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Effect of skimmed pasteurized milk and Hank's balanced salt solution on viability and osteogenic differentiation of human periodontal ligament stem cells

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Abstract – *Background/Aim*: The purpose of this study was to compare the effect of skimmed pasteurized milk and Hank's balanced salt solution on the viability and osteogenic differentiation potential of the human periodontal ligament stem cells at room temperature *in vitro*. *Material and methods*: Human periodontal ligament stem cells were obtained from extracted healthy third molars and conserved in skimmed pasteurized milk and Hank's balanced salt solution for 1, 2, and 4 h at room temperature to detect the viability of the cells and their osteogenic differentiation potential. *Results*: The efficacy of skimmed pasteurized milk on cell viability at 4 h was significantly higher than that of HBSS ($P < 0.05$), and cells stored in skimmed pasteurized milk showed significantly higher levels of mineralization than those in HBSS at 2 and 4 h ($P < 0.05$). *Conclusions*: Skimmed pasteurized milk was more effective than Hank's balanced salt solution in maintaining the viability and osteogenic differentiation potential of PDLSCs at room temperature *in vitro*.

Avulsion of permanent teeth is the most serious dental injury, resulting in severe damage to the periodontal ligament (PDL). The incidence of tooth avulsion is approximately 1–16% of all dental injuries (1). Studies have demonstrated that replantation of avulsed teeth occurs most frequently between 1 and 4 h after avulsion (2). Due to space and time limitations, replantation cannot always be carried out immediately. Therefore, a storage medium is often required to maintain the vitality of periodontal ligament cells during the extra-alveolar time (3, 4). An ideal storage medium should provide compatible pH, osmolarity, nutrition, and antibiotic properties, thus minimizing the risk of root resorption. It should not provoke immunologic reactions and should be available when dental trauma occurs. Unfortunately, such an ideal storage medium has not been discovered yet. A number of studies have examined various solutions as temporary storage media, including tap water, saliva, saline, milk, and Hank's balanced salt solution (HBSS) (5–18).

Tap water is not recommended as a storage medium as its hypotonicity leading to rapid cell lysis (5, 19). Saliva may be slightly more effective than tap water, but its non-physiologic osmolarity and the presence of microorganisms do not make it a suitable storage medium for avulsed teeth (6, 19, 20). Physiologic saline has been suggested as a short-term storage medium because of its physiologic osmolality (19, 21).

Hank's balanced salt solution (HBSS) has been recommended as the storage medium to maintain PDL cell viability for an extended period of time (up to 48 h) (19). Several studies have shown good results with HBSS (7, 16, 18, 22), but its widespread use is doubtful, because it may not be readily available in many occasions in which tooth avulsions are likely to occur.

Milk has been studied extensively (6, 7, 13, 16–19, 23–25) and has gained wide acceptance as a suitable medium for avulsed teeth. Milk has many advantages, such as low bacterial contamination, physiologic osmolality (230–270 mOsm Kg^{-1}), and almost neutral pH (6.5–6.8), and provides some nutrients (19) and growth factors to the cells (24, 25). In addition, skimmed pasteurized milk with its lower fat content may be more appropriate for maintaining PDL cell viability than whole milk (23). Numerous studies have demonstrated that its effectiveness can lost from 3 to as long as 120 h (7, 13, 17–19, 23). But there is no consensus regarding the effectiveness of milk as storage medium when compared with other media, mainly with HBSS (7, 13, 17).

As well known, the PDL contains heterogeneous cell populations that can differentiate into either cementum-forming cells (cementoblasts) or bone-forming cells (osteoblasts) (26). A recent study identified and characterized human PDL-derived mesenchymal stem cells as periodontal ligament stem cells (PDLSCs) for the first time (27). PDLSCs hold an important position in the PDL healing of replanted teeth, due to their capacity for self-renewal and multilineage differentiation (27). Although there are numerous studies on the effect of storage medium on PDL cells viability, the effect on PDLSCs differentiation potential has not been determined.

The objectives of this study were to compare the effectiveness of skimmed pasteurized milk and HBSS in maintaining PDLSCs viability and osteogenic differentiation potential at room temperature *in vitro*.

Materials and methods

Human PDLSCs were used in this study. Firstly, PDLSCs were isolated and identified. Secondly, the effect of skimmed pasteurized milk and Hank's balanced salt solution on viability and osteogenic differentiation of PDLSCs was assessed. Fig. 1 shows an overview over all procedures performed in our study.

Cell culture

PDLSCs were isolated from freshly extracted healthy third molars of the experimental individuals aged 18–30 years at Peking University School and Hospital of Stomatology, China, following approved guidelines set by the Health Science Center, Peking University (IRB00001052-11060).The isolation procedure was performed according to the method reported by Seo et al. (27), with minor modifications. Briefly, PDL was gently separated from the middle third of the root surface and digested in a solution of 3 mg ml^{-1} collagenase type I (Sigma, St Louis, MO, USA) and 4 mg ml^{-1} dispase (Sigma) at 37°C for 1 h. PDL samples from different individuals were pooled, and single-cell suspensions were obtained by passing the cells through a 70-μm strainer (Falcon, BD Labware, NJ, USA). To identify putative stem cells, single-cell suspensions (1×104–1×105 cells) were seeded into 100-mm culture dishes with growth medium and then incubated at 37°C in 5% carbon dioxide. Growth medium contained α-modified Eagle's medium (αMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mM glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Cells used in this study were at passages 1–4. Dental pulp stem cells (DPSCs) were isolated and cultured as previously described (28).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription (RT) with GoTaq polymerase (Promega, Madison, WI, USA). The primers used in PCR included scleraxis (SCX) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Primer sequences used in PCR are shown in Table 1. Amplification of GAPDH was used as an internal control.

Flow cytometry

Single-cell suspensions of PDLSCs (>1×10^6 cells) were washed and resuspended in phosphate-buffered saline (PBS). For indirect immunostaining, cells were incubated with 2.5 μg of STRO-1 antibody (R&D Systems, Minneapolis, MN, USA) or isotype-matched immunoglobulins (eBioscience, San Diego, CA, USA) for 20 min on ice. After washing, samples were incubated with PE-conjugated secondary antibody (R&D Systems) for 20 min on ice and then washed with PBS. For direct immunostaining, the cells were treated with 20 μl of PE-conjugated CD146 (BD Biosciences Pharmingen, San Jose, CA, USA) for 20 min on ice. After washing, all cells were analyzed with a FACS Calibur Flow cytometer (Becton Dickinson, Mountain View, CA, USA).

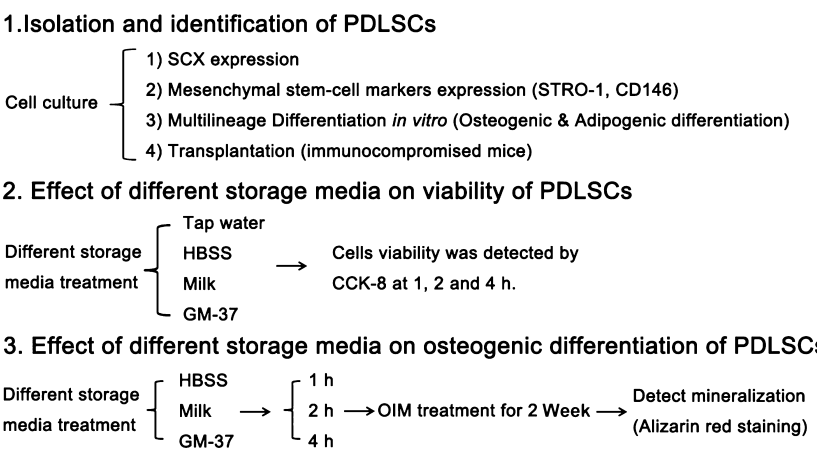

Fig. 1. Illustration depicting the isolation and identification of PDLSCs as well as different storage media treatment in PDLSCs. SCX: scleraxis, HBSS: Hank's balanced salt solution, Milk: skimmed pasteurized milk, GM-37: growth medium at 37°C, OIM: osteogenic induction medium.

Table 1. Primers used for reverse transcription-polymerase chain reactions

Genes	PCR primers sequence	Product
SCX	5′-CTGGCCTCCAGCTACATCTC-3′(F) 5′-CTTTCTCTGGTTGCTGAGGC-3′(R)	210 bp
GAPDH	5′-AGCCGCATCTTCTTTTGCGTC-3′(F) 5′-TCATATTTGGCAGGTTTTTCT-3′(R)	815 bp

Multilineage differentiation *in vitro*

We tested the *in vitro* multidifferentiation potential of the PDLSCs toward osteogenesis and adipogenesis as reported previously (29, 30).

Osteogenic differentiation
PDLSCs were seeded onto six-well plates, cultured to 80% confluence, and incubated in osteogenic induction medium (OIM) containing 10 nM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma), 0.1 mM L-ascorbic acid-2-phosphate (Sigma), 2 mM glutamine (Sigma), and 15% FBS for 2 weeks. After induction, cells were fixed and evaluated by staining with 2% alizarin red S (pH 4.2) (Sigma) to show calcium deposition.

Adipogenic differentiation
PDLSCs were seeded onto six-well plates, cultured to 80% confluence, and incubated with adipogenic induction medium (AIM) containing 1 μM dexamethasone, 10 mg l insulin (Sigma), 0.5 mM 3-isobutyl-1-methylxanthin (Sigma), 60 mM indomethacin (Sigma), 2 mM glutamine, and 15% FBS for 3 weeks. At the end of the culture period, cells were fixed, and lipid droplets were visualized by staining with 0.3% Oil Red-O (Sigma).

Transplantation

About 2.0×10^6 of *in vitro* expanded PDLSCs mixed with 40 mg of hydroxyapatite (HA) ceramic particles (Bio Osteon, Beijing, China) were transplanted subcutaneously into the dorsal surfaces of 8–10-week-old immunocompromised mice (CB-17/SCID; Vitalriver, Beijing, China) as previously described (27). These procedures were performed in accord with specifications of an approved animal protocol by the Health Science Center, Peking University (LA2011-045). The transplants were harvested after 8 weeks, fixed with 4% paraformaldehyde, decalcified with buffered 10% edetic acid (pH 8.0), and then embedded in paraffin. For histological analysis, 5-μm sections of implants were prepared and stained with hematoxylin and eosin (H&E) stain.

Experimental groups

Cultured PDLSCs were washed by phosphate-buffered saline (PBS) and then exposed to different experimental solutions. The storage media used in the experiment were as follows: tap water (pH 7.6), growth medium (pH 7.0), HBSS (Gibco; pH 7.2), and skimmed pasteurized milk (Sanyuan, Beijing, China; pH 6.8).

Microscopic examination and assessing cell viability by CCK-8 assay

PDLSCs (1×10^4 cells per well) were seeded in 96-well culture plates and incubated at 37°C in 5% carbon dioxide. At confluence, growth medium was removed, and the wells were filled with 100 μl of the different experimental solutions at room temperature (RT – approximately 25°C). After 1, 2, and 4 h, cells were washed with PBS for three times. The morphology of the PDLSCs in different groups was monitored during the experimental time using phase microscopy. Phase photomicrographs were obtained from a phase inverted microscope (Nikon Co., Tokyo, Japan). Then, 100 μl of growth medium containing 10 μl Cell Counting Kit-8 solution (CCK-8, Dojindo Laboratory, Kumamoto, Japan) was added to each well of the plate according to the manufacturer's instructions. The plates were incubated at 37°C for 2 h. Cell viability was determined by measuring the optical density at 450 nm on a spectrophotometer (Bio-Tek Instruments-Inc., Winooski, VT, USA). The cells stored in growth medium at 37°C (GM-37) were used as positive control. All experiments were repeated at least three times, and six wells were analyzed per experiment.

Osteogenic induction of PDLSCs after storage medium treatment

PDLSCs (4×10^5 cells per well) were seeded into 12-well culture plates, which were incubated at 37°C in 5% carbon dioxide. When PDLSCs were cultured to reach 80% confluence in growth medium, cells were washed with PBS for three times. Each well was added with 1 ml growth medium, HBSS, or skimmed pasteurized milk independently. After 1, 2, and 4 h, cells were washed with PBS three times. Then, the cells were treated with OIM for 2 weeks. The culture medium was changed every 3 days before alizarin red staining proceeded. The cells stored in growth medium at 37°C (GM-37) were used as positive control.

Alizarin red staining

After induction for 2 weeks, cells were fixed in 4% paraformaldehyde and stained with 2% alizarin red S to show calcium deposition. Calcium deposition was quantified by extracting the alizarin red staining with 100 mM cetylpyridinium chloride at room temperature for 3 h. The absorbance of the extracted alizarin red S stain was measured at 570 nm. All experiments were repeated at least three times, and six wells were analyzed per experiment.

Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments. Two-way ANOVA (analysis of variance) was used to test for statistical differences, and multiple comparisons were performed using Student–Newman–Keuls test (SNK test). Statistical significance was set at $P < 0.05$.

Results

Isolation and identification of PDLSCs

The PDL-derived cells could form adherent clonogenic cell clusters of fibroblast-like cells (Fig. 2a). *In vitro* expanded PDLSCs expressed the cell surface molecules of mesenchymal stem-cell markers STRO-1 (5.58%) and CD146 (64.98%) (Fig. 2f,g). Then, we assessed the expression level of scleraxis (SCX), a tendon-specific transcription factor. PDLSCs expressed higher SCX than DPSCs (Fig. 2e), which demonstrated that the isolated PDLSCs did originate from the PDL tissue. After induction, small, round alizarin red-positive nodules were formed, indicating calcium accumulation *in vitro* (Fig. 2b). In addition, after 4 weeks of induction of AIM, PDLSCs formed oil red-O-positive lipid droplets (Fig. 2c). Cementum/periodontal ligament-like structure was formed *in vivo* (Fig. 2d). The transplanted PDLSCs differentiated into cementoblast-like cells that formed cementum-like tissue on the surface of the carrier and formed dense collagen fibers connecting with newly formed cementum-like structures that mimicked physiologic attachment of Sharpey's fibers.

Morphological analysis

The PDLSCs cultured in growth medium at 37°C were shown in Fig. 2 (top left). Healthy cells have spindle-like morphology and remain attached to the plate, whereas unhealthy cells become rounded, shriveled, and detach from the plate. With the time prolonging, more and more cells incubated in HBSS shrunk and lost activity. In contrast, most cells stored in skimmed pasteurized milk kept almost the same spindle-like morphology as time passed (Fig. 3). The cells in tap water showed destruction of cell morphology in a low-osmolality condition within an hour (Fig. 3 bottom left). Many cells were floating around as a result of cell death.

Cell viability determined by CCK-8 assay

To determine the effect of storage medium on PDLSCs viability, CCK-8 assay was used. The mean absorbance values, which represent the PDLSCs viability for each tested medium and for storage periods, are shown in Fig. 4. The results indicated that HBSS, skimmed pasteurized milk, and growth medium performed significantly better than tap water (negative control) at all time periods ($P < 0.05$). The PDLSCs viability in skimmed pasteurized milk and HBSS was similar to GM-37 ($P > 0.05$) at 1 and 2 h. The efficacy of skimmed pasteurized milk at 4 h was significantly lower than that of control (GM-37) ($P < 0.05$), but still higher than that of HBSS ($P < 0.05$).

Mineralization of PDLSCs assessed by alizarin red staining

To test the effect of HBSS and skimmed pasteurized milk on mineralization of PDLSCs *in vitro,* we used alizarin red staining to determine the amount of matrix mineralization. Representative photomicrographs of mineralized PDLSCs subjected to different storage media for different time periods are shown in Fig. 5b. Results were expressed as absorbance among different groups (Fig. 5a). Overall, there were significant differences between HBSS and skimmed pasteurized milk groups at 2 and 4 h, but not for 1 h. Compared with GM-37, mineralization of HBSS group was significantly lower at 2 and 4 h ($P < 0.05$). However, skimmed pasteurized milk group only showed lower mineralization at 4 h ($P < 0.05$).

Discussion

Tooth avulsion is characterized by complete displacement of a tooth from its alveolar socket, and all fibers of the periodontal ligament (PDL) are damaged severely. The survival of the PDL cells especially peri-

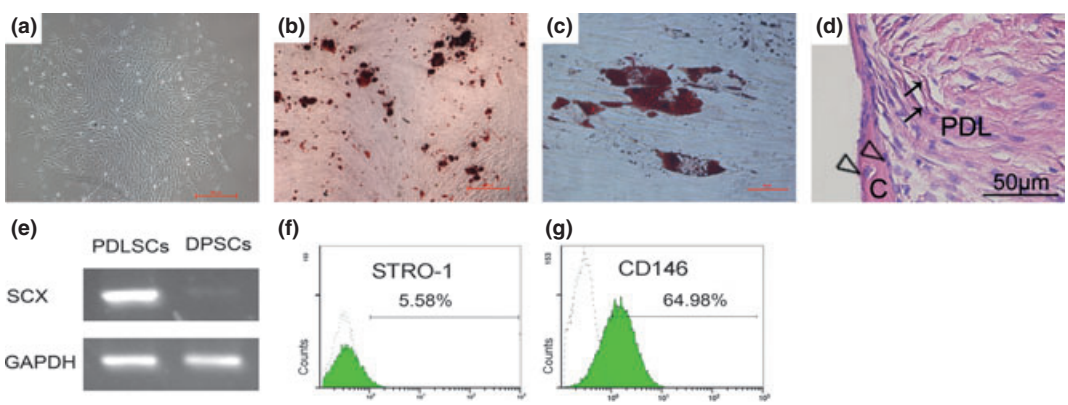

Fig. 2. Isolation and identification of PDLSCs (a) PDLSCs were capable of forming a single-colony cluster when plated at a low cell density. (b) When PDLSCs were cultured in osteogenic inductive medium for 2 weeks, mineralized nodules were found by Alizarin red staining. (c) Cultured PDLSCs formed Oil red O–positive lipid clusters after 4 weeks in adipogenic induction medium. (d) PDLSCs transplant showed a cementum-like structure (C)/PDL-like structures (PDL) *in vivo*, and differentiated into cementoblast-like cells (arrows) and cementocyte-like cells (triangles) that connected to newly formed collagen fiber (arrows). (e) RT-PCR showed that cultured PDLSCs expressed higher levels of scleraxis(SCX), a transcription factor specifically expressed in tendon cells, compared with DPSCs. GAPDH = glyceraldehyde phosphate dehydrogenase (control). (f, g) Flow cytometric analysis of *in vitro* expanded PDLSCs revealed expression of STRO-1 (5.58%) and CD146 (64.98%).

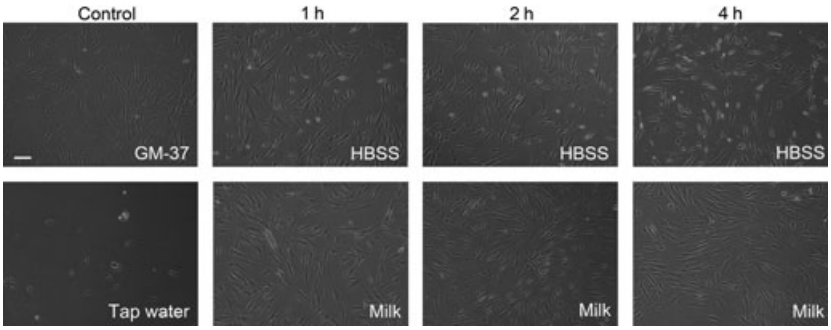

Fig. 3. Representative photomicrographs of PDLSCs obtained by transmission light microscopy after 1, 2 and 4 h of incubation under different storage medium at room temperature are shown. GM-37: growth medium at 37°C; Milk: skimmed pasteurized milk; HBSS: Hank's balanced salt solution. Bar 100μm.

odontal ligament stem cells (PDLSCs) on the root surface after injury plays a critical role in the periodontal healing of the replanted tooth. Previous studies usually used PDL cells or periodontal ligament fibroblast to test various storage media. In this study, we employed PDLSCs, which possessed the characteristics of clonogenicity, multipotency and self-renewal (27). As well known, PDL-derived mesenchymal stem cells could reflect the viability of cells responsible for periodontal healing more accurately.

In this study, we explored the effectiveness of different storage media in maintaining the viability and osteogenic differentiation potential of PDLSCs. Epidemiology of dental trauma has demonstrated that replantation of avulsed teeth occurs most frequently between 1 and 4 h after avulsion, and avulsed teeth were usually kept at room temperature(2). Given this, the longest time of incubation under different storage media was 4 h, and the present study was carried out at room temperature.

Generally, milk and HBSS have been recommended to be used as interim storage media for an avulsed tooth (6–9, 11, 13–20, 22, 23, 31–33). Milk is relatively cheap and can be easily obtained in places where avulsion commonly occurs (6–9, 13–20, 22, 23, 32, 33). Regular pasteurized milk has the advantage of lower bacterial content than bovine milk. Harkacz et al. (23) pointed out that the fat content of milk was found to have an effect on cell viability, and milk with lower fat content may be more appropriate for maintaining PDL cell viability than milk with higher fat content. Recently, Souza et al. (8) tested the effect of milk renewal on human periodontal ligament fibroblast viability *in vitro* and revealed that milk renewal promotes a negative effect. The authors speculated that the removal of growth factors and chemical cell mediators (proteins), by replacing the milk, may have disturbed cellular metabolism and the reversion of tetrazolium salts in formazan crystals.

HBSS, commercially available as Save-A-Tooth® (Save-A-Tooth, Inc., Pottstown, PA, USA), is a widely used standard solution recommended by the International Association of Dental Traumatology (IADT) as a storage medium for avulsed tooth (34). Its osmolality and pH are 270 to 290 mosmol kg^{-1} and 7.2, respectively (11). A shortcoming of HBSS is that it may not be available when tooth avulsion occurs.

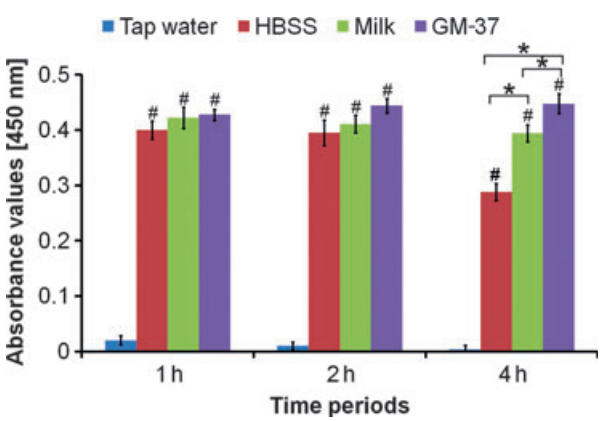

Fig. 4. Results for the viability of PDLSCs conserved at room temperature, obtained by the colorimetric CCK-8 method, expressed as absorbance values. GM-37: growth medium at 37°C, Milk: skimmed pasteurized milk, HBSS: Hank's balanced salt solution. #$P < 0.05$, compared with negative control (tap water). *$P < 0.05$, between two groups.

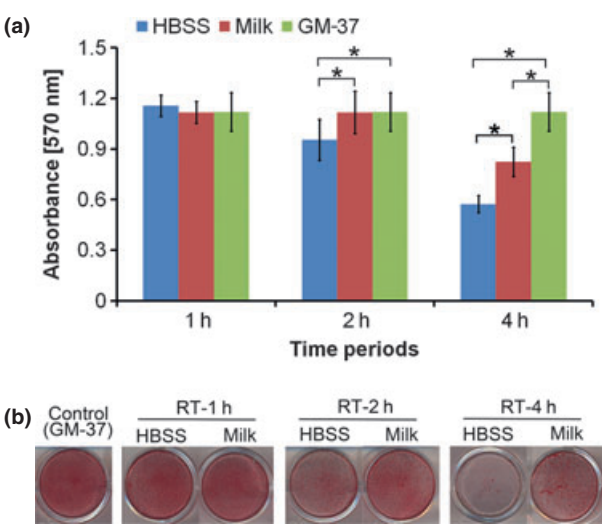

Fig. 5. Mineralization of PDLSCs assessed by alizarin red staining. Quantified data (a) representative photomicrographs (b) of alizarin red staining from PDLSCs after 1, 2 or 4 h of incubation under different storage medium and then cultured in osteogenic induction medium for 2 weeks. GM-37: growth medium at 37°C, Milk: skimmed pasteurized milk, HBSS: Hank's balanced salt solution. *$P < 0.05$.

In this study, PDLSCs viability was better in skimmed pasteurized milk than in HBSS at room temperature, in accordance with Casaroto et al. (14) and Souza et al. (10, 13). However, other authors obtained better results with HBSS (7, 16). The reason for the different results might be attributed to the type of milk and the temperature of HBSS employed.

We indicated that HBSS performed less effectively in maintaining osteogenic differentiation potential of PDLSCs than that of skimmed pasteurized milk. Recent studies showed that bovine milk contains growth factors, such as transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), and fibroblast growth factor (FGF), which are resistant generally to pasteurization (24, 25). These growth factors promote the proliferation and differentiation of periodontal ligament cells and periodontal regeneration (35–38). The remanent growth factors in skimmed pasteurized milk might contribute to the better preservation of the osteogenic differentiation potential of PDLSCs in our study. But the effect of bovine milk-derived growth factors on PDLSCs has not yet been established.

In conclusion, skimmed pasteurized milk was more effective than Hank's balanced salt solution (HBSS) in maintaining the viability and osteogenic differentiation potential of PDLSCs at room temperature *in vitro*. It seems that skimmed milk is a good storage medium for up to 4 h. Further *in vivo* studies are required to confirm this conclusion.

Acknowledgements

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