ORIGINAL ARTICLE

Cytotoxicity Analysis of Strontium Ranelate on Cultured Human Periodontal Ligament Fibroblasts: A Preliminary Report

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Background/Purpose: The aim of this study was to analyze the cytotoxicity of strontium ranelate (SR) on human periodontal ligament fibroblasts (PDL cells) *in vitro*.
Methods: PDL cells were obtained from healthy human third molars and cultured in Dulbecco's Modified Eagle's Medium. The experimental groups were: G1, cultures treated with fresh medium (control); and G2, G3, G4 and G5: treated with SR at 20, 10, 5 and 2.5 mg/mL, respectively. The experimental times were 1, 6, 12 and 24 hours (short-term) for viability, and 2, 4, 6 and 8 days (long-term) for cell survival. The cells were counted using a hemocytometer. Data were then analyzed by one-way ANOVA and Tukey's tests ($p < 0.05$).
Results: Cultures treated with the highest SR concentrations (G2 and G3) had significantly lower cell viability and cell numbers ($p < 0.05$) than those in G1, G4 and G5. SR at 2.5 mg/mL was non-cytotoxic to PDL cells.
Conclusion: SR was non-toxic at appropriate concentrations. Preclinical tests are needed to further assess its safety and effectiveness for tooth resorption prior to clinical use. [*J Formos Med Assoc* 2008;107(8): 609–615]

Key Words: cell culture, cytotoxicity, resorption, strontium ranelate, trauma

Tooth resorption is a common sequela of injury or irritation of the periodontal ligament and/or tooth pulp. The course of resorption involves an elaborate interaction among inflammatory cells, resorbing cells, and hard tissue structures.[1] Dental hard tissues (cementum, dentin, enamel) do not normally undergo resorption. When resorption of teeth is observed, it is usually the result of trauma, replantation therapy, chronic inflammation of the pulp and/or periodontal tissues, induced pressure in periodontal ligaments associated with orthodontic tooth movement, tumors, or tooth eruption, or chemical irritation by bleaching agents or other irritants.[2,3]

Resorption on the external root surface usually accompanies simultaneous reactions within the alveolar bone; the process of tooth resorption is considered to be similar to that of bone resorption. Resorption of alveolar bone occurs as a result of local inflammation and as part of life-long remodeling of the jaws.[2] Resorption is primarily by osteoclasts,[4] but other cells, such as macrophages[5] and monocytes,[6] have been reported to possess bone-resorbing activity. Osteoclasts resorb bone by releasing demineralizing agents and degradative enzymes into Howship's lacunae under the ruffled border, and then ingest the bone degradation products by phagocytosis.[7] Osteoclasts also

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have an important role in the inflammatory response to infection, as seen in resorption of necrotic bone in osteomyelitis[8] and root and bone resorption of teeth with pulp necrosis and/or infection.[9]

To inhibit osteoclastic activity, therapeutic agents such as calcium hydroxide,[10] fluoride,[11] dexamethasone,[12] calcitonin,[13] antibiotics,[14] corticosteroids,[14] and bisphosphonates[15] have been used in dental practice. Strontium ranelate (SR) (5-[bis(carboxymethyl)amino]-2-carboxy-4-cyano-3-thiophenacetic acid distrontium salt) is composed of an organic moiety (ranelic acid) and two atoms of stable strontium. Ranelic acid was chosen as the anion for the strontium salt from 26 other candidates because the resulting salt presented the most suitable physicochemical (e.g. percentage of strontium, solubility, no chelating properties, stability) and pharmacokinetic (e.g. bioavailability, exposure to strontium) characteristics for a therapeutic agent.[16] It has a dual mode of action, both increasing bone formation and decreasing bone resorption, which rebalances bone turnover in favor of bone formation and increases bone strength.[17] Therefore, it is possible that SR can be used for the treatment of tooth resorption.

Based on the anti-osteoclastic activity of SR, the use of this drug in the treatment of dental trauma is promising. However, for this purpose, SR must be biocompatible with periodontal tissue. Before testing this drug *in vivo*, the aim of this study was to analyze SR cytotoxicity in human periodontal ligament fibroblasts (PDL cells) in culture.

Methods

Primary culture of human PDL cells

PDL cells were obtained from fully erupted clinically healthy third molar teeth extracted for orthodontic purposes. Scaling and root planning were performed before extraction. The teeth were extracted as atraumatically as possible and washed twice in sterile saline solution to eliminate residual blood. Periodontal ligament tissues, attached to the middle third of the root surface only, were scraped with a #15 scalpel using aseptic techniques, and then transferred to culture medium.

The periodontal ligament tissue samples were cut into 1–2-mm^3 pieces, and then washed twice with HBSS (Hank's buffered salt solution). Thereafter, the biopsies were placed in tissue culture flasks (25 cm^2). The explants were incubated with culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St Louis, MO, USA), 10 mL HEPES, 4.5 g/L glucose, 3.7 g/L $NaHCO_3$, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2.5 mg/mL amphotericin (all from Biochrom KG, Berlin, Germany), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Pan Systems, Aidenbach, Germany). Cells were grown at 37°C in a humidified atmosphere of 5% carbon dioxide in air. The culture medium was renewed twice weekly until the cells reached confluence. For subcultivation, cells were detached from the culture flasks with 0.25% trypsin/EDTA solution (Sigma) for 3–5 minutes. The seventh passage of PDL cells was used in the experiment to achieve their maximum proliferative potential and homogeneity. All tissue manipulations were done under a sterile class I laminar airflow cabinet.

Exposure of PDL cells to different concentrations of SR

The cytotoxicity of 20, 10, 5 and 2.5 mg/mL SR (Protelos; Servier Laboratories, Paris, France) was measured *in vitro*. The experimental groups were: G1 (control), cultures grown on fresh DMEM and 10% FBS; G2, DMEM containing 20 mg/mL SR; G3, DMEM containing 10 mg/mL SR; G4, DMEM containing 5 mg/mL SR; and G5, DMEM containing 2.5 mg/mL SR. The immediate or short-term response and long-term survival that measured the retention of the self-renewal capacity of the cells were analyzed.

Short-term assay (cell viability)

A total of 1×10^3 cells were plated on 35-mm diameter petri dishes. Three days later, the culture medium was exchanged for medium that contained SR solution (G2–G5), while the control cultures

(G1) received fresh medium. The medium inside the dishes was discarded and replaced by solutions diluted in fresh DMEM containing 10% FBS. After 1, 6, 12 and 24 hours, the cells of five dishes per group were counted, and viability curves were plotted.

Long-term assay (cell survival, cell growth)

A total of 1×10^3 cells were plated on 35-mm diameter petri dishes. After 6 hours, when the cells were all attached, the culture medium was exchanged for medium that contained SR solution (G2–G5), while the control cultures (G1) received fresh DMEM. The attached cells were harvested from the dishes 2, 4, 6 and 8 days after seeding, using a 0.25% trypsin solution (Sigma). The cells of five dishes per group in suspension were then counted and growth curves were plotted. Maintenance of cell viability was obtained by exchanging half of the culture medium with fresh DMEM every other day.

Viability and cell growth curves

The cell number was determined by counting the viable cells in a hemocytometer using the trypan blue dye-exclusion assay. For each period, cells from five dishes of each group were harvested using 0.25% trypsin solution, and these cells in suspension were counted under a phase-inverted microscope (TMS; Nikon, Tokyo, Japan) with a hemocytometer at 200× magnification. The number of viable cells harvested from each petri dish was obtained by the following equation: $UC \times D \times 10^4/nSQ$, where UC is the unstained cell count (viable cells), D is the dilution of the cell suspension, and nSQ is the number of counted squares in the hemocytometer. The viable percentage of the cell population in each petri dish was obtained using the following equation: $UC/TC \times 100$, where UC is the unstained cell count (viable cells) and TC is the total cell count (stained plus unstained cells).

Statistical analysis

Statistical analysis was accomplished using one-way ANOVA complemented by Tukey's test. The level of significance was 5% ($p < 0.05$).

Results

The morphologic characteristics of the human PDL cells in the presence of SR at 8 days are shown in the Figure.

The mean percentage cell viability in the short-term assay is shown in Table 1. Control cells (G1) had stable cell viability of ~99% in the first 24 hours. PDL cells treated with 20 mg/mL SR (G2) presented a significantly smaller percentage cell viability than that in the other groups after 6 hours ($p < 0.05$). However, there was no significant difference between G1, G3, G4 and G5. At 12 and 24 hours, G5 presented a similar cell viability to the control cells (G1).

The mean numbers of viable cells in the long-term assay are shown in Table 2. At 2 days, there was no statistically significant difference between the groups ($p > 0.05$). At 4, 6 and 8 days, controls (G1), cells treated with 5 mg/mL SR (G4) and 2.5 mg/mL SR (G5) showed an increase in cell numbers.

Discussion

Toxicity testing of dental materials can be assessed either *in vitro* or *in vivo*. *In vitro* testing is accepted as a routine method for establishing cytotoxicity of dental materials.[18] When using *in vitro* methods, one can use established permanent cell lines such as HeLa or L929 cells, or primary cells, mainly oral fibroblasts, to test the dental materials.[19] Cells derived from a primary culture are distinguished by the fact that they have been cultured for the first time. Therefore, they are characterized, much like their original tissue, by a diploid set of chromosomes, a largely unchanged metabolic status, and a high degree of differentiation. In contrast, established cell lines are morphologically and physiologically more homogeneous, but because they have been passaged many times, they have lost the karyotype of their original tissue. The heterogeneity of primary cultures, which reflects different stages of physiologic aging, is much better suited to mimic the *in vivo*

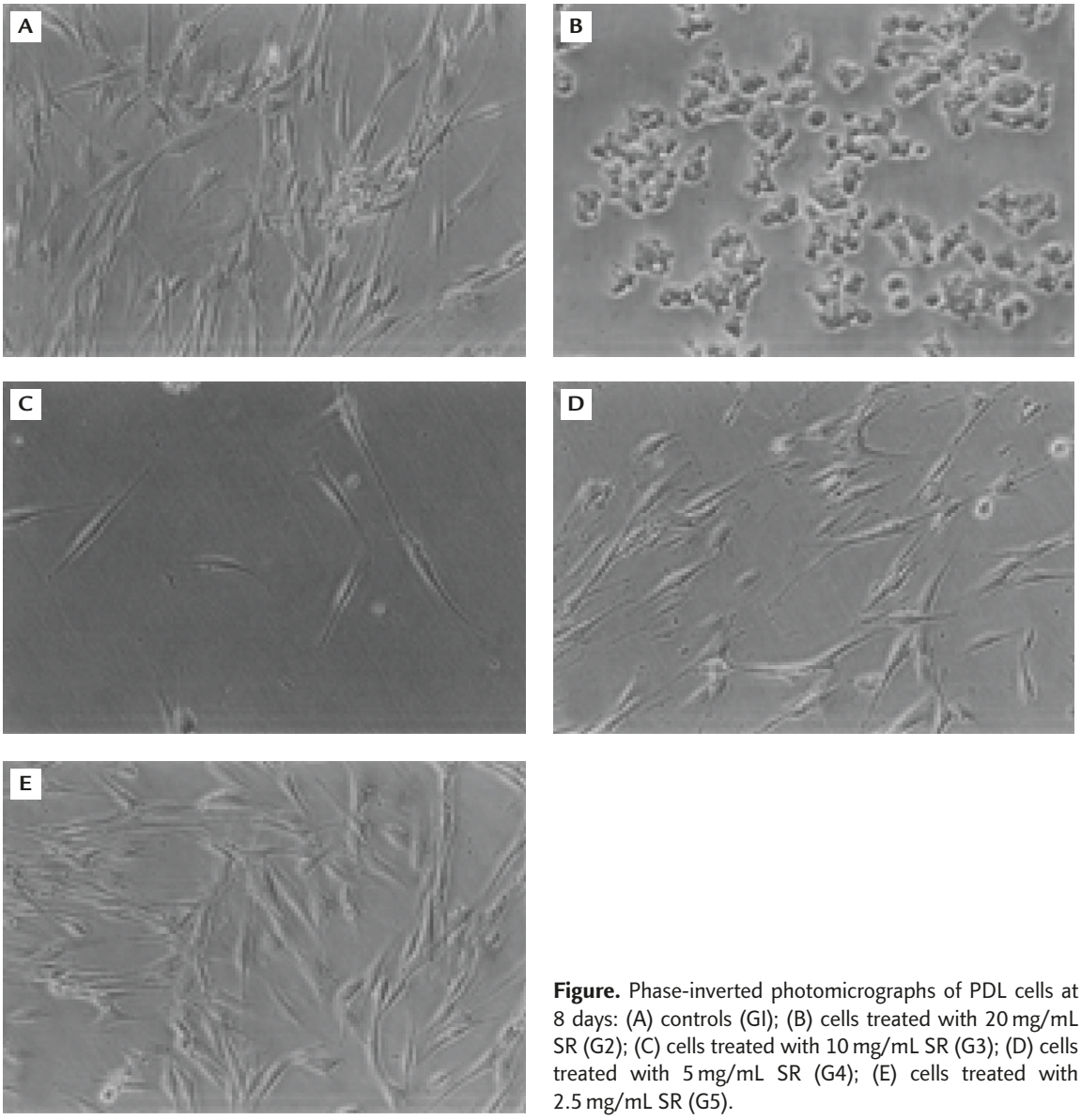


Figure. Phase-inverted photomicrographs of PDL cells at 8 days: (A) controls (GI); (B) cells treated with 20 mg/mL SR (G2); (C) cells treated with 10 mg/mL SR (G3); (D) cells treated with 5 mg/mL SR (G4); (E) cells treated with 2.5 mg/mL SR (G5).

Table 1. Percentage cell viability in the short-term assay*

Group	Total (*n*)	1 hr	6 hr	12 hr	24 hr
1 (Control)	5	99.50 ± 0.5^{a}	99.25 ± 0.9^{a}	99.25 ± 0.9^{a}	99.25 ± 0.5^{a}
2 (20% SR)	5	99.25 ± 0.9^{a}	90.00 ± 4.0^{b}	75.00 ± 1.6^{c}	58.75 ± 4.7^{b}
3 (10% SR)	5	99.25 ± 0.5^{a}	98.00 ± 0.8^{a}	85.00 ± 4.0^{d}	70.00 ± 1.6^{c}
4 (5% SR)	5	99.50 ± 0.5^{a}	99.25 ± 0.9^{a}	93.75 ± 2.6^{a}	88.00 ± 2.4^{d}
5 (2.5% SR)	5	99.50 ± 0.5^{a}	99.50 ± 0.5^{a}	98.00 ± 0.8^{a}	95.00 ± 0.8^{a}

**Data presented as mean ± standard deviation. The same superscript letters indicate statistically nonsignificant values ($p > 0.05$).*

situation than the homogeneity of established cell lines. Another important factor in this context is the selection of the cell type best suited for simulating the *in vivo* situation. Human uterine carcinoma cells or sarcoma fibroblasts from mice do not optimally simulate the cells found in the human oral cavity.[20] Therefore, a primary culture of human PDL fibroblasts was used in this study as target cells. These cells were obtained from clinically healthy, extracted third molar teeth.

Table 2. Number of viable cells in the long-term assay*

Group	Total (*n*)	2 d	4 d	6 d	8 d
1 (Control)	5	$4.0\times10^3\pm0.5^a$	$5.0\times10^3\pm0.5^a$	$6.4\times10^3\pm1.0^a$	$7.0\times10^3\pm1.0^a$
2 (20% SR)	5	$2.5\times10^3\pm0.5^a$	$1.5\times10^3\pm0.5^b$	$1.3\times10^3\pm0.2^b$	$0.1\times10^3\pm0.2^b$
3 (10% SR)	5	$3.0\times10^3\pm1.0^a$	$2.3\times10^3\pm1.0^b$	$2.0\times10^3\pm0.5^b$	$1.5\times10^3\pm0.5^b$
4 (5% SR)	5	$4.0\times10^3\pm1.0^a$	$5.5\times10^3\pm1.0^a$	$6.1\times10^3\pm0.5^a$	$6.5\times10^3\pm0.5^a$
5 (2.5% SR)	5	$4.3\times10^3\pm1.5^a$	$4.8\times10^3\pm0.2^a$	$6.3\times10^3\pm0.2^a$	$6.8\times10^3\pm0.2^a$

**Data presented as mean ± standard deviation. The same superscript letters indicate statistically nonsignificant values ($p > 0.05$).*

We chose the trypan blue exclusion staining technique because it is quick, easily performed, and distinctively differentiates nonviable from viable cells. However, the health of the viable cells and their ability to proliferate cannot be determined from this technique. We simply aimed to investigate the effects of experimental solutions on the viability of PDL cells.

Bacteria and bacterial products in the root canal act as stimulating factors of inflammatory root resorption. To date, calcium hydroxide[10] and ledermix[14] (a drug combining tetracycline and corticosteroids) have been used as intracanal medication to arrest this process.

Previous studies have indicated that inflammatory resorption and ankylosis, which are frequent sequelae after delayed tooth replantation, can be greatly reduced by treating the root surface with fluoride.[11,21] Fluorides induce the formation of fluorapatite crystals over the radicular surface, which inhibits the action of osteoclasts and thus reduces the incidence of resorption. Similar to fluoride, bisphosphonates are used for the treatment of osteoporosis. They inhibit osteoclast activity during bone resorption by binding to hydroxyapatite in bone, mainly in the surfaces affected by osteoclastic resorption.[22]

Several studies have shown favorable effects of topical or systemic antibiotics in avoiding root resorption.[23–26] The use of systemic penicillin after an avulsion injury has been recommended to decrease the occurrence of resorption complications.[23] Cvek et al[24] reported decreased root resorption after topical treatment of teeth with doxycycline before replantation, and suggested that the elimination of microorganisms from the root surface might decrease the frequency of ankylosis in replanted teeth. Additionally, systemic tetracycline treatment has been found to result in better root healing than systemic amoxicillin in replacement resorption.[25,26]

In the meantime, other therapeutic agents such as dexamethasone (anti-inflammatory drug),[12] calcitonin (hormone),[13] enamel matrix protein (emdogain),[27] and chlorophyllin (antioxidant)[28] have been used in dental practice to inhibit osteoclastic activity.

SR has a dual effect on bone metabolism. *In vitro* studies have suggested that SR enhances osteoblastic cell replication and activity.[29] Simultaneously, SR dose dependently decreases preosteoclast differentiation and osteoclastic activity.[29–31] Some recent studies have suggested that interaction of strontium with the calcium-sensing receptor is one of the explanations for its mechanism of action.[32] This potential mechanism of action and other possible modulations of bone turnover remain to be fully elucidated.

The aim of the present study was to analyze SR cytotoxicity *in vitro* using cell cultures that originated from human PDL cells. The SR concentrations of 20, 10, 5 and 2.5 mg/mL were based on the results observed in a pilot study in which concentrations > 20 mg/mL showed extreme cytotoxicity. In the short-term assay, the viability of cells treated with 2.5 mg/mL SR was similar to that of control cells. Additionally, cells did not lose their ability to enter the cell cycle, and they retained their proliferation activity, as demonstrated by the long-term assay.

Based on our results, we suggest that administration of SR at appropriate concentrations might

be non-cytotoxic to PDL cells and should be used for treating tooth resorption. Besides, the measurement of cytotoxicity, as carried out in the present study, is a purely cellular event. Thus, it does not reflect *in vivo* situations that have complex pharmacokinetics of drug exposure. New studies, using animals as well as human subjects, are needed to investigate the treatment and control of tooth resorption.

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