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ORIGINAL ARTICLE

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Effect of cDMEM media containing Ectoine on human periodontal ligament mesenchymal stem cell survival and differentiation

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Abstract

Background/Aim: Ectoine is an amino acid that can preserve osmotic balance and has more preservative activity than other osmoregulators. There are no published reports on the osmoregulators' effects on viability or differentiation of dental stem cells. The aim of this study was to investigate the effect of Ectoine as a storage media on the viability and differentiation potential of human periodontal ligament mesenchymal stem cells (hPDLMSCs).

Materials and Methods: hPDLMSCs were obtained from impacted third molar teeth. The cells were isolated, submitted to trilineage differentiation, and characterized by flow cytometer (FC). hPDLMSCs were incubated with different media containing Ectoine, complete DMEM (cDMEM), Ectoine+cDMEM, milk, and tap water for 2, 6, 12, and 24 h. The cells were analyzed by FC for viability, early-apoptosis, late apoptosis, and necrosis rates. Osteogenic and fibroblastic differentiation in hPDLMSCs were investigated by Alizarin red stain and vimentin expression.

Results: All treated groups showed significant decreases in cell viability after 2 h. Significant increases were detected in the number of dead cells between 2 and 12 h in all groups except the Ectoine+cDMEM group. The deposition of mineral matrix nodules was significantly higher in cells cultured with Ectoine+cDMEM compared with the other media. Higher vimentin expressions were detected in cells cultured with cDMEM and Ectoine+cDMEM media, respectively.

Conclusions: Ectoine added to cDMEM media, promoted cell survival plus osteogenic and fibroblastic differentiation of hPDLMSCs.

KEYWORDS
cell viability, ectoine, fibroblastic differentiation, osteogenic differentiation, periodontal ligament cells

1 | INTRODUCTION

Avulsion is the complete displacement of a tooth from its socket due to trauma.[1] The prognosis of an avulsed permanent tooth is mainly dependent on the procedures after the avulsion and the condition of the periodontal ligament cells at replantation.[1,2] The emergency management is important, and the avulsed tooth should be replanted as quickly as possible.[2] When immediate replantation of the avulsed tooth is not possible, the tooth needs to be stored in a transport medium that can maintain periodontal ligament cell viability.[3]

Periodontal ligament (PDL) cells have an important role in periodontal regeneration following periodontal tissue damage.[4,5] The stem cells and progenitor cells in a healthy tissue proliferate and migrate to the damaged area. These migrated cells differentiate into fibroblasts, osteoblasts, or cementoblasts and form bone and cementum.[4]

An ideal storage medium should be easily accessible, ready to use, and cheap. Besides these characteristics, it should have a long shelf life, neutral pH, osmolality, and antioxidant capacity.[6] Additionally, an ideal storage medium needs to maintain the viability and clonogenic capacity of the PDL and pulp cells. The most important factor for the storage medium is to have physiological osmolality, which should be around 290-300 mOsmol/kg.[7]

Osmotic solutes are nonionic, water-soluble, non-toxic, dipolar, uncharged, or anionic organic compounds that preserve cellular function and osmotic balance even at high concentrations. They have low molecular weight and protect proteins against denaturation.[8]

Ectoine is a natural osmoregulating amino acid produced by bacteria that can survive under unfavorable conditions. Ectoine is used for treatment of eye, nose, and skin dryness.[9] As a storage medium should have the same osmolality as the intracellular environment in treating avulsion cases, this study was designed to test Ectoine as a storage medium for avulsed teeth.

It was hypothesized that Ectoine might induce viability and differentiation potential of human periodontal ligament mesenchymal stem cells (PDLMSCs). The aim of this study was to investigate the effect of Ectoine as a storage medium on the viability and differentiation potential of human PDLMSCs.

2 | MATERIALS AND METHODS

The study was approved by the ethics committee of Istanbul University, Faculty of Dentistry (56/2015). Human periodontal ligament tissue explants were obtained from 10 freshly extracted impacted third molar teeth with informed consent (donors aged 18-25 years).

The PDL tissues were separated from the middle third of the root surface, minced using sterilized scalpels under a laminar hood for primary cell cultures. The tissue fragments were transferred to a 25-cm^2 flask with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco), and cultured at 37°C in a humidified atmosphere containing 5% CO_2 with medium exchange. The culture plates and flasks were monitored daily for cell growth with medium changes every 3 days. After 1-2 weeks of culture, when 70% to 80% confluence was reached, the cells were detached using 0.05% trypsin and passaged. The total cell number was determined using a hemocytometer. In this study, cells from all 10 donors and third-passage cells were used in each assay.

Phenotype of isolated periodontal ligament mesenchymal cells was determined by flow cytometry analysis for expression of CD146-FITC, CD45-FITC, CD29-APC, CD25-APC, CD105-PE, CD90-PE, CD73-PE, CD34-PE, CD28-PE, and CD14-PE markers. The cells were washed in cold PBS, and aliquots of 1×10^5 cells were labeled (30 min in the dark at 4°C) with monoclonal antibodies specific for human markers associated with mesenchymal and hematopoietic lineages. Flow cytometry was performed using a Flow Cytometer (BD, FACSCalibur, San Jose, CA, USA).

To determine the trilineage differentiation potential of *hPDLMSCs*, cells were seeded in 6-well plates (1×10^5 cells per well) cultured in growth medium until reaching 70-80% confluence. Growth medium was replaced with specific differentiation media [Osteogenic Stimulatory Kit (Human Stem Cell Technologies, USA), Adipogenesis Differentiation Kit (Gibco), and Chondrogenesis Differentiation Kit (Gibco)], to induce osteogenic, adipogenic, and chondrogenic differentiation of hPDLSCs. The media were replaced every 3 days. The calcium deposition and extracellular matrix mineralization were confirmed with Alizarin red staining (Sigma-Aldrich, USA) assay performed after four weeks of osteogenic treatment. The cells were incubated with chondrogenic differentiation media for 14 days and, chondrogenic differentiation of the cells was assessed via staining with Alcian Blue (Sigma-Aldrich). For adipogenic differentiation, the cells were incubated with adipogenic differentiation media for 14 days, and adipogenic differentiation of the cells was assessed with oil red O (Sigma-Aldrich) staining. Cells were photographed using a binocular microscope (Olympus, BH2-RFCA, Olympus, Tokyo, Japan).

The cells from the third passage were frozen at −196°C and kept in liquid nitrogen for subsequent experiments. hPDLMSC cells were retrieved from storage, thawed in a 37°C water bath, and cultured at 37°C in a humidified atmosphere containing 5% CO_2. 1×10^5 cells were seeded into 24-well culture plates, and the cells were allowed to attach to the plate overnight. The culture medium was removed after 24 h, and 2 mL of the five different experimental solutions was added for 2, 6, 12, and 24 h.

The experimental storage solutions used in the experiments were as follows:

1. Ectoine (Oly-Nal Ectoin Nasal Sprey; 2% Ectoine, 1% sea salt, water, phosphate buffer, Johnson &Johnson, Germany),
2. Milk (Pınar Milk, Low-fat (0,1%), cow milk, UHT processed, Long shelf life, Yasar Inc, Turkey),
3. Ectoine+cDMEM (1/3 Ectoine+2/3 complete DMEM, 10% FBS (fetal bovine serum),%1 Penicillin / streptomycin containing DMEM (Dulbecco's modified Eagles Medium, Gibco),
4. cDMEM
5. Tap water (negative control group).

All groups were evaluated by cell viability (flow cytometry analysis), osteogenic differentiation (Alizarin red staining), and fibroblastic differentiation potential (flow cytometry analysis and morphological investigation).

To determine the levels of viability, apoptosis, and necrosis, an Annexin V-FITC Apoptosis Detection Kit (eBioscience, BD Biosciences, San Jose, CA, USA) was used at 2, 6, 12, and 24 h following exposure of the cells to the test solutions. 1×10^5 cells were

seeded in 24-well plates and incubated after adding 1 mL of the culture medium. After 24 h, the culture medium was removed, and 1 mL of the five different experimental solutions was added for 2, 6, 12, and 24 h. At the end of each observation period (2, 6, 12, and 24 h), cells were harvested by centrifuging at 1500 rpm for 5 min, washed once in PBS, and stained with the eBioscience kit. The viable, apoptotic, and necrotic cell populations were determined by flow cytometry. Markers for apoptosis (annexin V, AV) or necrosis (propidium iodide; PI) were assayed. The percentages of viable (AV^-/PI^-), early apoptotic (AV^+/PI^-), late apoptotic (AV^+/PI^+), and necrotic (AV^-/PI^+) hPDLMSCs were determined. The results were quantified using CellQuest software with FCS (Becton-Dickinson). A total of 40 000 cells were analyzed. All experiments were performed in triplicate wells for each condition and repeated at least twice. 3×10^5 cells were seeded in 6-well plates and incubated after adding 2 mL of the culture medium to determine the osteogenic differentiation. When cells had grown to confluence, the culture medium was removed, and 2 mL of the five different experimental solutions was added. At the end of each observation period, the culture media were replaced with specific differentiation media (StemPro Osteogenesis Differentiation Kit, Life Technologies, Toronto, ON, Canada). The cells were washed once in PBS, and then 2 mL of differentiation media was added to the wells. The media were replaced three times a week. The cells were fixed in 2% paraformaldehyde neutral buffer solution. Alizarin red S method performed for observation of mineralization nodules after four weeks of osteogenic treatment. The samples were observed using a binocular microscope (Olympus, BH2-RFCA).

hPDLMSCs were seeded at a concentration of 3×10^5 in 6-well plates containing 2 mL of the culture medium. When confluent, the medium was aspirated from the wells, and then 2 mL of the five different experimental solutions was added to each well. The cells were washed once in PBS, then 2 mL of fibroblastic differentiation culture medium was added to the wells at the end of each observation period (2, 6, 12, and 24 h).

To induce fibroblastic differentiation, the cells were washed once in PBS, and then they were treated with the 1 ng/mL basic fibroblast growth factor (bFGF, BioSource, Thermo Fisher, Waltham, MA, USA)-containing differentiation medium (DMEM supplemented 10% FBS and 1% penicillin-streptomycin). After 7 days, 5 ng/mL transforming growth factor-β (TGF-β) (Gibco Invitrogen, NY, USA) was added to the differentiation media. The medium was changed three times a week. The cells were cultured for 3 weeks. At the end of this period, the culture medium was removed, cells were trypsinized and washed once in PBS. The cells were transferred into polystyrene tubes. After centrifuging at 1500 rpm for 5 min, the supernatant was removed, and 1 μL of vimentin antibody (Thermo Fisher, NB500-512PE) was added to the tubes, and the labeled cells were analyzed using flow cytometry (BD, FACSCalibur).

Statistical analysis was performed using SPSS Statistics 22 (IBM SPSS Inc., Turkey). The Shapiro-Wilks test was used to check the normality of the data. The Kruskal-Wallis test was used to study differences among groups in non-normally distributed data (95% CI, $P < .05$). Friedman's test was used to compare the distributions of the two or more non-normally distributed quantitative variables. The Kruskal-Wallis test was used as a post hoc test to study differences

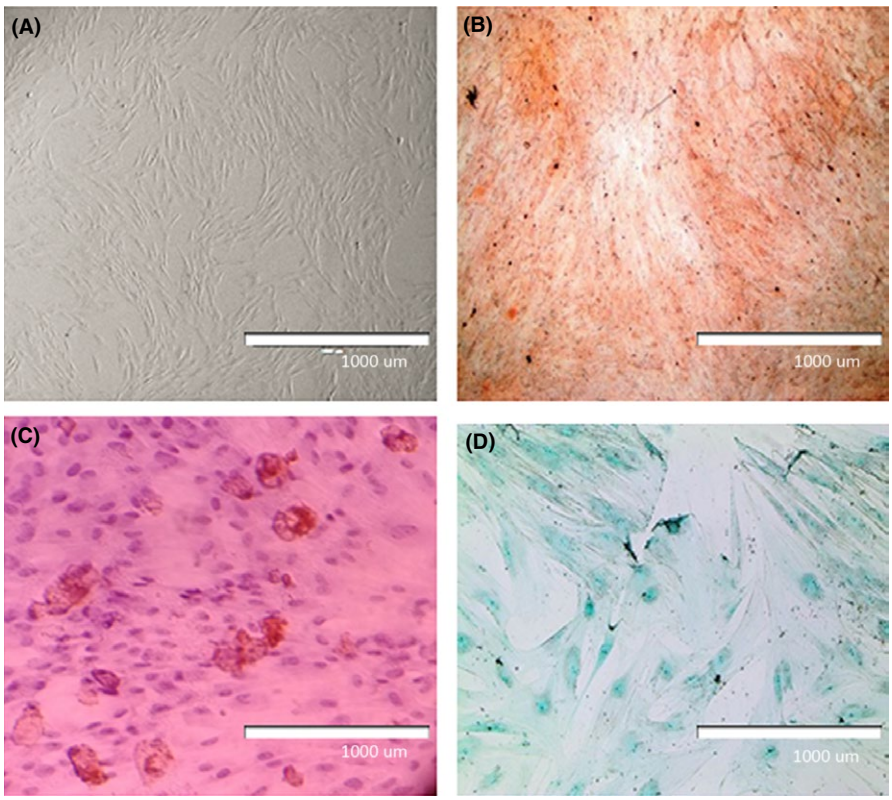

FIGURE 1 A, PDLSCs on third passage exhibited long processes and displayed a spindle-shaped fibroblast-like morphology, (×10). B, Differentiation of PDLMSCs into osteoblasts was confirmed by Alizarin red stain, (×40). C, Differentiation of PDLMSCs into adipocytes was confirmed by oil red O, (×40). D, Differentiation of PDLMSCs into chondrocytes was confirmed by Alcian blue staining, (×40)

among groups in non-normally distributed data (95% CI). $P < .05$ was considered as the significance level. Spearman's correlation test was used for intragroup analyses with the significance level $r > .4$.

3 | RESULTS

Adhesion of cells was detected between two and three days and primary cell cultures reached a confluence of 70-80% after 1-2 weeks. Third-passage cells exhibited long processes and showed a spindle-shaped fibroblast-like morphology (Figure 1A).

HPDLSCs were cultured in osteogenic, adipogenic, and chondrogenic conditions to assess the multipotent capability. Differentiation into osteoblasts was confirmed by Alizarin red (Figure 1B). Osteogenic differentiation was demonstrated by the presence of mineralized nodules. The extracellular calcium deposits were stained in bright orange-red color. Differentiation into adipocytes was confirmed by oil red O (Figure 1C).

Adipogenic differentiation was demonstrated by the presence of fat vacuoles and red-stained lipid droplets in PDLSCs after 2 weeks of induction. Differentiation into chondrocytes was confirmed by Alcian blue staining (Figure 1D). Chondrogenic differentiation was demonstrated by the presence of proteoglycans and chondrocyte-like cells.

The high expression of surface markers related to mesenchymal stem cells (CD 105, CD 146, CD 90, CD 29, and CD 73) was detected by flow cytometry. The cells were negative for hematopoietic stem cell markers, such as CD45, CD34, CD25, CD28, and CD14 (Figure 2).

After exposure to the different experimental solutions at different time courses, the percentages of viable hPDLMSCs were represented in Table 1.

All groups demonstrated decreased cell viability in a time-dependent manner. A statistically significant difference was found in the percentage of viable cells at 2 and 24 h in all experimental groups ($P < .05$). There was a significant decrease in the percentage of viable cells for each observation period in the tap water-containing group, when compared with other experimental solutions ($P < .05$). The higher percentage of viable cells was detected in cells cultured with Ectoine+cDMEM medium at 2, 6, and 12 h ($P < .05$).

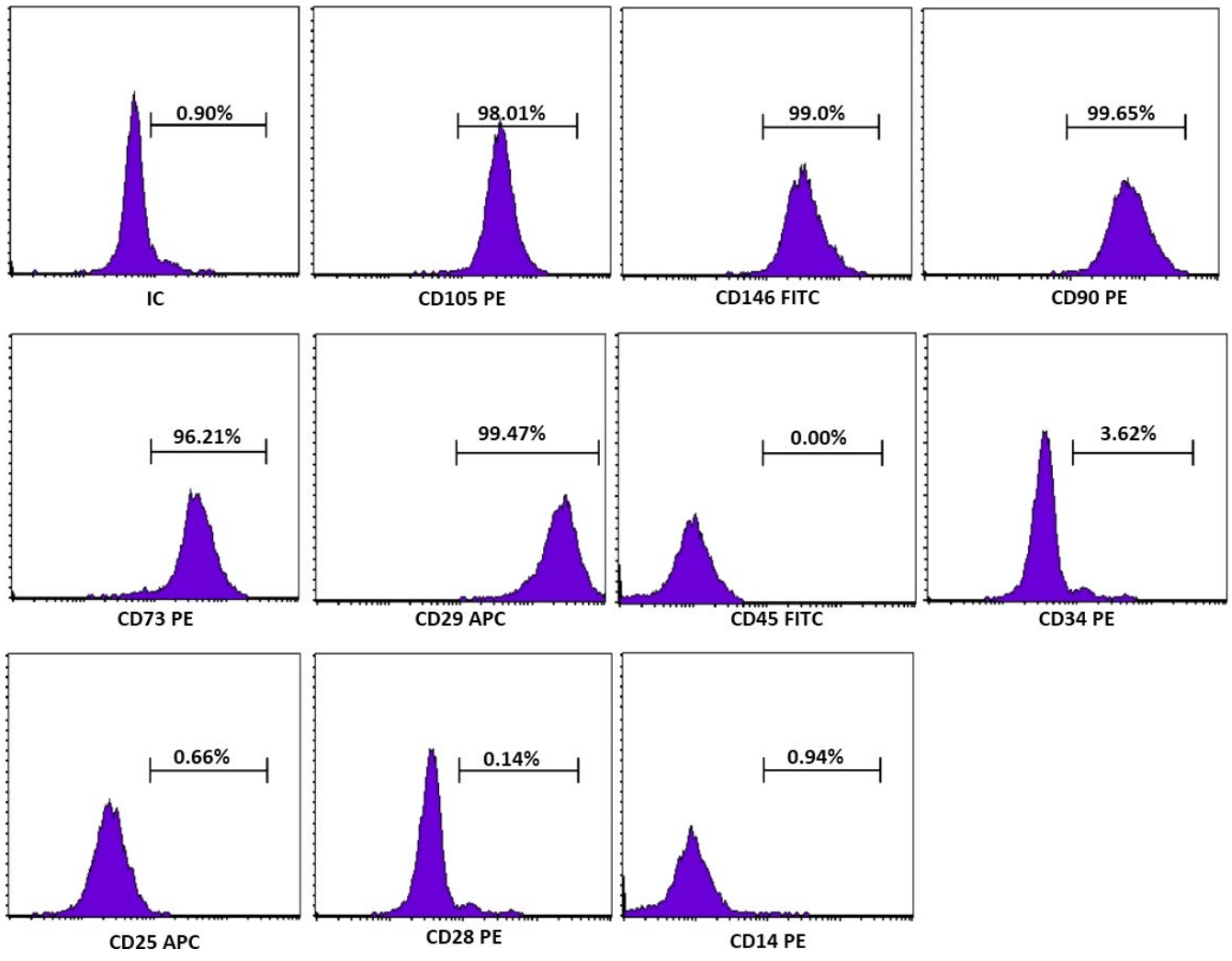

FIGURE 2 Determination of the immunophenotypic characteristics of PDLMSCs by flow cytometry

TABLE 1 The percentages of viable hPDLMSCs after exposure to the different experimental solutions for different time intervals. The data represent means ± SD. All experiments (n:6) were performed in triplicate wells for each condition and repeated at least twice

Cell Viability (%)					
	2 h Mean ± SD (Median)	6 h Mean ± SD (Median)	12 h Mean ± SD (Median)	24 h Mean ± SD (Median)	P^1
Ectoine (1)	69.64 ± 10.83 (71.54)	32.22 ± 14.57 (31.58)	17.62 ± 11.17 (15.82)	12.25 ± 5.41 (11.07)	(2-24 h)* P = .01
Milk (2)	73.32 ± 8.33 (73.80)	54.74 ± 4.98 (55.36)	69.02 ± 17.11 (69.10)	31.47 ± 1.06 (31.52)	(2-24 h, 12-24 h)* P = .01
Ectoine+cDMEM (3)	80.27 ± 1.33 (80.36)	71.41 ± 1.06 (71.48)	74.09 ± 14.09 (73.68)	34.86 ± 3.80 (35.23)	(2-24 h)* P = .01
cDMEM (4)	79.77 ± 6.49 (80.39)	63.39 ± 4.84 (63.81)	70.70 ± 20.94 (70.59)	36.70 ± 9.68 (37.01)	(2-24 h)* P = .04
Tap water (5)	14.67 ± 10.40 (13.99)	10.67 ± 10.95 (9.56)	6.06 ± 3.21 (6.06)	8.48 ± 2.49 (8.50)	P = .47
P^2	(4-5, 3-5)* P = .005	(4-5, 1-3, 3-5)* P = .000	(2-5, 4-5, 3-5)* P = .001	(4-5, 3-5)* P = .001	

P^1: Friedman test represents a significant difference between groups. P^2: Kruskal-Wallis test represents a significant difference among groups. *(P < .05).

TABLE 2 The percentages of early apoptotic hPDLMSCs after exposure to the different experimental solutions for different time intervals. The data represent means ± SD. All experiments (n:6) were performed in triplicate wells for each condition and repeated at least twice

Early Apoptosis (%)					
	2 h Mean ± SD (Median)	6 h Mean ± SD (Median)	12 h Mean ± SD (Median)	24 h Mean ± SD (Median)	P^1
Ectoine (1)	9.72 ± 3.96 (10.06)	23.29 ± 17.42 (22.30)	24.41 ± 7.42 (24.33)	26.78 ± 5.75 (26.76)	(2-24 h, 2-24 h)* P = .05
Milk (2)	9.80 ± 5.85 (9.37)	13.17 ± 4.25 (12.47)	15.41 ± 8.93 (15.43)	18.52 ± 3.24 (18.37)	(2-24 h, 2-24 h)* P = .01
Ectoine+cDMEM (3)	9.67 ± 3.36 (10.21)	11.96 ± 2.60 (12.57)	18.04 ± 14.63 (18.32)	27.45 ± 3.97 (26.66)	P = .12
cDMEM (4)	8.63 ± 1.44 (8.52)	14.89 ± 1.93 (14.17)	21.91 ± 20.95 (21.03)	27.45 ± 2.48 (28.00)	P = .06
Tap water (5)	25.01 ± 5.69 (25.94)	26.72 ± 13.82 (26.77)	43.18 ± 29.36 (43.05)	25.41 ± 3.20 (25.36)	P = .67
P^2	(4-5)* P = .02	P = .083	P = .456	(2-3)* P = .03	

P^1: Friedman test represents a significant difference between groups. P^2: Kruskal-Wallis test represents a significant difference among groups. *(P < .05).

The percentages of early apoptotic, late apoptotic, and necrotic cells are represented in Tables 2, 3, and 4, respectively. Milk, Ectoine, Ectoine+cDMEM, and cDMEM all had a low percentage of early apoptotic cells, and no significant difference (P > .05) was found among these treatment groups after 2 h. A higher percentage of early apoptotic cells was detected in the tap water-containing group after 2 h (P = .02). On the other hand, a significant increase in the percentage of early apoptotic cells was observed after 2 h for both the Milk and the Ectoine groups (P < .05).

All groups demonstrated increased late apoptotic cell rates in a time-dependent manner (P < .05). The tap water-containing group represented the higher percentage of late apoptotic cells for each observation period. A significant increase in the percentage of late apoptotic cells was observed after 2 h for the Ectoine group (P = .01).

Ectoine+cDMEM had the lowest percentage of necrotic cells among the treatment groups after 2 h (P < .05). The percentages of necrotic cells in the Ectoine and the tap water-containing groups were significantly higher (P < .05) when compared with the Ectoine+cDMEM group at 2 h (P < .05). A significant increase in the percentage of necrotic cells was observed at 24 h for the Milk, cDMEM, and Ectoine+cDMEM groups (P < .05).

TABLE 3 The percentages of late apoptotic hPDLMSCs after exposure to the different experimental solutions for different time intervals. The data represent means ± SD. All experiments (n:6) were performed in triplicate wells for each condition and repeated at least twice. Friedman test represents a significant difference between groups ($P < .05$). Kruskal-Wallis test represents a significant difference among groups *($P < .05$).

Late Apoptosis (%)					
	2 h Mean ± SD (Median)	6 h Mean ± SD (Median)	12 h Mean ± SD (Median)	24 h Mean ± SD (Median)	P^1
Ectoine (1)	13.39 ± 5.14 (13.17)	35.96 ± 10.19 (34.59)	46.88 ± 17.24 (47.00)	46.53 ± 4.23 (45.96)	(2-24 h, 2-12)* $P = .01$
Milk (2)	10.61 ± 7.03 (10.10)	22.03 ± 0.36 (22.06)	13.04 ± 7.22 (12.97)	40.61 ± 6.54 (40.52)	(2-24 h, 12-24 h)* $P = .01$
Ectoine+cDMEM (3)	8.06 ± 4.75 (6.40)	12.72 ± 1.70 (12.66)	5.38 ± 2.39 (5.38)	26.09 ± 2.32 (25.32)	(2-24 h, 12-24 h)* $P = .01$
cDMEM (4)	8.39 ± 5.69 (5.79)	12.11 ± 4.07 (11.24)	4.63 ± 1.01 (4.85)	19.54 ± 4.09 (20.96)	(12-24 h)* $P = .02$
Tap water (5)	46.62 ± 17.43 (47.10)	56.74 ± 22.70 (56.89)	41.23 ± 23.88 (41.58)	47.97 ± 1.27 (47.33)	$P = .15$
P^2	(3-5, 4-5)* $P = .007$	(1-4, 3-5, 4-5)* $P = .000$	(1-4, 1-3, 4-5)* $P = .002$*	(1-4, 4-5)* $P = .000$*	

TABLE 4 The percentages of necrotic hPDLMSCs after exposure to the different experimental solutions for different time intervals. The data represent means ± SD. All experiments (n:6) were performed in triplicate wells for each condition and repeated at least twice. Friedman test represents a significant difference between groups ($P < .05$). Kruskal-Wallis test represents a significant difference among groups *($P < .05$).

Necrotic cells (%)					
	2 h Mean ± SD (Median)	6 h Mean ± SD (Median)	12 h Mean ± SD (Median)	24 h Mean ± SD (Median)	P^1
Ectoine (1)	9.91 ± 4.77 (9.76)	8.54 ± 9.43 (8.89)	11.08 ± 4.50 (12.24)	14.42 ± 6.23 (15.82)	$P = .35$
Milk (2)	6.27 ± 0.91 (6.61)	10.06 ± 1.17 (9.76)	2.53 ± 1.30 (2.45)	9.42 ± 7.07 (7.90)	(6-12 h)* $P = .01$
Ectoine+cDMEM (3)	2.01 ± 0.87 (2.16)	3.91 ± 1.81 (3.90)	2.49 ± 1.70 (1.81)	11.61 ± 2.99 (11.75)	(2-24 h, 12-24 h)* $P = .01$
cDMEM (4)	3.22 ± 1.37 (3.39)	9.60 ± 3.31 (11.26)	2.76 ± 1.11 (3.48)	16.30 ± 10.91 (14.91)	(12-24 h)* $P = .04$
Tap water (5)	13.70 ± 9.03 (12.97)	5.86 ± 3.50 (5.57)	9.54 ± 13.78 (4.91)	18.14 ± 6.73 (18.77)	$P = .32$
P^2	(1-3, 3-5)* $P = .006$	$P = .098$	$P = .065$	$P = .295$	

The results of early apoptotic, late apoptotic and necrotic cells were combined and shown as total cell death in Table 5.

All groups demonstrated increased total cell death rates after 2 h ($P < .05$). Ectoine+cDMEM containing group showed the lowest total cell death rates among the treatment groups after 2, 6, and 12 h ($P < .05$). The tap water-containing group represented the highest total cell death rates for each observation period.

The results confirmed that the number of viable hPDLMSCs was negatively correlated with the number of dead hPDLMSCs ($r > .4$).

The mineralization of hPDLMSCs was qualitatively assessed by Alizarin red staining. hPDLMSCs without extracellular calcium deposits (undifferentiated cells) were slightly reddish, whereas cells with extracellular calcium deposits (differentiated/mineralized cells) were bright orange-red. The osteogenic differentiation of hPDLMSCs into osteoblasts was confirmed by intense staining for Alizarin red staining. The nodular aggregates of cells stained with Alizarin red demonstrated that the deposits observed were calcified. Images of PDLMSCs that were transferred to the osteogenic differentiation environment after incubating in different storage media for 2, 6, 12, and 24 h are shown in Figures 3-7, respectively.

The lowest amount of intracellular calcium deposition and many apoptotic cells was observed in the tap water-containing group. Osteoblast-like cells and calcified nodule-like structures

TABLE 5 The percentages of total cell death in hPDLMSCs after exposure to the different experimental solutions for different time intervals. The data represent means ± SD. All experiments (n:6) were performed in triplicate wells for each condition and repeated at least twice. Friedman test represents a significant difference between groups ($P < .05$). Kruskal-Wallis test represents a significant difference among groups *($P < .05$).

Total cell death (%)					
	2 h Mean ± SD (Median)	6 h Mean ± SD (Median)	12 h Mean ± SD (Median)	24 h Mean ± SD (Median)	P^1
Ectoine (1)	33.00 ± 9.38 (29.00)	67.40 ± 14.86 (68.00)	82.40 ± 10.74 (84.00)	87.40 ± 5.59 (89.00)	(2-12 h, 2-24 h)* $P = .011$
Milk (2)	26.40 ± 8.47 (27.00)	45.20 ± 5.07 (44.00)	30.80 ± 17.12 (30.00)	68.60 ± 1.67 (69.00)	(2-12 h)* $P = .007$
Ectoine+cDMEM (3)	19.80 ± 1.30 (20.00)	28.80 ± 1.30 (29.00)	25.60 ± 13.67 (26.00)	65.20 ± 3.27 (65.00)	(2-24 h, 12-24 h)* $P = .007$
cDMEM (4)	20.40 ± 6.58 (20.00)	36.40 ± 4.83 (36.00)	29.60 ± 20.55 (30.00)	63.20 ± 9.76 (63.00)	(2-24 h, 12-24 h)* $P = .028$
Tap water (5)	85.00 ± 10.58 (86.00)	89.20 ± 10.85 (90.00)	94.00 ± 3.61 (94.00)	91.60 ± 2.30 (92.00)	$P = .472$
P^2	(3-5, 4-5, 2-5)* $P = .002$	(3-5)* $P = .000$	(3-5, 1-3, 4-5, 2-5)* $P = .001$	(4-5, 3-5)* $P = .001$	

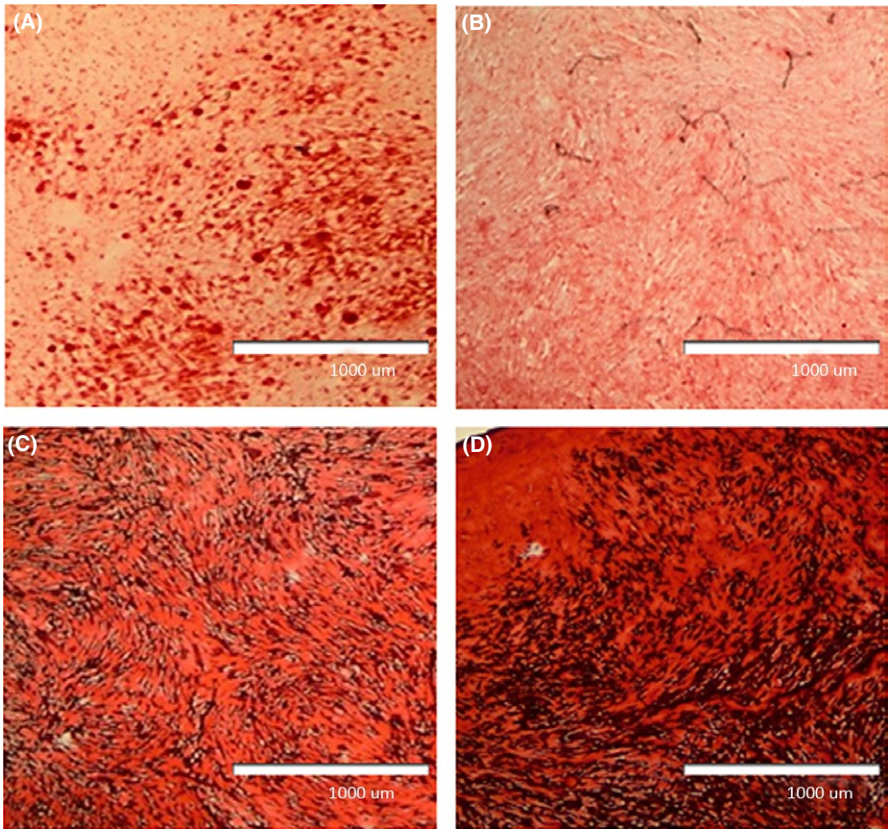

FIGURE 3 Alizarin red staining images of PDLMSCs that were transferred to the osteogenic differentiation environment after incubation in Ectoine for different time intervals. (A) 2 h; (B) 6 h; (C) 12 h; (D) 24 h

were observed only in the 2-h samples of the Ectoine group. Some apoptotic cells and irregular calcium deposits were observed in the Ectoine group over time.

The hPDLMSCs were slightly stained with Alizarin red in the milk group, but some deposits were observed in calcified nodule-like structures. The cells differentiated into osteoblast-like cells in the cDMEM and Ectoine+cDMEM groups for all time intervals.

The fibroblastic differentiation of hPDLMSCs was confirmed by vimentin expression and morphological investigation. The vimentin

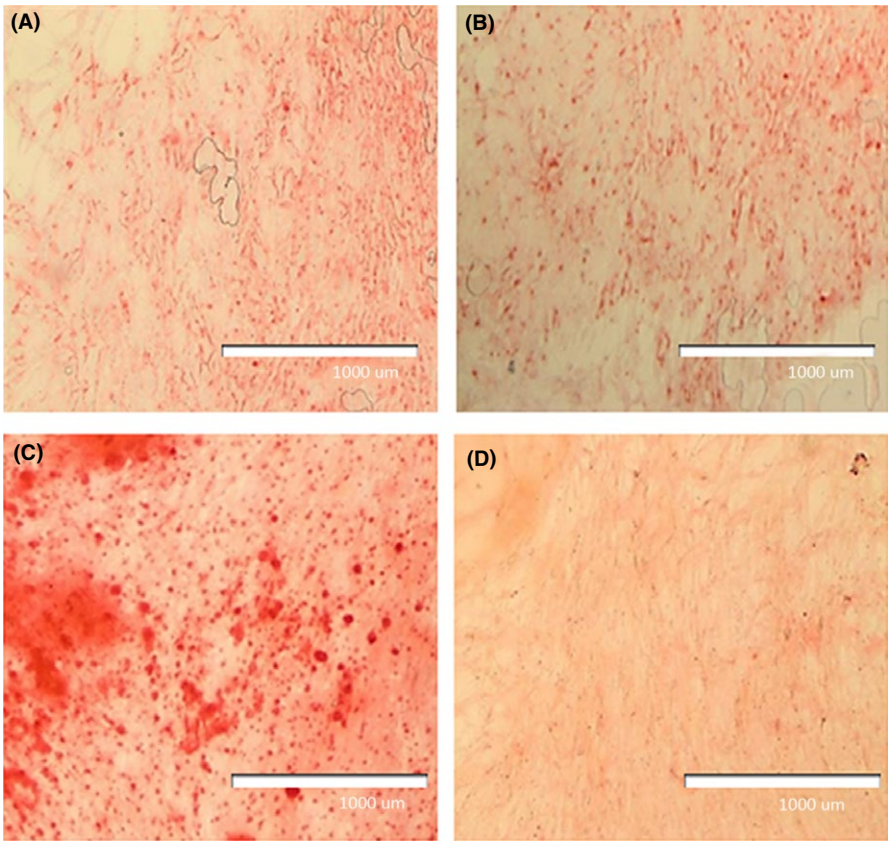

FIGURE 4 Alizarin red staining images of PDLMSCs that were transferred to the osteogenic differentiation environment after incubation in Milk for different time intervals. (A) 2 h; (B) 6 h; (C) 12 h; (D) 24 h

expression of hPDLMSCs for each experimental group is shown in Figure 8.

The highest vimentin expression was observed in the cDMEM group and later in the Ectoine+ cDMEM group. The lowest levels were determined in the tap water, Ectoine, and milk groups, respectively.

4 | DISCUSSION

Mesenchymal stem cells (MSCs) can be obtained from a variety of different tissues.[10] MSCs show fibroblast-like morphology, can differentiate into different lineages, and carry some specific cell surface markers.[10,11] The presence of MSCs can be demonstrated with the negativity (CD14, CD34, and CD45) and positivity (CD105, CD73, and CD90) of some hematopoietic stem cell markers.[12,13] Stem cells obtained from tooth pulp and PDL tissue are multipotent mesenchymal stem cells having high proliferation, regeneration, and differentiation capacity.[10]

In this study, hPDLMSCs were characterized by detecting specific surface antigens of cultured cells. The cells isolated from PDL tissue showed a fibroblast-like structure morphologically and also strongly expressed MSC markers immunophenotypically. It was also shown that these cells were able to show trilineage differentiation, and their MSC characteristics were proven.

The viability of periodontal ligament cells is essential for the clinical healing of replanted avulsed teeth.[14-21] This can be achieved by immediate replantation or by placing the tooth in a transport medium that is able to preserve the viability, mitogenicity, and clonogenic capacity of the injured periodontal ligament cells and their progenitors.[22,23]

In studies regarding the storage conditions for avulsed teeth, a variety of different experimental setups have been tested to find an ideal storage medium.[14-21]

Considering the ability to preserve cellular functions and osmotic balance, this study evaluated the potential of Ectoine as a storage medium. The effects of Ectoine on the viability and the differentiation potential of hPDLMSCs were compared with milk and cDMEM.

In storage media having appropriate osmolality and pH, the cells can preserve their normal physiology (related to cell homeostasis system), and nutrients in the storage media have less effect on cell viability in the 1-h time interval.[6]

Milk contains many amino acids, carbohydrates, vitamins, and growth factors, plus it has physiological osmolality.[24,25] These nutrients provide an efficient environment for cell growth.[24] It has been shown that milk is effective in maintaining the viability of PDL cells for 1-6 h.[24-27] Even though milk is better than water and saline as a storage medium, it has been reported that milk had low capacity to preserve the morphological integrity of PDL cells.[24] Additionally, the risk of bacterial growth is also high in milk.[28]

The results of this study demonstrated decreased viable cell rates in all groups in a time-dependent manner. Milk increased the number of viable hPDLMSCs for 12 h, but the cell number decreased to approximately 50% after 24 h. cDMEM provided an ideal environment for cell growth and survival.

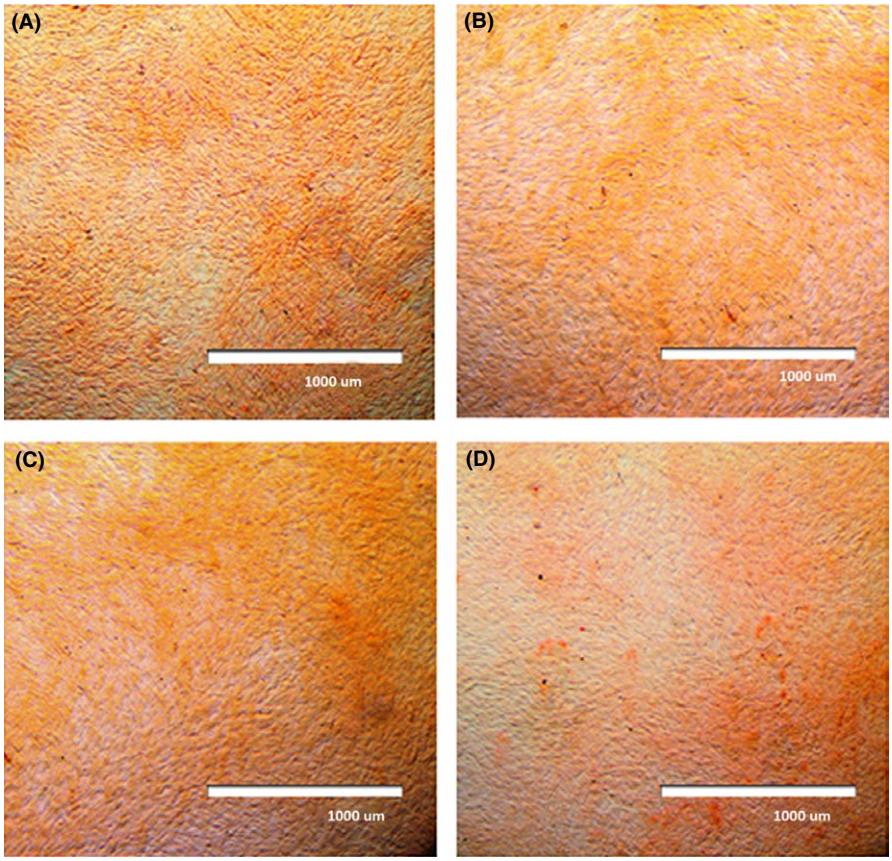

FIGURE 5 Alizarin red staining images of PDLMSCs that were transferred to the osteogenic differentiation environment after incubation in Ectoine+cDMEM for different time intervals. (A) 2 h; (B) 6 h; (C) 12 h; (D) 24 h

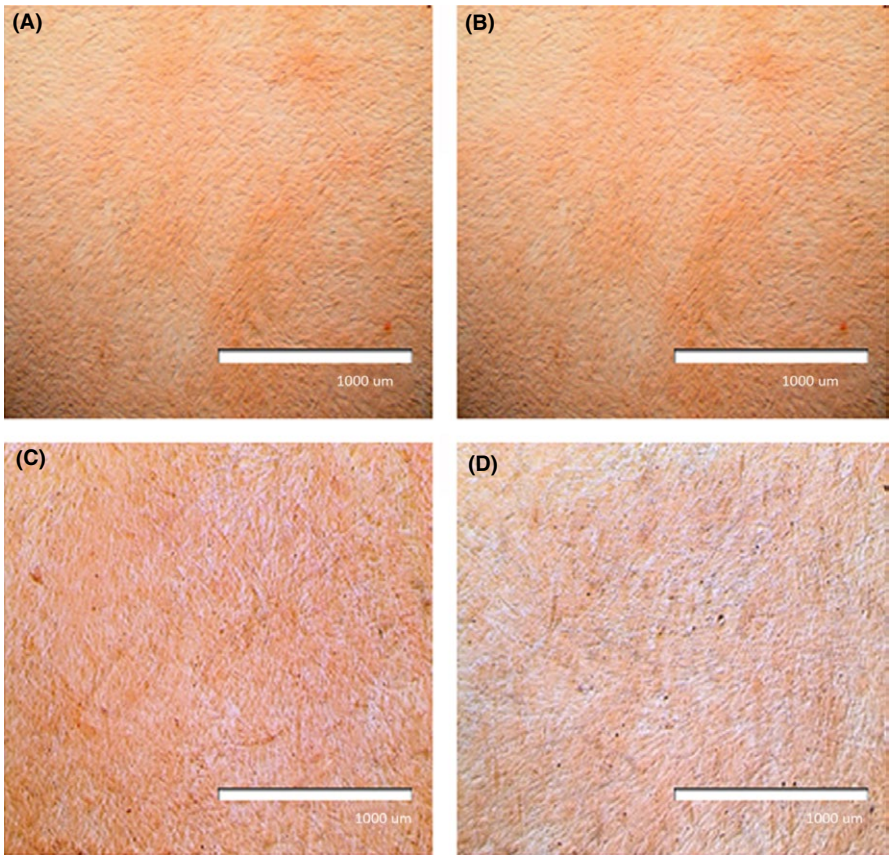

FIGURE 6 Alizarin red staining images of PDLMSCs that were transferred to the osteogenic differentiation environment after incubation in cDMEM for different time intervals. (A) 2 h; (B) 6 h; (C) 12 h; (D) 24 h

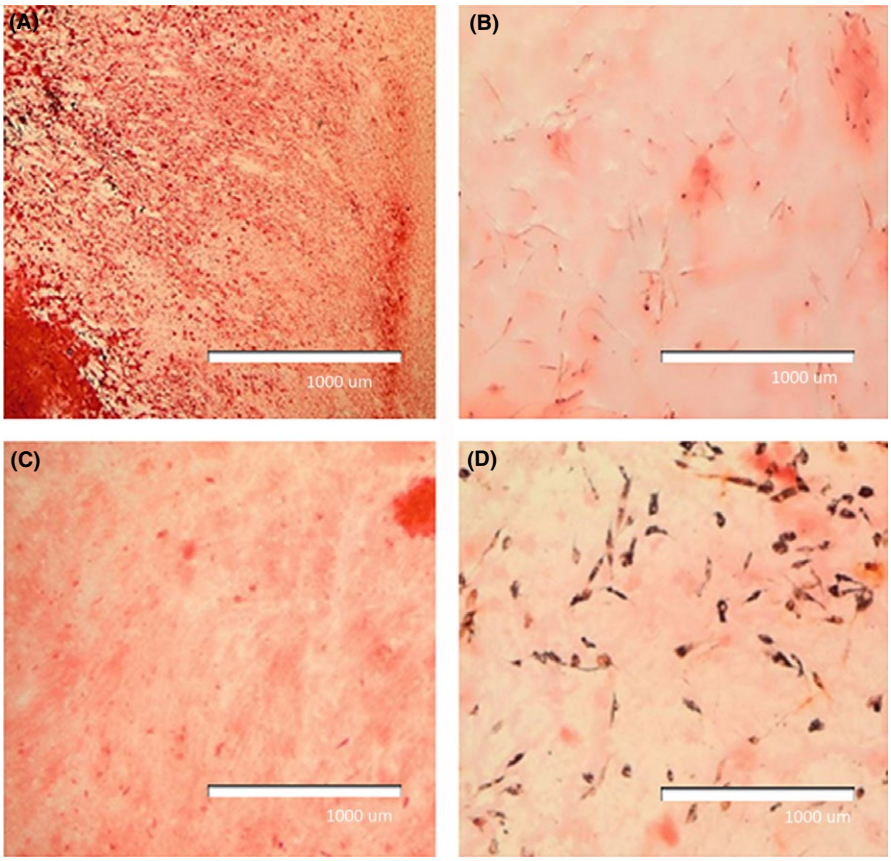

FIGURE 7 Alizarin red staining images of PDLMSCs that were transferred to the osteogenic differentiation environment after incubation in tap water for different time intervals.. (A) 2 h; (B) 6 h; (C) 12 h; (D) 24 h

In this study, a preparation containing 2% Ectoine, 1% sea salt, water, and phosphate buffer (nasal drop) was used in the Ectoine-containing group. This preparation was effective on hPDLMSCs for 2 h. However, it did not provide a suitable microenvironment for cell growth and survival. The positive response of cells in the Ectoine medium for 2 h was thought to be related to the cell homeostasis system.

Ectoine when added to cDMEM had the highest cell viability and decreased cell death rates at 12 h. The positive response of hPDLMSCs for Ectoine added to cDMEM can be explained by the enhanced microenvironment for cell growth and survival. The results demonstrated that action added to cDMEM promoted the survival of hPDLMSCs.

In this study, all groups demonstrated low percentage of early apoptotic cells after 2 h. The increase in the percentage of early apoptotic cells after 6 h for both the Milk and the Ectoine groups may be related to low capacity of these media to preserve the morphological integrity of cells. At all time intervals, the lowest cell viability rate and highest dead cell rate were observed in the tap water-containing group.

The differentiation potentials of dental pulp and PDL-derived stem cells are important for the repair mechanism. It has been stated that PDLMSCs had an important role in the PDL repair mechanism with their self-renewal and multilineage differentiation capacities.[29-31]

Culture environment and methods are very important for the proliferation of PDLMSCs without losing their characteristics.[32] In this study, differentiation of hPDLMSCs was stimulated by adding β-glycerol phosphate, ascorbic acid, and dexamethasone for 3 weeks, and the osteogenic differentiation capacities of the experimental groups were qualitatively evaluated with Alizarin red staining.

The cells stored in Ectoine+cDMEM had the highest mineralized calcium accumulations. The effect of Ectoine on the cell viability of hPDLMSCs was similar to its effect on osteogenic differentiation. Ectoine was not effective alone after 2 h. The weak differentiation capabilities of cells in the Ectoine-containing group were thought to be related to low nutrient intake. Ectoin increased the osteogenic differentiation capacity of hPDLMSCs when it was applied with cDMEM.

The results of this study showed that milk preserved the viability of the periodontal ligament cells, but it had a limited positive effect on both mitotic activity and osteogenic differentiation capabilities of periodontal ligament cells.

In the experimental groups where osteogenic differentiation was detected, a high apoptotic rate was also observed. This result supports the other studies[33,34] that have suggested a positive correlation between apoptosis and osteoblastic differentiation.

Although several studies in the literature are related to dental and bone marrow-derived MSCs, only a few studies can be found on fibroblastic differentiation of PDLMSCs.[35-37] Studies investigating the roles of different growth factors in the fibroblastic differentiation of PDLMSCs showed that TGF-β1 and CTGF increased fibroblastic differentiation by regulating type I collagen, α-actin, and pericytes.[35,38] Maegawa et al. reported that sequential usage of

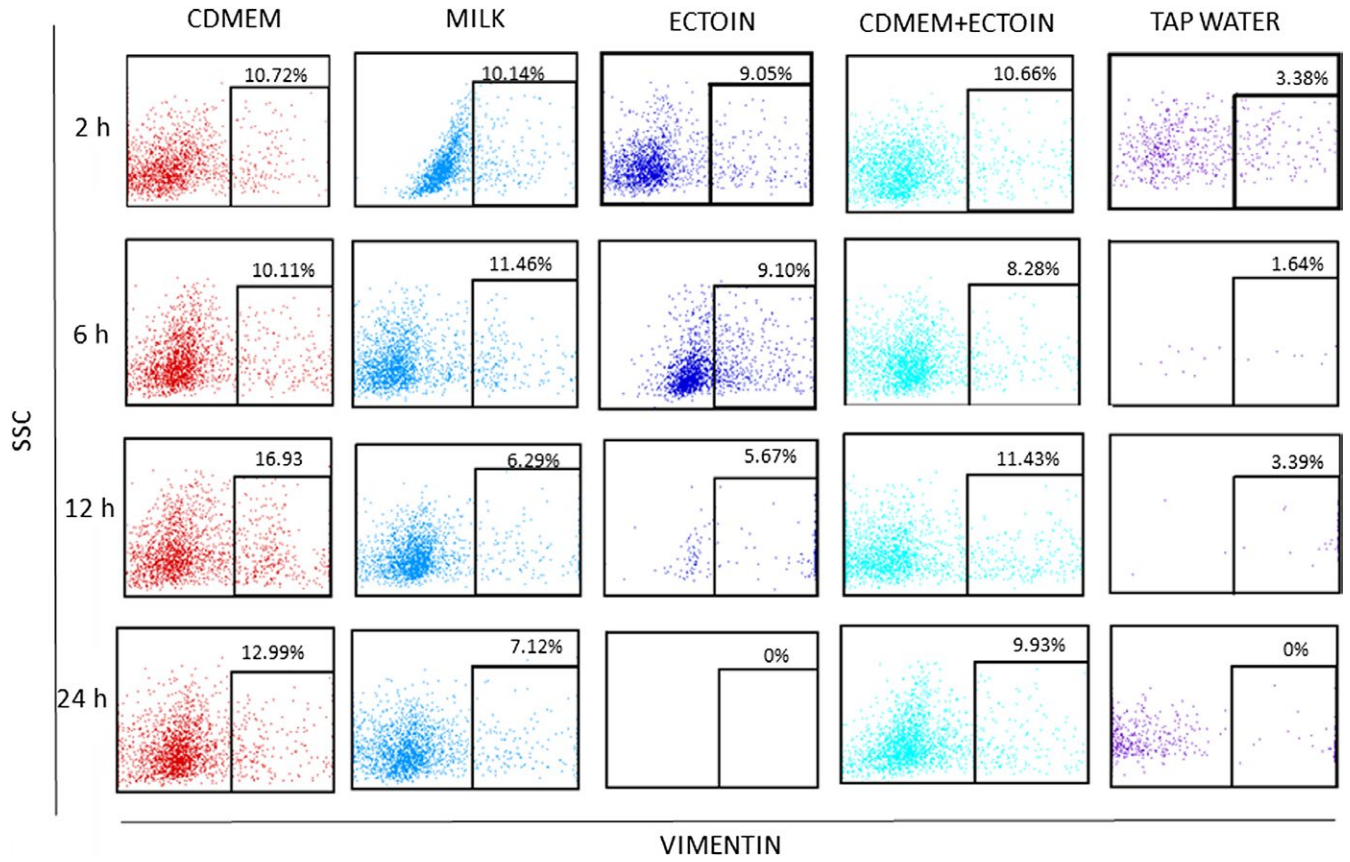

FIGURE 8 Vimentin analysis of the PDLMSCs that were transferred to the fibroblastic differentiation environment after incubation in different storage media for 2 h, 6 h, 12 h, and 24 h using flow cytometry

FGF-2 and BMP-2 caused increased bone formation and the fibroblastic differentiation of PDLMSCs.[36]

In this study, fibroblastic differentiation was induced with low-dose bFGF (1 ng/mL) and sequential usage of bFGF and TGF-β culture medium.

It is quite hard to differentiate fibroblasts that show structural characteristics and antigenic profile depending on their location and functions from fibroblast-like cells.[39] As all of these cells have a similar fibroblast origin, they were collected under the same title. Because of a limited number of fibroblast/ fibroblast-like cell markers, it is hard to define cells that belong to fibroblast family based on the strict criteria.[40] Vimentin, type I collagen, and α-SMA markers were used for fibroblast characterization.[41]

In this study, the cells that showed vimentin expression were defined as fibroblasts. The highest vimentin expression was detected in cDMEM medium. The fibroblastic differentiation of hPDLMSCs was negatively affected by Ectoine exposure over time.

In the current study, the highest ratio of hPDLMSCs that showed fibroblastic differentiation was detected as 17%. It can be explained by the heterogeneous nature of hPDLMSCs and possible stability in the differentiation process of stem cells.

In this study, the higher vimentin expressions were detected in cells cultured with cDMEM and Ectoine+cDMEM. As fibroblasts or fibroblast-like cells sustain their viability depending on a substrate that cells are cultured on, the best results were obtained in the culture environments that provided the required conditions.

The milk group also gave close results to that of the Ectoine+cDMEM and the cDMEM groups regarding cell viability, but insufficient results for fibroblastic differentiation after 6 h. It can be concluded that milk, which is easily accessible, ready to use, and cheap, is a good storage medium prior to early replantation of avulsed teeth.

Further studies taking into account all parameters such as structure and different doses of the osmotic solutes are necessary to confirm the findings of this study and to provide data for comparing the effects of Ectoine on PDL cells.

5 | CONCLUSIONS

Ectoine had a positive effect on both cell viability and differential potential of hPDLMSCs for 2 h. However, Ectoine alone was not ideal after 2 h. Ectoine with cDMEM increased the cell viability and differentiation potential of the hPDLMSCs.

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DECLARATION OF INTEREST

The authors reported that they had no conflict of interests. The authors alone are responsible for the content and writing of the manuscript.

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