Original

The Effect of Strontium on Osteoblastogenesis and Osteoclastogenesis in Dental Stem Cells-induced Epidermal Growth Factor at Molecular Level: In Vitro Study

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Abstract: For a reconstructive surgeon in oral and maxillofacial surgery, repair of massive bone defects in craniofacial region and mandible caused by different types of trauma, extensive bone destruction by cancer or metabolic diseases is amongst one of the most difficult tasks to handle. The aim of this study was to assess the effectiveness and potential effect of incorporating Strontium (Sr) with locally delivered epidermal growth factor (EGF) on osteoclastogenesis and osteoblastogenesis gene expressions of dental stem cells (DSCs) in an in vitro study. Sr, EGF, and dental stem cells (DSCs) with all materials (reagents and drugs) were commercially purchased from companies. Viability test (cytotoxicity test) was carried out to determine compatibility and optimal concentrations of Sr scaffold and EGF in accordance with the protocol of the current study. DSCs were treated with three modalities of drugs base time points, control-0 concentration, DSCs treated with Sr only, DSCs treated with EGF only DSCs treated with Sr/ EGF. RNA from DSCs were extracted from all treated groups. RT-PCR was used to amplify the specific osteoblast/ osteoclast genes markers. Electrophoresis of the RT-PCR products was followed by gel image capturing. Significant enhanced osteoblast markers responsible for bone healing were observed in the DSCs group that were treated with Sr/ EGF as compared with the other studied group. This strategy seems to be a reliable new tool for bone tissue engineering by mean using bone graft material potentiates inducing high capacity of bone wound healing and could be effective strategy in reconstructive surgery when used in in vivo study used stem cells-based therapy.

Key words: Osteoblastogenesis, Strontium, Epidermal growth factor, Dental stem cells, In vitro

Introduction

Successful surgical reconstruction protocol of massive bone defect using all previous types of bone grafts in Oral & Maxillofacial Surgery, Craniofacial Surgery and Orthopaedic can pose serious problems in clinical surgery and still needs attention5. Repair process of bone defect depends on specific sequential interaction representing the scaffold of tissue matrix and stem cells or pluripotent cells with certain growth factors as protein within the standard local conditions or environment6-8. Bone tissue is capable of complete regeneration without scarring. This property has enabled the development of bone tissue engineering (BTE)9. Certain definition of bone wound healing in literature review with more simplified meaning of a complicated biological process that is initiated whenever tissue integrity is breached9. Wound healing involves cellular interactions to promote processes such as phagocytosis, chemotaxis, mitogenesis, angiogenesis, apoptosis and synthesis of numerous factors (e.g., collagen and extracellular matrix components)9. Strontium (Sr) is an element that has an approximate volume of distribution of 11/kg. The Sr binding capacity to human plasma proteins is low (~25%), but display high affinity for bone tissues. Studies showed that Sr elimination is performed by the kidneys and gastrointestinal tract, independent of time or dose7. The biological process of bone tissue repair is regulated through a large number of growth factors (GF) and cytokines through complicated intracellular events9. These factors provide signals that induces osteoprogenitors migration. Osteoprogenitor cells migration promotes the cellular differentiation, multiplication, tissue revascularization and extracellular matrix production. Effective regenerative process has been demonstrated after including growth factors, epidermal growth factor (EGF), bone morphogenetic proteins (BMPs), and fibroblastic growth factors (FGFs) in scaffolds seeded with stem cells9. However, the use of these GF in clinical applications has been restricted due to their limited availability in terms of price, shelf life during storage and manipulation, and the short time in effectiveness after their application due to hydrolysis, neutralization, and degradation9. Strontium is an osteoprototic anabiotic drug currently prescribed to treat bone diseases in elderly patients (old-old) and postmenopausal period9. Imbalance between the bone formation and resorption results in osteoporosis. The problem of such imbalance could be considered as the...
main causative factor behind the reduction or decrease in bone strength and increased bone fragility with the risk of fracture at the end(10). Sr is considered a unique dual effect of simultaneously promoting bone formation while inhibiting bone resorption(10), thereby improving bone strength. EGF plays an enhancer role on osteogenic differentiation of stem cells because it is capable of increasing extracellular matrix mineralization. EGF triggers endothelial cells, responsible for angiogenesis or vascularization. Grafting of massive bone defect or atrophic non-unions with mesenchymal stromal cells (MSCs) based graft is not a fruitful treatment protocol, in spite of autogenous type bone graft remain the gold standard graft(11). There are many shortcomings associated with allografts, xenografts and tissue-engineered based grafts. Some of the problems associated with these grafts include aging population, mortality, morbidity, insubstantial harvestable autographs, disease transmission and immunogenic response. Incorporated bone marrow mesenchymal stromal cells (BM-MSCs) in osteoconductive scaffolds with the help of BTE has not shown any promising clinical usage(15). Using human BM-MSCs, for the reconstruction of critical-sized bone defect (CSBD), have failed to fill large sized bone defects due to the insufficient amount of bone production(13). New dimensions for the development of biomaterials have been explored that have faster and better regeneration of bone with massive defect besides availability of graft amount and sufficient to reconstruct the defect in one session and no need secondary surgical intervention.

One of the most commonly used filling materials is the synthetic calcium phosphate (CaP) ceramics. It is used as a substitute of autograft and allografts. Other features of synthetic CaP include resemblance with inorganic state of bone and osteoconduction. CaP based materials have shown very weak stimulation of bone cells, lower tendency of producing fragments and particles that may induce immune response. The induced pro-inflammatory immune response can be detrimental leading to the short survival of the implant(14,15). Bisphosphonates (BPs) are effective antiresorptive agents used to treat bone disorders including metabolic bone diseases and bone metastases. They have shown promising results as antioestrogenotic agent and used in tissue regeneration research. However, the single action of BPs in osteoclastogenesis and not in osteoblastogenesis impede it to be a drug of choice in bone tissue regeneration and looking for alternative drugs(16).

Sr is an anabolic drug with dual action that acts on both osteoblasts and osteoclasts. It is widely used as a medication for osteoporosis due to its role in the formation of new bone and osteolytic inhibition. Bone replacement material development in the form of a composite graft with anabolic effect is an important objective in Oral & Maxillofacial Surgery, Craniofacial Surgery and Orthopedic Surgery. There is a dire need of developing BTE based therapeutic modalities for accelerated bone healing within a single surgical operation. So far, no other study is looking at the use of MSCs obtained from dental tissue like used in our design of the study DSCs incorporated into Sr loaded with EGF protein in controlled release of drug using fibrin glue (FG) as a natural delivery vehicle.

Materials and Methods

This research project was carried out and completed in at Craniofacial Science Laboratory, School of Dental Sciences, Health Campus, Universiti Sains Malaysia, Kelantan. Materials used in the cell culture are given in Table 1. All chemicals and reagents used in cell culturing were obtained from (Gibco, USA). Drugs, growth factors, reagent kits were prepared according to the supplier’s protocol. Dulbecco’s Modified Eagle Medium (DMEM) (high glucose 4.5 g/l) enriched with fetal bovine serum (FBS) 10% was used to explain the DSCs.

| Table 1. List of cell line, media, buffers, reagents, drugs, antibiotics and analytical kits were used in cell culture |
| Supplier | Name |
| Invitrogen, USA (GIBCO) | Epidermal Growth Factor (EGF) 10μg:pk |
| Sigma, St. Louis, MO | Strontium (Sr), Powder |
| BioSUN Biotechnology, Malaysia | Primer Synthesis |
| (Cusabio Biotech Co., Ltd, Wuhan, China) | Human Epidermal Growth Factor (EGF), enzyme-linked immunosorbent assay (ELISA) KIT,96T |
| CellGen, LLC Company, Emeryville, CA, USA | Dental Stem Cells (DSCs) |
| Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA | Dulbecco’s modified Eagles medium (DMEM) high glucose(4.5g/l) |
| Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA | Fetal bovine serum (FBS) |
| Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA | Dulbecco’s PBS (without Ca²⁺ and Mg²⁺) |
| Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA | Penicillin/streptomycin, liquid |
| Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA | Triple-Th Express Stable Trypsin Replacement Enzyme without Phenol Red |
| (Biophotometer, Eppendorf AG, Hamburg, Germany) | Spectrophotometer |
| (Analytik Jena AG, Konrad-Zuse-Strasse 1, 07745 Jena, Germany) | innuPREP RNA Mini Kit-850-KS-2040050 |
| QIAGEN, USA | one-step Reverse Transcriptase Polymerase Chain Reaction RT-PCR Kit |
| Baxter Healthcare Corporation, Westlake Village, CA- 91362 USA, US License No.140 | Tissel Fibrin Sealant (FG) KIT |
| Ajax Finechem Pty Ltd – Australia | Dimethyl Sulphoxide (DMSO) |
| Invitrogen Corporation, (San Diego, California, USA) | PrestoBlue® Cell Viability Reagent |

Viability and cytotoxicity test

When using any chemical or physical material in tissue engineering, it is important to consider factors such as biocompatibility and minimal non-toxicity in order to promote cell migration, cellular differentiation, multiplication and cell adhesion without interference with other vital organs or living tissues (ISO/EN 10993-5)(17). Determination of optimal concentrations of Sr and EGF by cytotoxicity analysis was done. Different concentrations of Sr extract treated-DMEM media were used in viability assays (1.0 mM, 2.0 mM, 4.0 mM, and 6.0 mM) with four different time intervals (day 1, 3, 7, 14) for incubation of the cells. Untreated media were used for the control group (Figs. 1 and 2).

EGF-treated media

To reconstitute the lyophilized EGF for working solution, sterile distilled water or RNase-free water was used and stored at ≤ –20°C after reconstitution. Concentration of EGF in each vial was 10 μg/mL. When needed, 10,000 ng/ml volume per 1 ml (1,000 μl) was obtained by adding 1ml distilled to each vial. According to the experimental protocol, different concentrations of EGF media were used (Figs. 1 and 2).
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Figure 1. A, EGF in lyophilized form was stored at 2°C to 8°C, preferably desiccated. B, Sr powder. C, DSCs treated Sr-added EGF. D, PCR machine

Figure 2. A, Sr powder loaded with 20 ml solvent in 50 ml centrifuge tube, B, the supernatant was filtered through a membrane 0.2 μm, C, centrifugation of the extract after filtration process and cytotoxicity test using 96 well plate, D, pure extract was ready to make serial dilutions and used. Preparation with aliquots of EGF and stored at ≤ –20°C., E, F, FG matrix carry EGF inside the prepared flack contain DSCs treated Sr powder.
Preparation of synthetic page purified-type primers

Ten μM primer was prepared by mixing 5μl of 100 μM primer with 45 μl RNase-free water. From the prepared 10 μM primer, 1.5 μl was added to the PCR master mix. One step RT-PCR was carried out to osteoblastogenesis and osteoclastogenesis target gene bone formation markers like osteoprotegerin (OPG), collagen type 1 (CO1-1), Osteoblast enzymes, Alkaline phosphatase (ALP) and bone resorption marker receptor activator of nuclear factor kappa-B ligand (RANKL) according to the manufacturer’s instructions. Specific primers i.e. human GAPDH (F): 5’-CAGAACAACATCATCCTGC CCTCT-3’ (R): 5’-GTTGACAAA- GTGTTGCGTGAG-3’(18), human OPG (F): 5’-GCTCCAACACATAGGAGCTG-3’ (R): 5’- GTTTAC CTGGTGCCAGG -3’(18), human RANKL (F): 5’-CAGTAATAGGCACCGAC-3’ (R): 5’- GGTTAGGAGACCTGGATTT-3’(18), human Collagen Type-I (Col-I) (F) 5’-GATGATTCCAGTGTCGATG G-3’ (R): 5’- GTTTAGG GTTCTGGTCTGTTG -3’(18), human ALP (F): 5’-TGGAGCCTCAGAACCTACACCA-3’ (R): 5’-ATCTCGTTGTCTGAGTAC CAGTCC-3’ were used in the RT-PCR(19).

Preparation of the extracts of Sr powder

International standardized conditions (ISO/EN 10993-5)(17) were maintained during the preparation of Sr powder extract for indirect tests(17). In labeled tubes, 50 ml samples were mixed with 20 ml of DMEM solvent and were scaled with paraffin. A low rotational speed (50 rpm) shaking plate was used for the incubation of all the tubes for 7 days. After 7 days, tubes were filtered through 0.2 μm filter membrane to obtain the supernatant in a 50 ml tube. All the tubes were then centrifuged at 2,000 rpm for 4 minutes and sufficient amount of the supernatant was collected carefully (Figs. 1 and 2). Serial dilutions of the prepared pure extract were made using DMEM media.

Cell culture and cytotoxicity assay

DSCs were treated with different concentrations of 1.0 mM, 2.0 mM, 4.0 mM and 6.0 mM Sr powder extract. After lysis, standardized number of cells were transferred into a 96-well plate and incubated in 5% CO2 and 95% humid atmosphere at 37°C. Untreated media with 0 Sr concentration, was used for the control group. As per protocol, the viability tests were carried out after incubation at different intervals i.e. day 1, 3, 7, and 14. Ten μl PrestoBlue® cell viability reagent (PB) was added to each well and incubated according to the above-mentioned specifications for 2 hours. Samples were protected from light exposure and kept in dark. Absorbance of each well was read at 570 and 600 nm using enzyme-linked immunosorbent assay (ELISA) reader scan. In DSCs the induced regulated gene expression by EGF (at concentrations of 5, 10, 15 and 20 ng/ml) was evaluated. After collection, DSCs passage 4 were cultured in T-25 cm2. After incubation, the EGF genes were amplified by RT-PCR. After electrophoresis of the PCR products, the bands were stained and images were obtained AlphaEaseFC™ (Alpha Innotech, USA). Image J 1.46r software (National Institutes of Health, USA) was used for the analysis of the gel images. Untreated media was cultured with cells and considered as control group. Harvesting of cells was done on day 1, 3 and 7. InnuPREP RNA Mini Kit (Analytikjena, Germany) was used to isolate RNA from both treated and non-treated samples. The isolated RNA was stored at –80°C (Figs. 1 and 2). Each experiment was triplicated.

Preparation of fibrin glue (FG) matrix and measurement of EGF delivery

Varying concentrations of fibrin glue (FB) were prepared according to the manufacturer’s instructions in a heating water bath. Aprotinin solution was used after diluting it from 3,000 KIU/ml to 100 KIU/ml. EGF-specific enzyme-linked immunosorbent assay (ELISA) kit was used to measure the potency and constant release of EGF from FG matrix(19). To validate the analytical procedure and statistical analysis, the tests were run in triplicate (Fig. 2).

Osteoblastogenesis and osteoclastogenesis gene expressions experiments

Optimal concentrations of Sr and EGF were determined and three different modalities were used to expose DSCs to the Sr and EGF. The three modalities were Sr alone, EGF alone and Sr-added EGF. Harvesting of cells were carried out on day 7, 14 and 21. Then RNA was isolated from the harvested cells using RNA Mini Kit (Analytikjena, Germany). Osteoblastogenic and osteoclastogenic genes were amplified by RT-PCR (Fig. 2).

Figure 3. Cytotoxicity test of DSCs treated with different concentrations of Sr, cytotoxic effect of Sr treatment increased significantly over time in culture and the most dramatic increase at the highest drug concentration 2.0 mM of Sr (P<0.05). 0 concentration of Sr did not show a significant change in control groups at all-time points. *, a significant difference when compared to other concentrations and control group (0), the experiment was repeated 3 times.

Figure 4. Optimizing EGF concentration by (proliferation assays using one-step-RT-PCR), 10 ng/ml was considered an optimal concentration treated on DSCs when compare it with other concentrations and 0 (P<0.05). The levels of EGF express on DSCs treated with EGF at different concentrations, 10 ng/ml showed the best result compared with 5 ng/ml and no significant difference can be seen between 15 ng/ml and 20 ng/ml (P<0.05).
Statistical analysis

Statistical software PASW® Statistics 20.0 (SPSS Inc, Chicago, IL, USA) was used for data analysis. One-way analysis of variance (ANOVA) was used to compare the cytotoxicity of varying concentrations of Sr treated DSCs. Post hoc test was used to test the optimal concentration with a p value of <0.05. Independent t-test was used to determine the optimal concentration of EGF.

For osteoblastogenesis and osteoclastogenesis gene expressions, the GAPDH-normalized band intensities of DSCs samples were compared with that the control. Data were collected by quantifying band intensity of GAPDH values divided by band intensity of DSCs values for each sample using Quantity One® 1-D analysis software (USA). Data were analyzed using Kruskal Wallis test. The p-values of less than 0.05 were considered statistically significant if less than 0.05. Mann Whitney U test followed with multiple comparison analysis was also employed when indicated.

Results

Cytotoxicity assay of Sr treated DSCs

Significant proliferation of DSCs were observed at a concentration of 2.0 mM Sr treated media in comparison with other concentrations i.e. 1.0 mM, 4.0 mM and 6.0 mM and control 0 concentration (p<0.05). Cells viability was 100% at 2.0 mM (Fig. 3). Viability test of Sr powder at different concentrations was expressed as relative cell viability that is a percentage of the readings (or activities) in the absence of Sr drug. Non-treated media (control) were assigned a value of 100%. No cell death was detected under light microscope for the optimal concentration group with high cell proliferation (positive biological cell activity) during the four-time points of DSCs differentiation in Sr treated media.

Optimization assay of EGF treated DSCs at different concentrations

The optimal concentration of EGF treated DSCs using RT-PCR was 10 ng/ml with a p value of < 0.05 (Fig. 4), compared with other concentrations used in our experiment. Ten ng/ml expressed higher EGF gene level among other concentrations.

Sr added EGF upregulated the osteoblastogenesis-related gene expression

Gene expressions of OPG, RANKL, ALP and COI-1 at mRNA level in three groups of treatment modalities was analyzed using RT-PCR. Our data showed that the normalized expressions of OPG, RANKL, ALP and COI-1 in the Sr-added EGF group in controlled release manner were 941.8 ± 19.1%, 155.22 ± 5.3%, 898.4 ± 1.2%, and 855.7 ± 3.9% respectively, which were significantly higher than those of the Sr-only group (OPG, RANKL, ALP and COI-1) 396.33 ± 23.4%, 130.34 ± 7.5%, 320.2 ± 3.8%, and 370.4 ± 6.2% respectively, and higher than those of the EGF-only group (OPG, RANKL, ALP and COI-1) 280.75 ± 14.2%, 250.34 ± 3.6%, 260.75 ± 2.4%, and 280.22 ± 4.3% respectively, P<0.05 for all). This suggests that the expression of osteoblastogenesis genes markers (OPG, ALP and COI-1) was increased in response to Sr-added EGF treatment group (2.0 mM/ 10 ng/ml) at mRNA levels and less expression was seen in osteoclastogenic cytokine RANKL. Interestingly, it has been shown that high expression of OPG can be seen in
Sr-added EGF treatment group and less band intensity of RANKL seen in the same group of study. In addition, the expression levels of RANKL and OPG mRNA were evident due to decrease in RANKL mRNA expression and increase in OPG mRNA expression (Fig. 5).

Discussion

The volume of bone and amount of minerals with micro-architecture are under the control of both osteoblasts and osteoclasts action. The process of bone remodeling, bone resorption and bone wound healing mainly depend on the quality and quantity of the main bone cells which also considered osteoblasts, osteoclasts, and endothelial cells. Systemic and local factors control the mechanism of bone remodeling and bone healing followed by trauma, which tightly regulates the cellular migration, proliferation, differentiation, activity and apoptosis of both osteoblasts and osteoclasts. For osteoblastogenesis and osteoclastogenesis, different types of drugs and growth factors have been discovered and have been used widely by many researchers. Sufficient data is available showing advantages and disadvantages of each drug.

Sr is a selective anabolic drug which has dual action of bone resorption (inhibition) and bone formation. It can trigger each gene related to bone resorption/bone formation in crosstalk mechanism. This dual role tends to increase bone remodeling in addition to excellent bone regeneration with good quality when used Sr added EGF in controlled released manner by sophisticating this biomaterial in composite form like scaffold (Fig. 6).

Besides, the role of Sr in stimulating osteoblastic proliferation, it is well recognized that Sr induces the osteoblastic differentiation more than proliferation. Our results stressed that Sr has an enhancing effect only in DSCs differentiation but not in proliferation phase. As well as, it has an inhibitory effect on proliferation of DSCs that confirmed the gene expression at mRNA level. On the other hand, EGF enhanced the proliferation of DSCs but not differentiation phase. The evidence in our results was represented by bone forming/bone resorption genes, with increased ALP, OPG, and COI-1 genes expression in the group treated with Sr added EGF as compared with other treatment modalities. These findings are consistent with that of Wu et al. and Li et al. They stressed that the Sr treatment inhibited the proliferation of rat BM-MSCs with no significant difference with and without added Sr to culture media (P>0.05). Subsequently, over the past decade, researches in bone field, have explored new insights in the regulation of bone remodeling. There is a growing evidence of the pivotal role of three major contributors i.e. RANK-RANKL-OPG. It is well known that RANKL induces the differentiation of pre-osteoclasts into mature osteoclasts. It is achieved by increasing the production of OPG. Interaction of RANKL on osteoclasts with the RANK present on the surface of the osteoclasts is also suppressed by the Sr. This results in hypo proliferation, differentiation and survival of the osteoclasts. EGF can induce pre-osteoclastic proliferation. It can also inhibit the differentiation of preosteoclasts into osteoclasts (Fig. 6). On the other hand, osteoblasts produce RANKL and OPG. Increased expression of RANKL and RANKL/OPG ratio have been observed in the bone tissues of patients with osteoporosis. For the evaluation of osteoblastogenesis, RANKL/OPG ratio is an important index interestingly, due to fracture susceptibility associated with high serum RANKL levels, and high OPG levels are correlated with low risk of fracture. OPG a specific marker for active osteoblasts was used as a key factor to analyze the osteoclast regulation. Expression of OPG was found to be increased in Sr-added EGF group when compared with other groups of treatment and 0 concentration (control) group. At the onset of the mineralization, the expression of OPG was increased that remained at high level continuously. It resulted in a lower ratio of RANKL/OPG in the mature osteoblasts. From these changes it could be concluded that the osteoblasts originated from DSCs may have other different roles that maintains the balance of the bone remodeling. ALP and COI-1 genes are other markers of osteoblasts. Their expression was found to be significantly high showing the mineralization of bone. Mineralization was high in the Sr-added EGF group when compared to the EGF-only, and Sr-only groups (P<0.05). Sr effectively stimulates the formation of bone and inhibition of bone resorption simultaneously. Our findings showed significantly decreased bone resorption due to the less expression can be seen with RANKL gene as observed in the RT-PCR image electrophoresis findings in Sr-added EGF group compared with other modalities of treatment (P<0.05). These findings were in agreement with other published study that had also demonstrated the positive role of Sr on the osteoblastic differentiation into osteocytes. As mentioned above, EGF enhances the proliferation of DSCs but not differentiation phase, in our design, using EGF insures both proliferation and differentiation enhancing of the DSCs in reciprocal interaction. This results in the enhanced migration of osteoblasts into the tissues. Additionally, EGF can potently induce the formation of osteoclasts by regulating the expression of OPG and MCP-1 in osteoclasts. Sr-added EGF may be a promising approach in bone tissue engineering strategy. Further in vivo and in vitro studies are needed using small animal as a screening sample before using large animal sample for the confirmation of these parameters and to evaluate the role of such biomaterials in a more complex system before resorting it clinically.

These findings indicate a role for new formula Sr-added EGF in controlled release manner may enhance our understanding of the mechanisms involved in bone tissue engineering in supporting bone regeneration during the process of skeletal repair in general, and, more specifically, when cell therapies are applied. We found that Sr potently replicates preosteoblasts and differentiates osteoblasts via expression of specific markers like ALP, OPG and COI-1. Promising results observed by combining Sr-added EGF open up a new strategy for modulating bone turnover, formation and resorption and as a scaffold for bone wound healing.

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Conflict of Interest

The authors declare that there are no conflicts interest.

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