RJ on human PDLF viability and proliferation may be modulated by the MRJPs, for which future studies would be needed.

Taken together, our results showed the positive effect of RJ solution in maintaining the viability and proliferative abilities of PDLF in an *in vitro* tooth avulsion condition.

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