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Storage media enhance osteoclastogenic potential of human periodontal ligament cells via RANKL-independent signaling

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Abstract – *Background:* Hank's balanced salt solution (HBSS) and milk have gained wide acceptance as storage media for avulsed tooth. However, the effect of the media and storage time on the periodontal ligament (PDL) cells involvement in the development of root resorption is still unclear. The purpose of this study was to evaluate whether precultured PDL cells in HBSS, milk, or modified Eagle's medium alpha (α-MEM) would affect osteoclastogenesis. *Materials and methods:* PDL cells were precultured in HBSS, milk, or α-MEM for 1 h or 6 h before being co-cultured with RAW 264.7 cells for an additional 3 days for mRNA analysis and 11 days for osteoclastogenesis assay. *Results:* Cyclooxygenase-2 (COX-2) mRNA was detected immediately in PDL cells precultured in the three storage media. The expression was up-regulated markedly in all co-cultures when compared with RAW cells alone. As a result of the co-culture, interleukin-1β (IL-1β) expression was detectable in both PDL and RAW cells. TRAP+ multinucleated, osteoclast-like cells developed in all co-cultures; the number of TRAP+ cells was highest ($P < 0.05$) in the co-cultures that PDL cells precultured in milk for 6 h. The mRNA level of receptor activator of nuclear factor-kappa B ligand (RANKL) was not detected in PDL cells. Osteoprotegerin (OPG) mRNA expression reduced with increased preculture time, but the difference was not significant ($P > 0.05$). *Conclusions:* PDL cells kept in the three storage media led to TRAP+ multinucleated, osteoclast-like cells formation via RANKL-independent signaling. The ability to induce osteoclastogenesis may be considered as one of the factors to evaluate the ability of storage medium to maintain PDL viability after tooth avulsion.

Tooth avulsion is characterized by complete displacement of a tooth from its alveolar socket. It ranges from 0.5 to 3% of all traumatic injuries to the permanent dentition (1). After avulsion, immediate replantation in the socket is the ideal procedure for maintaining the viability of periodontal ligament (PDL) cells (2). However, it is not always possible, so that choosing a suitable storage medium for maintaining the viability of PDL cells is of great importance to successful replantation (1). Hank's balanced salt solution (HBSS), milk, and modified Eagle's medium are the widely recommended solutions for this purpose because they have neutral pH and physiological osmolarity and contain essential nutrients (3–5).

PDL cells not only play an essential role in periodontal tissue regeneration but are also associated with bone remodeling (6) and physiological (7) and pathological root resorption (8). After replantation of avulsed tooth, the most common complication is root resorption (1), as the result of the action of odontoclasts (9, 10). The morphological and functional characteristics of odontoclasts are similar to those of bone-resorbing osteoclasts, with both odontoclasts and osteoclasts being differentiated from monocyte and macrophage lineage cells (11, 12). Increasing amount of evidence suggests that PDL cells closely resemble osteoblasts that can induce osteoclast-like cell formation if co-cultured with osteoclastic precursors, such as peripheral blood mononuclear cells (PBMC) (13, 14), mouse bone marrow cells (15, 16), and monocyte/macrophage lineage (RAW 264.7 cells) (17). It is well established that osteoclastogenesis is mainly through RANKL/OPG pathway (18). The receptor activator of NF-kappa B ligand (RANKL), a tumor necrosis factor (TNF) family member, is expressed on the surface of osteoblasts and is essential for osteoclast differentiation. Binding of RANKL to its receptor, RANK, expressed on osteoclasts, activates a cascade of transcription factors to promote osteoclastogenesis (19).

Osteoprotegerin (OPG), a decoy receptor for RANKL, also expressed on osteoblasts, neutralized RANKL, and therefore prevented RANKL–RANK interaction and the subsequent bone resorption (19). However, the effects of storage media and time on the potential of PDL cells to induce osteoclastogenesis are largely unknown, although the two factors are said to be important in the successful replantation of an avulsed tooth. The aim of this study was to examine the effects of these two variables on the PDL cells by preculturing them at different conditions and then inducing osteoclastogenesis by co-culturing them with osteoclast precursors of RAW 264.7 cells.

Materials and methods

Cell culture

Human PDL tissue was obtained from extracted healthy premolars for orthodontic reasons with informed consent and following an approved protocol by the ethics committee of Fujian Medical University. The PDL tissue was gently scraped from the middle third of the root surface and then digested in a solution of 3 mg ml^{-1} collagenase type I (Sigma, St Louis, MO, USA) and 4 mg ml^{-1} dispase (Roche, Mannheim, Germany) for 1 h at 37°C. Single-cell suspensions of PDL were obtained by passing the cells through a 70-μm strainer (Falcon; BD Labware, Franklin Lakes, NJ, USA), which were seeded into 10-cm culture dishes in a modified Eagle's medium alpha (α-MEM) (Gibco, Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U ml^{-1} penicillin (Invitrogen, Carlsbad, CA, USA), 100 μg ml^{-1} streptomycin (Invitrogen), and 250 ng ml^{-1} fungizone (Gibco) at 37°C in 5% CO_2 atmosphere. The cultures were switched to the same, freshly prepared medium every 3 days. When the cells had reached confluence, they were subcultured for several passages, and those cells from passage 4 were used in this study.

The murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC) and cultured with Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% FBS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 250 ng ml^{-1} fungizone.

Storage medium

The storage media examined were fresh whole milk (Trappist Dairy, Hong Kong), HBSS (14025-050; Invitrogen), and α-MEM.

Co-culture

PDL cells (1.5×10^4 cells cm^{-2}) were seeded in six-well plates and incubated at 37°C with 5% CO_2 in α-MEM. At confluence, the culture medium was removed, and the wells were filled with 2 ml HBSS, milk, or α-MEM. After 1-h or 6-h storage in the designated medium, the cells were washed three times with PBS, and then RAW 264.7 (1×10^5 cells cm^{-2}) was seeded on the top of the PDL cells. They were co-cultured in α-MEM for an additional 3 days for mRNA analysis, or 11 days for osteoclastogenesis assay.

Reverse transcription and real-time PCR for RANKL, OPG, cyclooxygenase-2 (COX-2), and interleukin-1β (IL-1β)

Total RNA from each sample was extracted with RNeasy Mini kit (Qiagen, Hilden, Germany) and purified by removing genomic DNA with RNase-Free DNase set (Qiagen). One microgram of total RNA was used to synthesize cDNA by SuperScript III Reverse transcriptase (Invitrogen). The concentration of the cDNA produced was quantified using a Nanodrop ND-1000 spectrophotometer. Quantitative real-time PCR was carried out using the ABI Prism 7000 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) with SYBR green (Applied Biosystems). All samples were run in triplicate in 96-well plates, with each well containing 1.25 μl cDNA, diluted 1:10 to a total reaction volume of 20 μl. Reactions were carried out at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR primers were designed specifically to amplify human or mouse mRNA, see sequences in Table 1. For data analysis, the STEPONE™ software v2.0.2 (Applied Biosystems) was used to calculate the level of target gene expression in samples relative to the level of expression in the control (α-MEM) sample with comparative CT method (ΔΔCT). Expression values for target genes were normalized to the expression of GAPDH.

Tartrate-resistant acid phosphatase staining

After 11 days of co-culturing, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity, which is a specific marker typical of osteoclastic

Table 1. Human- and mouse-specific primers used for real-time PCR

Primer	Sequence 5′ → 3′	Reference
Human		
Osteoprotegerin	CTGCGCGCTCGTGTTTC ACAGCTGATGAGAGGTTTCTTCGT	(14)
Receptor activator of nuclear factor-kappa B ligand	CATCCCATCTGGTTCCCATAA GCCCAACCCCGATCATG	(14)
IL-1β	CTTTGAAGCTGATGGCCCTAAA AGTGGTGGTCGGAGATTCGT	(14)
COX-2	TCCCTGAGCATCTACGGTTTG AACTGCTCATCACCCCATTCA	(20)
GAPDH	GGCATGGACTGTGGTCATGAG TGCACCACCAACTGCTTAGC	(21)
Mouse		
IL-1β	CGC AGC AGC ACA TCA ACA AGA GC TGT CCT CAT CCT GGA AGG TCC ACG	(22)
COX-2	TGA GTA CCG CAA ACG CTT CTC TGG ACG AGG TTT TTC CAC CAG	(22)
GAPDH	GAGCCAAACGGGTCATCA CATATTTCTCGTGGTTCACACC	(23)

differentiation, using the leukocyte acid phosphatase kit (386-A; Sigma). Images were obtained with a light microscope (Nikon TE2000-S, Japan) using a SPOT RT camera (USA). TRAP+ cells appeared as dark purple. The number of TRAP+ multinucleated (with three or more nuclei) cells was counted and expressed as average number of TRAP+ multinucleated cells per three random sites in one well under light microscope. Three independent experiments were performed.

Statistical analysis

One-way ANOVA was performed to compare the difference in gene expression and osteoclasts formation between groups. The number of osteoclast-like (TRAP+ multinucleated) cells was given as mean ± standard error of the mean (SEM), and mRNA expression was represented as mean ± standard deviation (SD). A P value of < 0.05 was considered as statistically significant.

Results

Expression of osteoclastogenesis-related genes after different storage medium treatment

Effect after preculture with storage media on PDL cells
The expression of OPG was decreased slightly after storage of the PDL cells in HBSS or milk for 1 or 6 h compared to α-MEM, but the difference was not statistically significant (Fig. 1). The expression of COX-2 was augmented significantly after keeping the PDL cells in HBSS or milk ($P < 0.05$). Longer storage time (6 h) elevated the COX-2 expression significantly for both HBSS and milk (Fig. 2a). The expression of RANKL and IL-1β was undetectable in all PDL cell monocultures (Fig. 2b).

Effect of co-culturing with RAW 264.7 for 3 days
As IL-1β and prostaglandin G2 (PGE2) are produced by both osteoblasts and osteoclast precursor cells (24), we used species-specific probes to determine the contribution of the human PDL and mouse RAW 264.7 cells to the production of the respective cytokines in the co-cultures.

Co-culturing of pretreated PDL and RAW 264.7 cells led to constant expression of COX-2 in both PDL and RAW 264.7 cells. There was no significant difference in COX-2 expression by human PDL cells in any groups of the co-culture. However, expression of mouse COX-2 was elevated significantly in all co-culture groups, compared with RAW 264.7 cells alone. PDL cells that had been stored in HBSS or milk for 6 h showed significant up-regulation of the COX-2 expression by RAW 264.7 cells in the co-culture ($P < 0.05$) (Fig. 2a).

Interestingly, IL-1β mRNA expression was undetected when PDL cells were cultured singly. Under co-culture condition, IL-1β mRNA was expressed in both human PDL and RAW 264.7 cells. PDL cells pretreated for 6 h induced higher levels of IL-1β mRNA expression by RAW 264.7 than those by RAW 264.7 monoculture (Fig. 2b).

There was no difference in OPG mRNA expression between different co-culture groups (Fig. 1). RANKL was not detected in human PDL cells in the presence of murine RAW cells.

Formation of osteoclast-like cells

Based on the changes in mRNA expression observed after temporal incubation with culture medium, HBSS or milk, we wondered whether this could affect osteoclast formation. TRAP+ multinucleated, osteoclast-like cells developed in all co-cultures. Markedly increase in the

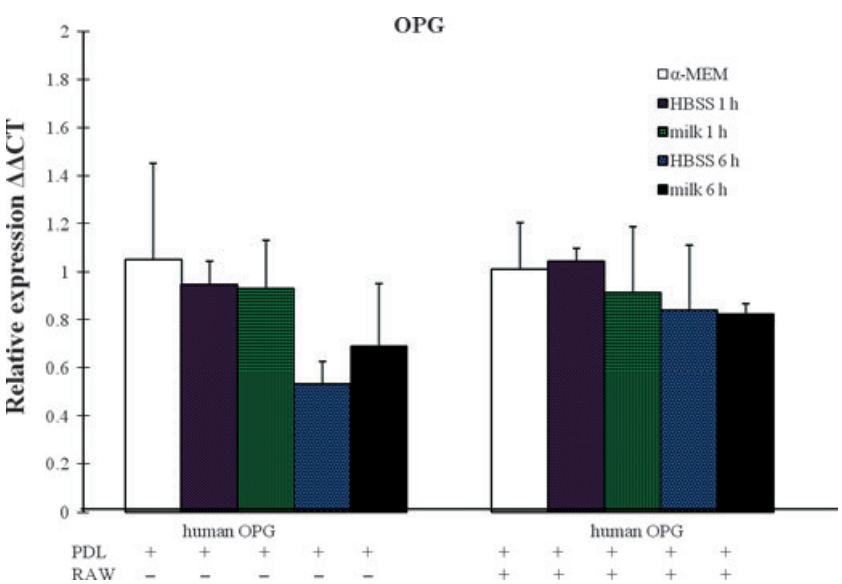

Fig. 1. Osteoclastogenesis-related gene osteoprotegerin expression in periodontal ligament cells was analyzed, respectively, after storage in modified Eagle's medium alpha, Hank's balanced salt solution, or milk for 1 or 6 h and followed by co-culturing with RAW 264.7 cells for additional 3 days ($^{\star}P < 0.05$, $^{\star\star}P < 0.01$). Data were presented as mean ± standard deviation (SD) from three independent experiments and each analyzed in triplicate.

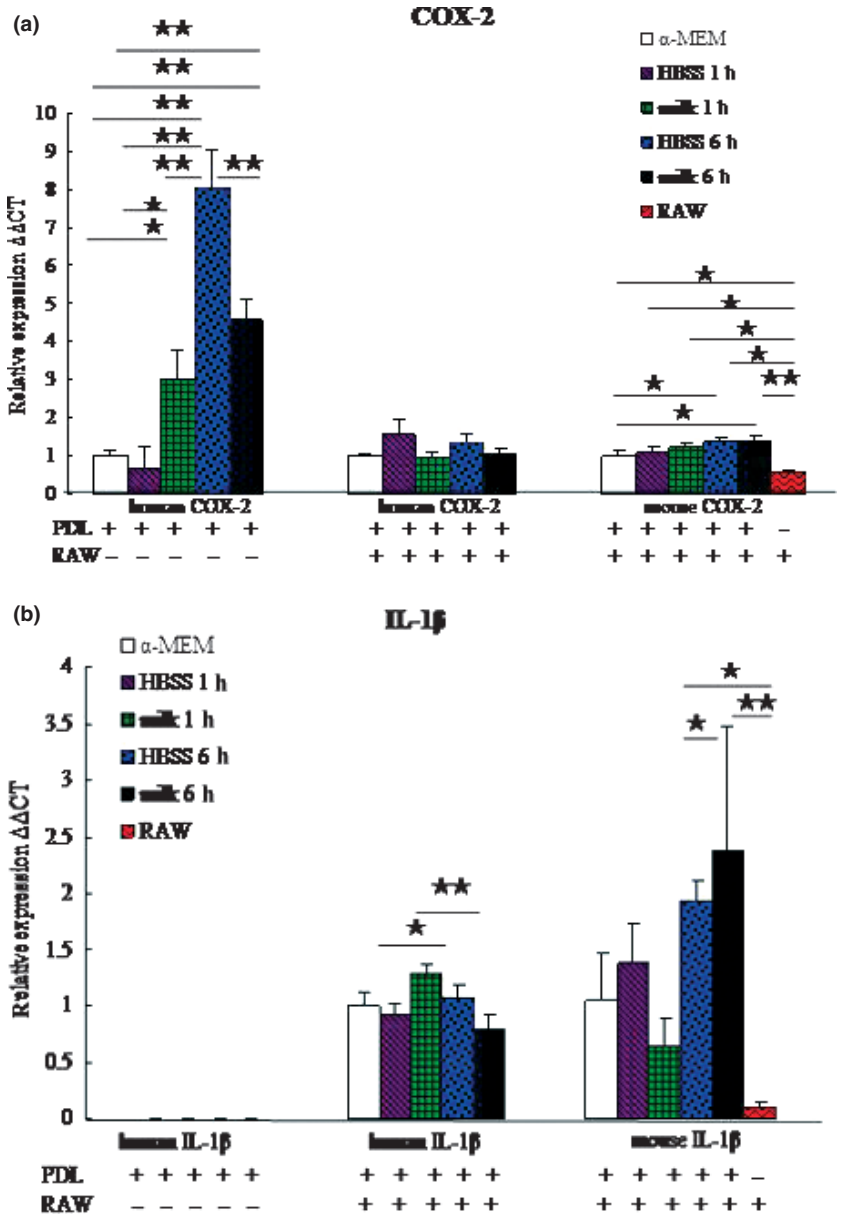

Fig. 2. Inflammatory cytokine genes, (a) COX-2 and (b) IL-1β, were analyzed by species-specific probes for human periodontal ligament cells in modified Eagle's medium alpha, Hank's balanced salt solution, or milk for 1 or 6 h and co-cultured with RAW 264.7 cells for additional 3 days. Data were presented as mean ± standard deviation (SD) from three independent experiments and each analyzed in triplicate. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$ for the difference between two groups linked by a horizontal line.

production was observed in co-culture groups with the PDL cells having been kept for 6 h in milk beforehand ($P < 0.05$) (Fig. 3).

Discussion

Many solutions have been studied as possible storage media for avulsed teeth. HBSS, fresh milk, and modified Eagle's medium (MEM) are among the most extensively examined and highly recommended solutions (3, 4). However, the effects of these storage media and storage time on maintaining the viability of PDL cells are still controversial (5). Furthermore, after storage in the storage media, the potential for PDL cells to induce osteoclastogenesis remains largely unknown.

In this study, we pretreated human PDL cells with HBSS, fresh milk, and α-MEM for 1 h or 6 h and then co-cultured them with osteoclastic precursor cells (murine RAW 264.7). The results showed that TRAP+ multinucleated, osteoclast-like cells developed in all co-cultures. Storage in milk for 6 h induced significantly more TRAP+ multinucleated, osteoclast-like cell formation than in α-MEM, HBSS, or 1 h in milk.

TRAP+ multinucleated, osteoclast-like cells developed without any exogenous osteoclast differentiation factor in all co-cultures, which was not only observed in

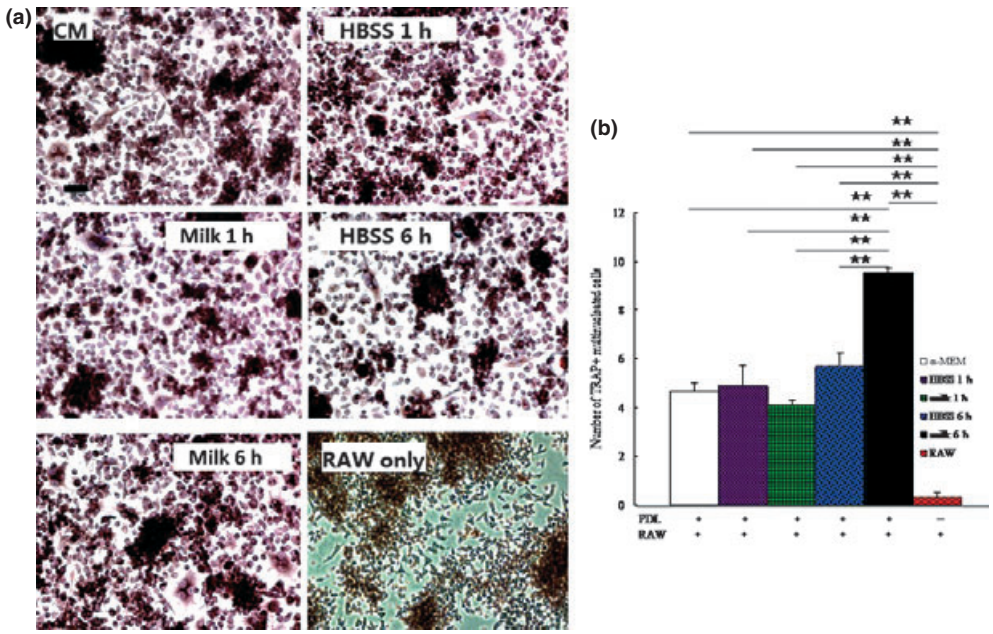

Fig. 3. (a) Storage media enhance the formation of TRAP+ multinucleated, osteoclast-like cells. After precultured with three storage media for 1 or 6 h, periodontal ligament cells were co-cultured with RAW 264.7 for 11 days. Cultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity (TRAP staining; magnification ×20, bar = 50 μm). (b) The number of multinucleated cells (with three or more nuclei) was counted and expressed as average number of TRAP+ multinucleated cells per three random fields in each well. Data were presented as mean ± SEM from the data obtained from three independent experiments. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$ for the difference between two groups linked by a horizontal line.

the present study but also by others (13, 17, 25). PDL cells showed a considerable influence on osteoclastic precursors' activity by direct cell–cell contact, consistent with the widely accepted view that signals from osteoblasts would induce osteoclast differentiation (26). However, the precise molecular mechanism of PDL cell-induced osteoclastogenesis is controversial. Lossdörfer et al. (17) showed that human PDL cells supported mouse osteoclastic differentiation via RANKL/OPG system in co-cultures with RAW 264.7 cells. Conversely, de Vries et al. (13) found that formation of TRAP+ multinucleated cells can occur in the absence of RANKL when human PDL cells were co-cultured with PBMC. In addition, Uchiyama et al. (25) demonstrated that multinucleated osteoclasts were formed when $CD14^{+}$ monocytes were co-cultured with human PDL cells, suggesting, at least in part, a RANKL-dependent mechanism, because OPG would strongly suppress but not completely prevent osteoclast formation. Our results showed a consistent expression of OPG but not RANKL, which indicated that osteoclastogenesis induced by PDL cells pretreated with storage media took place in a RANKL-independent manner.

It is well established that inflammatory root resorption and replacement resorption are important causes of failure after replantation of the avulsed tooth (27). Clinical studies have shown that inflammatory resorption has an early onset and progresses rapidly (28). Inflammatory cytokines such as IL-1β and PGE2 play an important role in the formation and activation of osteoclasts (29). For example, IL-1β as key mediators of immune and acute-phase inflammatory responses has been shown to be involved in bone resorption (30); PGE2 can induce osteoclast formation from RAW 264.7 cells co-cultured with mouse osteoblasts (31).

In the present study, COX-2 expression was detected soon after the PDL cells pretreated with a storage medium. 3 days after co-culture, murine COX-2 expression in co-cultures was greatly enhanced compared with RAW cells alone, which was in line with the higher number of osteoclast-like cell formation in co-cultures. COX-2 is an inducible enzyme, which is responsible for the production of PGE2. The finding indicated that PGE2 is involved in osteoclastogenesis under the present co-culture condition. Previous studies have shown that PGE2 stimulates osteoclastic bone resorption through the induction of RANKL expression in osteoblasts (31–33). Our data are contradictory to these previous results, which means that another mechanism may participate in inducing osteoclastogenesis.

Previously, Kim et al. (34) demonstrated that osteoclast formation occurred in mice lacking RANKL, where IL-1 and TNF-α could substitute for RANKL. Bloemen et al. (14) also showed that IL-1β may induce osteoclastogenesis at the very low level of RANKL expression for PDL cells co-cultured with PBMC. It has been demonstrated previously that the IL-1β action in osteoclastogenesis was mediated by PGE2 (24). Induction of osteoclast formation by IL-1β in mice can be largely blocked by indomethacin (35). Moreover, it was demonstrated that an important target for the effects of PGE2 on osteoclast formation was the osteoblast (26). In this study, IL-1β expression was detected as a result of co-culture, following COX-2 expression in PDL cells

precultured in the storage media. This suggested that IL-1β expression may be mediated by PGE2. After 3 days of co-culture, compared with RAW 264.7 monoculture, mouse IL-1β expression was up-regulated in the co-culture to a significant amount only for the groups that had been kept in milk for 6 h. The results may be due to the time frame of 3 days. Haynes et al. (24) have reported that when murine stromal cells were cultured with human PBMC, the levels of human and mouse IL-1β mRNA were highest at day 1 and then gradually declined. This suggested that the effects of storage media on IL-1β expression might have been more pronounced if we had analyzed it earlier. Further studies are needed to clarify the mechanism in more detail.

In conclusion, the present study showed that PDL cells can lead to osteoclastogenesis after being soaked in α-MEM, HBSS, or milk for 1 h or more. The effect would be more pronounced for storage in milk up to 6 h. The possible mechanism may be related to the secretion of COX-2 and IL-1β in the co-culture that mediates osteoclastogenesis in RANKL-independent manner. The ability to induce osteoclastogenesis may be considered as an important factor to consider the suitability of storage medium to maintain PDL viability after tooth avulsion.

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