Strontium Effects on Human Gingival Fibroblasts

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Strontium is a naturally occurring alkaline earth metal that has been shown to be useful not only in the treatment and prevention of osteoporosis but also in the treatment of dentinal hypersensitivity in the oral cavity; strontium is also an effective cariostatic, antiplaque, antigingivitis agent. Relatively little is known, however, about the effects of strontium on gingival fibroblasts. The purpose of the present investigation was to conduct in vitro studies on the potential for strontium to positively affect the activity of these cells such that it might be effective in the enhancement of gingival attachment to surfaces, such as healing abutments in implants in the oral cavity. The results indicate that strontium added as strontium citrate (0.5–1.0 mM), both in the absence and presence of a healing abutment, increases human gingival cell activity and decreases apoptosis in these cells. Scanning electron microscopy studies also reveal that the addition of strontium increases attachment of gingival fibroblasts to the surfaces of healing abutments. These studies provide the basis for further investigations on the use of strontium in the prevention and treatment of peri-implantitis by maximizing the formation of a peri-implant soft-tissue barrier.

Key Words: strontium human gingival fibroblasts healing abutment

INTRODUCTION

Strontium is the 15th most abundant alkaline earth metal and belongs to the same chemical family as calcium.[1] It is naturally present in the adult human body at approximately 0.3–0.4 g, with 99% found in the skeleton as bone or teeth.[2] Strontium is available as citrate, chloride, carbonate, lactate, and ranelate and has been shown to be equally delivered to bone regardless of the analogs. Studies have reported that supplementation with strontium in its various forms is well tolerated and generally safe at approved doses.[3] Strontium ranelate is presently an approved therapy for osteoporosis treatment in Europe and Australia, and in the United States strontium citrate is a Food and Drug Administration–approved bone support supplement.[4] In the field of dentistry, strontium-incorporated dentifrices have been used to treat dentinal hypersensitivity for over 50 years.[5] Recent studies have demonstrated that when strontium is administered at concentrations reported to be functionally effective, there is no apparent toxicity to periodontal ligament fibroblastic cells.[6] Furthermore, studies have reported lower caries prevalence and plaque levels associated with strontium.[7] Strontium has been shown to be an effective cariostatic, antiplaque, antigingivitis agent because it can displace calcium in the plaque and prevent its mineralization.[7] Moreover, studies conducted with strontium oxide–enriched bioactive glass show an increase in gingival fibroblast cell proliferation compared with bioactive glass without added strontium.[8] These previous observations suggest a potential role for strontium in the enhancement of gingival attachment to healing abutments used in dental implants.

Bacterial penetration of the periodontal soft tissues in teeth is naturally limited by the gingival mucosa and the seal that it creates around the tooth. In addition, the epithelium can release various antimicrobial agents and cytokines as a result of innate immunity and inflammatory responses and so create a physiological barrier to bacterial invasion.[9,10] With the use of dental implants, the barrier created by the junctional epithelium and the periodontal ligament is compromised. For long-term success of such implants, studies have suggested that epithelial and fibroblastic cells must form a cuff of keratinized tissue, thereby creating a peri-implant mucosal seal.[11] Epithelial cells have been shown to be present in layers on titanium implant surfaces in oral mucosal tissues[12] and to adhere to titanium implant surfaces via hemidesmosome structures.[13] It appears, therefore, that in addition to osseointegration of the host bone tissue and the implant surface, integration of the abutment with the adjacent soft tissues is a critical component for successful oral implants.[14] Although a great deal of research has concentrated on osseointegration, there is also the need for further development of biomaterials and agents that enhance the formation of a peri-implant soft-tissue barrier.[15]

It has been well documented that at the site of attachment of an implant abutment the mucosal tissue includes an area of fibrous connective tissue.[13,16,17] It has also been noted in several studies that gingival fibroblasts, by their attachment to the abutment, are the primary cells that form the collagen-rich connective tissue needed to repopulate the wound created by

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the placement of an oral implant.[18,19] If the fibroblastic cell layer is compromised, it can contribute to peri-implant disease, so it is reasonable to suggest that increased attachment of these cells to abutment surfaces can help prevent peri-implantitis.[20]

Oral implant abutments are exposed to the mucosal microenvironment of saliva and gingival exudate containing a complex array of microorganisms. It has been shown that oral bacteria can quickly colonize the surfaces of the abutments and compromise the attachment of epithelial and connective tissue cells.[21]

Strontium, with its antibacterial effects and apparent gingival fibroblast proliferative activity, appears to be particularly well suited to enhance an implant-mucosal seal. The aim of this present study is to further characterize the effects of strontium on human gingival fibroblasts in order to elucidate its potential use in the enhancement of mucosal attachment to healing abutments surfaces.

Materials and Methods

Cell cultures

Human gingival fibroblast cells (HGFs) were isolated from discarded gingival tissue obtained from healthy subjects undergoing routine clinical dental procedures (institutional review board protocol 663292-1). An outgrowth method was used for the isolation procedure. Tissue was collected immediately and washed in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Dublin, Ireland) containing 10× antibiotics, followed by washing 3 times in medium containing 1× antibiotics. The pieces were then minced into smaller specimens, placed in 6-well tissue culture plates, and allowed to attach to the wells 30 minutes before the addition of 1 mL fresh DMEM medium plus 10% fetal bovine serum (FBS) (Neuromics, Edina, MN) with antibiotics. These specimens were incubated at 37°C, 5% CO_2 with replacement of fresh medium every 3 days until cells were noted to migrate from the tissue explant. At approximately 60% confluency, cells were detached using 0.25% trypsin/0.05% ethylenediaminetetraacetic acid (EDTA) and reseeded into larger flasks for growth of large-scale culture. Routinely, HGFs were passaged using 0.05% trypsin/EDTA and cultured at the appropriate cell density for each assay. Cells used in the experiments described here came from cultures that originated from 4 different patients.

Cell assays

MTT Assay

Cell metabolic activity was evaluated by a colorimetric assay based on reduction of the tetrazolium salt sodium 3I-[1-phenylamino-carbonyl-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) (MTT) (Sigma-Aldrich, St Louis, Mo) by mitochondrial dehydrogenase of viable cells to a formazan dye. The MTT test was performed after 48 hours of strontium exposure. In order to optimize the concentration of strontium to the cells, the cells were seeded in complete media (alpha modified Eagle's medium [MEM],10% FBS, and 1% antimycotic), and after 80% confluency, the HGFs (n = 3 samples for each group) were treated with various concentrations (0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM, and 0.0312 mM) of strontium for the indicated time. Strontium was added as strontium citrate dibasic (anhydrous) (Jost Chemical Co, Overland, Mo). The cells were then incubated with 200 μL clear (without phenol red) MEM (GIBCO) and 13 μL MTT assay reagent for an additional 3 hours and with 200 μL dimethylsulfoxide at the end of the incubation period. The supernatants were then transferred to a new 96-well plate for recording the absorbance at 490 nm using a 96-well plate reader (Flexstation 3, Molecular Devices, San Jose, Calif).

Cell apoptotic and adenosine triphosphate activity

At approximately 5000 cells/well, HGFs were seeded in a flat 96-well microplate as with the 5 groups (n = 4 samples per group) mentioned previously. After 5 days of exposure to strontium, old medium was removed from all the wells and 100 μL of fresh medium was added. Subsequently, 100 μL of Caspase-Glo 3/7 reagent (Promega, Madison, Wis) was added to each well, briefly mixed by orbital shaking at 300–500 rpm for 30 seconds, and then incubated at room temperature for 30–180 minutes. Luminescence was measured using a Perkin Elmer Victor$^{3}{}_{TM}$V Wallac plate reader with the luminescence (1.0 seconds; Molecular Devices) protocol, which is proportional to the amount of caspase activity present.

Adenosine triphosphate (ATP) activity was assayed using the cell titer 2.0 assay (Promega, USA), which involved adding the reagent to the cells (n = 3 samples for each group) followed by incubation for 10 minutes and measurement under luminescence as noted previously.

Incubations of HGFs with healing abutments

In order to test the effects of strontium on cells attached to abutment surfaces, HGFs were seeded with or without 1 mM strontium in MEM in the presence of healing abutments (Straumann NC 3.6 mm × 3.5 mm; conical shape) for the predetermined time periods and were assayed as follows. Cells associated with the healing abutment surfaces were obtained by incubation in trypsin/EDTA for 2 minutes and the solution was centrifuged and added to 100 μL of clear, no phenol red medium in a 96-well plate. The assays were then conducted as described previously for the cells incubated without the presence of the abutments.

For the scanning electron microscopy study, the healing abutments, after completion of a 4-day incubation with HGFs in the strontium-containing or control medium, were fixed in 2% glutaraldehyde and dehydrated through alcohol washes, exchanged in 100% hexamethyldisilazane to control dryness, and viewed with a scanning electron microscope (Hitachi S-4000 Field Emission scanning electron microscope, Hitachi High Technologies, Clarksburg, Md).

Statistical analyses

The data were analyzed using Microsoft Excel software (version 15.37, Microsoft Corporation, Redmond, Wash). After verifying normality with a Fisher's exact test, the 1-way analysis of variance test was used to test for differences among groups or

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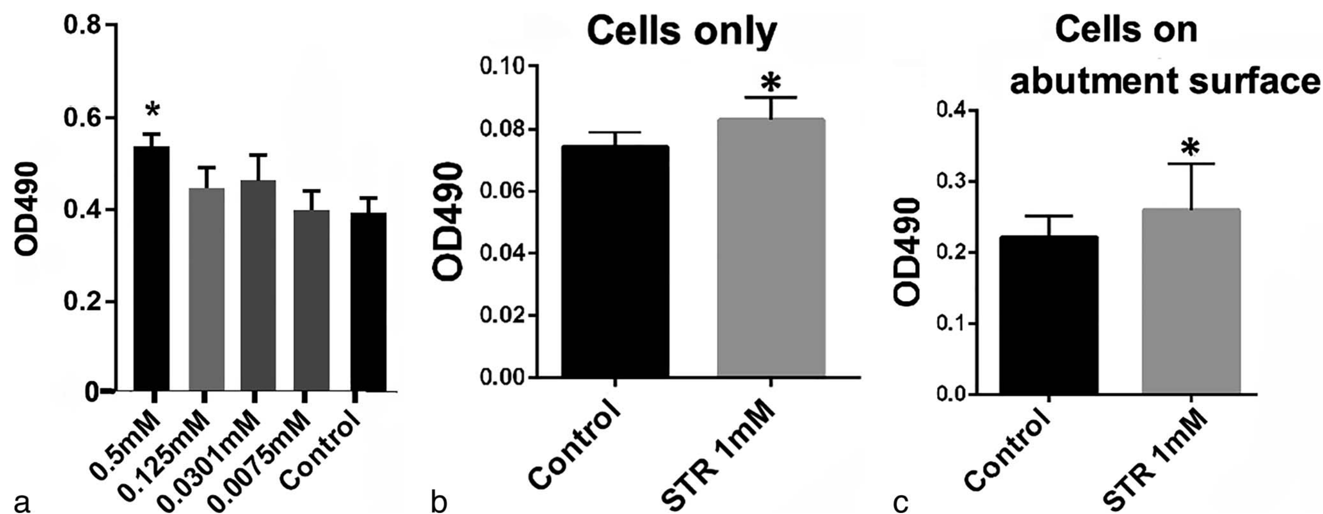

Figure 1. Strontium can increase human gingival fibroblast (HGF) activity: tetrazolium salt sodium 3l-[1- phenylamino-carbonyl-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) (MTT) assay. (a) HGF cells were seeded in HGF media containing strontium concentrations of 0.5–0.0075 mM or no added strontium for the controls. Results shown represent mean/culture well ± SEM (n = 3). *Significantly higher activity in the 0.5 mM strontium (STR) group compared with controls. There were no significant differences in the metabolic activities between the other groups. Analysis of variance: degrees of freedom (*df*) (4 between groups; 10 within groups); F = 10.50; $P < .001$; Tukey HSD post hoc = 0.5mM STR group compared with control; $P < .004$. (b) HGF cells were seeded in medium without added STR (control) and with 1 mM STR added (STR group). Results shown represent mean/culture well ± SEM (n = 3). *Significantly higher cell MTT activity in 1 mM STR group compared with controls. Student *t*-test: $t = 4.242$; $df = 2$; $P < .023$. (c) HGF cells were seeded with the healing abutment in medium alone and with 1 mM STR. Results shown represent mean/culture well ± SEM (n = 7). *Significantly higher cell activity in the HGF media with 1 mM STR compared with the control. Student *t*-test: $t = 1.342$; $df = 6$; $P = .045$.

a 2-sided Student *t*-test with a *P* value < .05 considered as a significant difference.

Results

Effect of strontium on cell activity, ATP activity, and apoptotic activity

Using the MTT assay to assess cell activity, increasing concentrations of strontium in the medium resulted in increases in the percent of viable cells in a dose-dependent manner from 0.032 to 0.5 mM strontium. The concentration of 0.5 mM strontium resulted in a significantly higher cell activity compared with the other treated groups and controls without added strontium. (Figure 1a). Figures1b and c show that 1.0 mM strontium added to the medium produced increases in MTT cell activity compared with controls without added strontium when cells were incubated alone (Figure 1b) or in the presence of a healing abutment (Figure 1c).

Figure 2 shows the results of an Caspase-Glo Assay (Promega, Madison, Wis) used to assess the effects of strontium on apoptosis in the HGF cells under the conditions used in this study. This assay measures caspase 3/7 activity as an indication of apoptosis. The levels of the caspases are significantly decreased by the presence of strontium at 1 mM both in the presence and absence of the healing abutment in the 5-day incubation period used in this study.

Measurements of ATP levels also indicated that adding strontium to the medium increases HGF metabolic activity. Figure 3 shows that the cells attached to the healing abutment surface exhibited a robust significant increase in ATP levels when incubated in the presence of 1 mM strontium.

Strontium increases the HGFs associated with the healing abutment surface

The SEM analysis demonstrated a marked increase in the number of HGFs present on the surfaces of a healing abutment in the strontium group compared with the untreated cells group (Figure 4). Because all the samples were initially cultured with HGFs at identical cell numbers and conditions except for the presence of strontium in the treatment group, and were then washed and fixed under identical conditions, the presence of more cells remaining associated with the abutment surfaces in the strontium treated group is presumed to be indicative of increased cell attachment due to strontium in the culture medium. Images shown are representative of 4 analyzed surfaces from each experimental condition.

Discussion

There has been a great interest in the effects of strontium on bone over the past several decades, and this research has led to the development of strontium ranelate, which has been approved in some countries as an antiosteoporotic therapy.[22–24] Likewise, there have been several studies on the enhancement of implant materials with strontium to enhance osseointegration.[25–29] Studies on the effects of strontium on fibroblastic cells, such as periodontal or gingival fibroblasts,

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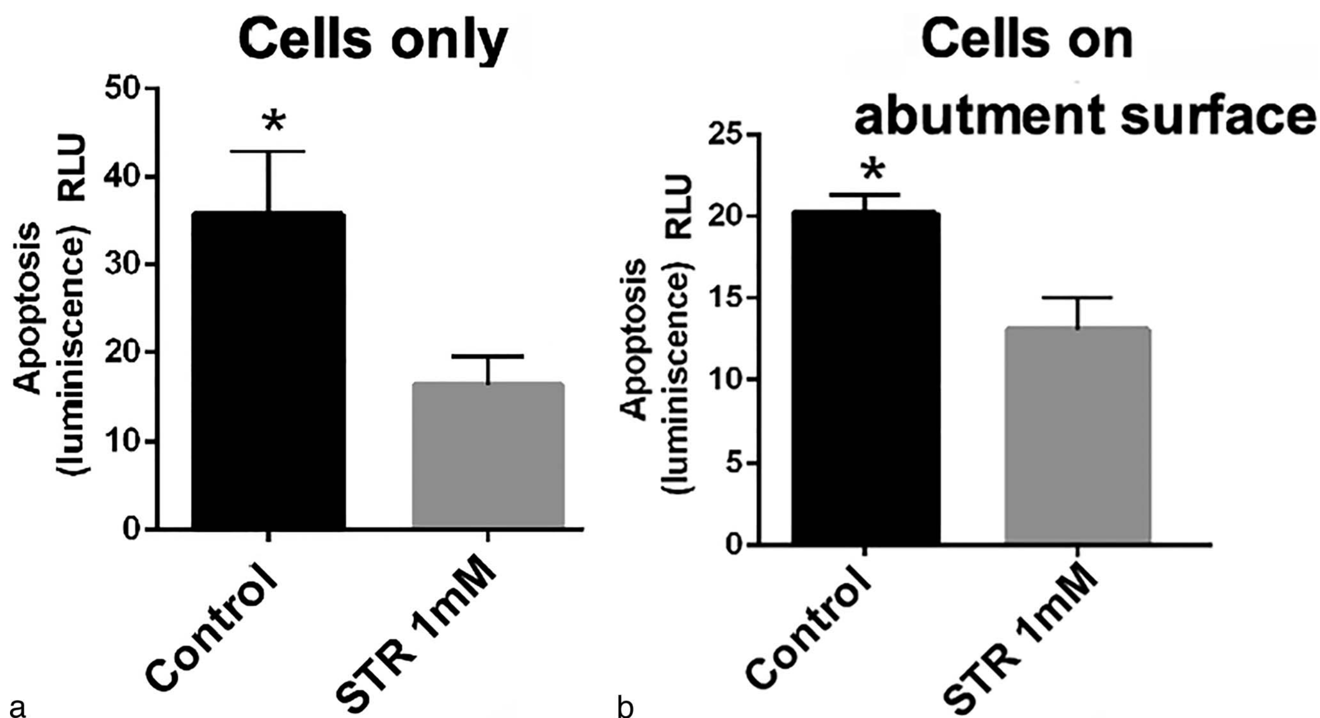

FIGURE 2. Strontium decreases apoptosis in human gingival fibroblast (HGF) cells. Apoptosis assay using APO-live GLO assay. (a) HGF cells were seeded in medium alone (control) or with added 1 mM strontium (STR group). Results represent mean/culture well ± SEM (n = 4). *Significantly higher increase in apoptotic activity in the control group compared with the 1 mM STR group. Student *t*-test: t = 2.353; degrees of freedom (*df*) = 3; $P < .050$. (b) HGF cells were seeded with the healing abutment in medium alone or with 1 mM STR. Results represent mean/culture well ± SEM (n = 3). *Significantly higher increase in apoptotic activity in the control group compared with the 1 mM STR group. Student *t*-test: t = 4.242; *df* = 2; $P = .013$.

found in the oral cavity have been limited. However, because of the potential of a strontium-enriched microenvironment to increase fibroblastic cell attachment to implant surfaces, experiments here were focused on the effects of strontium on HGFs.

The ultimate goal of these studies is to elucidate the role of strontium in the establishment of more stable implant placements with less chance of developing peri-implantitis. This is a destructive condition due to a progression of active inflammatory events. It affects both soft and hard tissue and results in bone resorption and loss of the supporting structures surrounding the dental implant. Present treatment options for peri-implantitis are varied, and there are not many well-designed studies comparing the efficacy of these different treatment approaches. A recent Cochrane Database review concluded that more research is needed to establish effective approaches for preventing and treatiing this condition.[9] Local administration of different biological material has been proposed in several studies for management of this local inflammatory process, but little research appears to focus on the development of approaches to increase fibroblast cell attachment to the implant to form a soft-tissue barrier.

The data presented here are supportive of a role for strontium in the enhancement of cellular activity of HGFs as evidenced by the MTT assay measurements as well as ATP activity. It is also important to note that strontium at the effective concentrations for enhancing cell activity also decreases apoptotic enzymes in the HGF cells associated with the abutment surfaces. Inhibition of programmed cell death can be a very important mechanism in the development of an active soft-tissue barrier that will be maintained. However, in vivo studies in which the rate of apoptosis of connective tissue cells in the mucosal tissue layer is analyzed in relation to the rate of normal cell turnover will have to be conducted to understand the relative importance of the cellular events involved in maintaining tissue stability. Results of experimental periodontitis animal models have suggested that apoptosis in gingival fibroblasts plays a role in long-term inflammatory processes with antiapoptotic mechanisms activated in shorter-term reactions.[30] Extrapolation of these data to the formation of a stable mucosal attachment at abutment surfaces, due to changes in the extracellular environment, requires further study. However, the SEM observations presented here that show evidence for an increase in HGF attachment to the surface of the abutments with strontium in the medium are suggestive of a role for this ion in establishing optimal growth of soft-tissue cells in the implant microenvironment.

The method of obtaining the HGF cells used here is based on the technique established by Somerman et al[31] and shown to be effective in isolating a heterogeneous population of cells with differential characteristics from periodontal ligament cells in the oral cavity. In vitro studies with HGF cells, such as used

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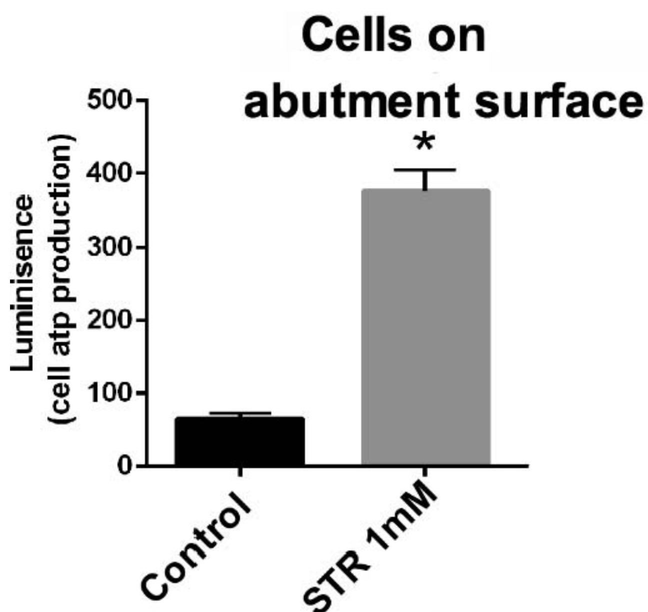

FIGURE 3. Adenosine triphosphate assay: human gingival fibroblast (HGF) cells were seeded in the presence of a healing abutment either in medium without added strontium (STR) (control) or with 1 mM STR added. Results are mean/culture well ± SEM (n = 3). *Significantly higher adenosine triphosphate production (four-fold) in the HGF cells treated with 1 mM STR in the medium compared with the control group without added strontium. Student *t*-test: t = 15.037; degrees of freedom = 2; *P* = .000114.

here, have made extensive contributions to the understanding of the cellular and molecular nature of such events as wound healing, tissue regeneration, and biocompatibility of materials in the oral cavity, although limitations to the in vitro approaches must always be recognized.[32,33]

The results of the experiments presented here may not be truly indicative of the clinical situation at an abutment interface where the microenvironment is complex given the presence of saliva and its associated proteins as well as gingival exudate with various microorganisms. Another limitation of the in vitro studies conducted here is that the HGF cells were incubated under conditions in which the concentrations of strontium were easily controlled, and this might prove to be difficult in vivo clinical situations. Since it appears that there is a limited range of concentrations over which strontium is effective in these studies, as well as in others with strontium in different systems,[6,22] careful attention will no doubt have to be paid to the delivery of optimum local concentrations of strontium in clinical situations where it might be used therapeutically to prevent or treat peri-implantitis.

Antibiotics such as minocycline can be delivered effectively via microspheres at oral sites in clinical therapy.[34] Another study has suggested that controlled release of metronidazole can be achieved by use of an alginate ring placed on an implant.[35] It is anticipated that effective methods can be developed for the local administration of effective concentrations of strontium. Studies have shown that titanium can be modified by incorporating strontium, which can then increase osteoblastic cell growth and eventually enhance osseointegration.[25–29] However, in peri-implantitis, other approaches, such as placement of a strontium-releasing device on the implant abutment, might be more effective in creating the desired effect of enhanced fibroblastic cell growth at the site that can be colonized by oral bacteria. Future studies should address methods by which the strontium concentration can be controlled at appropriate concentrations at the implant abutment site in preclinical models. In vivo studies are therefore necessary to overcome the inherent limitations of in vitro studies where the microenvironment is experimentally controlled, particularly when, as with strontium, the effective concentration range is limited.

Conclusion

Strontium citrate increased the cell activity of HGFs and decreased apoptosis in these cells under relatively standard in vitro tissue conditions. The same concentration of strontium increased the attachment of HGFs to implant abutment surfaces. The in vitro studies conducted here with a well-established system for understanding human gingival cell growth and regeneration provided an approach to further delineate the direct effects of strontium on this cell type. These studies suggest that further experiments, particularly in preclinical animal models, are warranted to investigate the potential use of strontium to enhance an implant-mucosal seal and aid in the management of peri-implantitis.

Abbreviations

ATP: adenosine triphosphate
DMEM: Dulbecco's modified Eagle's medium
EDTA: ethylenediaminetetraacetic acid
FBS: fetal bovine serum
HGF: human gingival fibroblast
MEM: modified Eagle's medium
MTT: tetrazolium salt sodium 3I-[1-phenylamino-carbonyl-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate)

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Note

There are no conflicts of interest for any of the authors with any of the work involved in the study.

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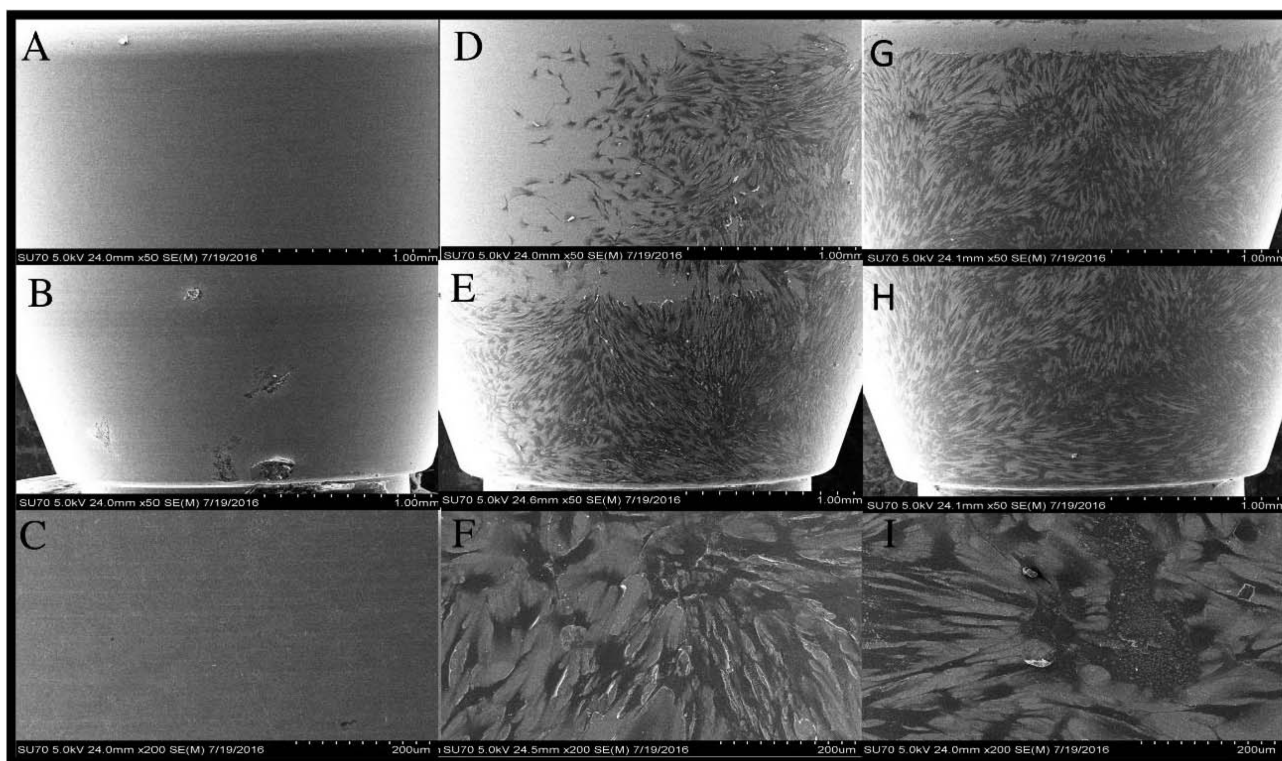

FIGURE 4. Scanning electron microscopy analysis: Strontium (STR) increased human gingival fibroblast (HGF) cells on the surfaces of the healing abutment surface. HGF cells were seeded at equal concentrations in HGF media alone and with 1 mM STR for 4 days. First and second rows represent 50× magnification and third row represents 200× magnification. (a,b,c) Surface of a healing abutment with no cell incubation (negative control). (d,e,f) Surface of a healing abutment incubated with HGF cells in media alone (positive control). (g,h,i) Surface of a healing abutment with HGF cells incubated in HGF media with 1 mM STR. Images shown are representative of 4 analyzed surfaces from each experimental condition.

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