CHAPTER 2

METHODS OF STUDY

2.1 The expression of OPG/RANKL mRNAs in osteoblast-like cell MG-63

2.1.1 Osteoblast-like cell MG-63 cell culture

MG-63 human osteoblast-like cells from ATCC (Rocksville, MD) were used at passage 13. They were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Gaithersburg, Md) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, Md), 50 U/ml mycostatin, 100 μg/ml kanamycin, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine in humidified atmosphere of 95% air / 5% CO_2 at 37^0C. After 3 days of culture, the cells were passaged with Trypsin-EDTA (Gibco BRL, Gaithersburg, Md), counted and plated at a density of $2x10^4$cells/cm^2 in 60-mm petri dishes. The cells were maintained in 3 ml DMEM and condition as described above. The media were changed every 2 days until cell reach 70 % confluent. All the dishes were washed three times with phosphate buffer saline (PBS), and the media were changed to DMEM without FBS containing 50 U/ml mycostatin, 100 μg/ml kanamycin, 100 μg/ml streptomycin, 100 U/ml penicillin, 1% L-glutamine and left for 24 h.

Three different medium conditions were tested: DMEM containing 0% FBS, DMEM containing 10% FBS, DMEM containing 100 μg/ml EMD. Cells were cultured in these conditions for 0, 6, 12, 24, 48 h (Table 2.1). The EMD proteins (30 mg) were first dissolved in 100 ml of 10 mM (0.1%) acetic acid to get a better solubility. Then they were diluted in DMEM to the end concentration (100 μg/ml). After 0, 6, 12, 24, 48 h. The medium was removed and wash twice with PBS. The cells were harvested by trypsinization and collected as a cell pallet for RNA extraction.

Table 2.1 Groups of study in the study of the effect of Emdogain on expression of mRNAs in osteoblast-like cell MG-63

Groups of study	Descriptions	Numbers of samples	Investigated parameters	Investigating time
A	MG-63 + 0% FBS	2	OPG and RANKL mRNAs expression by semiquantitative RT-PCR	0, 6, 12, 24, 48 h after incubation
B	MG-63 + 10% FBS	2		
C	MG-63 + EMD 100 μ g/ml	2		

2.1.2 RNA extraction procedure

RNA extraction was performed by using RNeasy Mini Kit from Qiagen (Qiagen, Valncia, CA) according to the manufacturer's instructions. Lysis of the cell in RNeasy Mini Kit was followed by disrupted cell pallet using 350 μl of Buffer RLT and pipetted to mix them. After that homogenized lysated cells by added 350 μl of 70% ethanol, mixed well by pipetting. Placed 700 μ l of lysated cell in to RNeasy mini column and centrifuged for 15 s at 10,000 rpm. Then added 700 μl of Buffer RW1 and centrifuged at 10,000 rpm for 15 s to wash the RNeasy mini column. Transferred the RNeasy mini column into a new 2 ml collection tube and added 500 μl of Buffer RPE to wash column and centrifugation at 10,000 rpm for 15 s. Added another 500 μl of Buffer RPE and centrifuged for 2 min at 10,000 rpm to dry the RNeasy silica-gel membrane. Used 50 μl of RNase-free water to elute RNA from the RNeasy silica-gel membrane by centrifugation at 10,000 rpm for 1 min (Fig. 2.2). The extracted RNA was determined by qualitative and quantitative analysis. For qualitative analysis, 5 μl of extracted RNA was run on 1% agarose gel and for quantitative analysis, 2 μl of extracted RNA was measured OD with UV visible spectrophotometer.

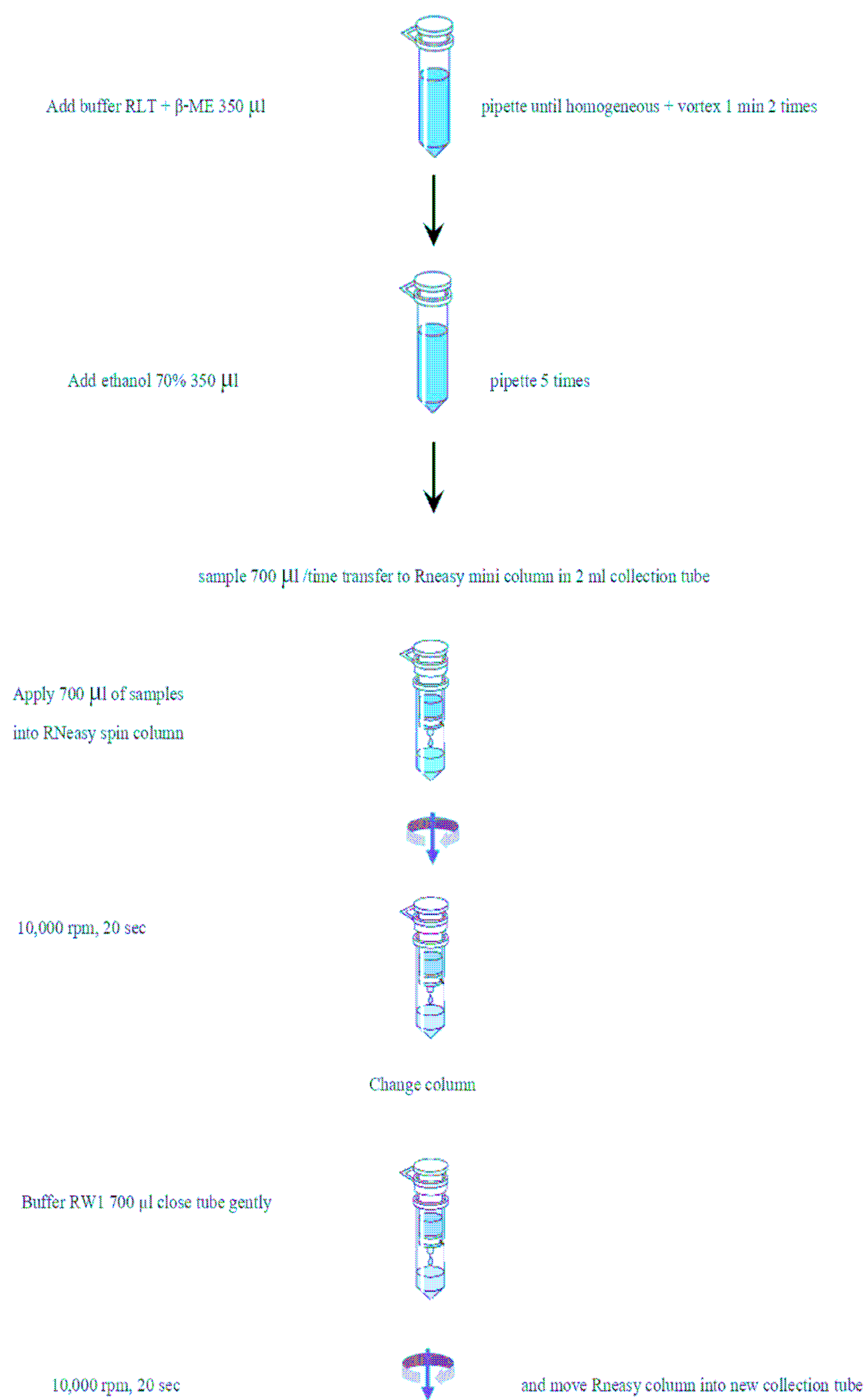

Figure 2.1 RNA extraction procedures

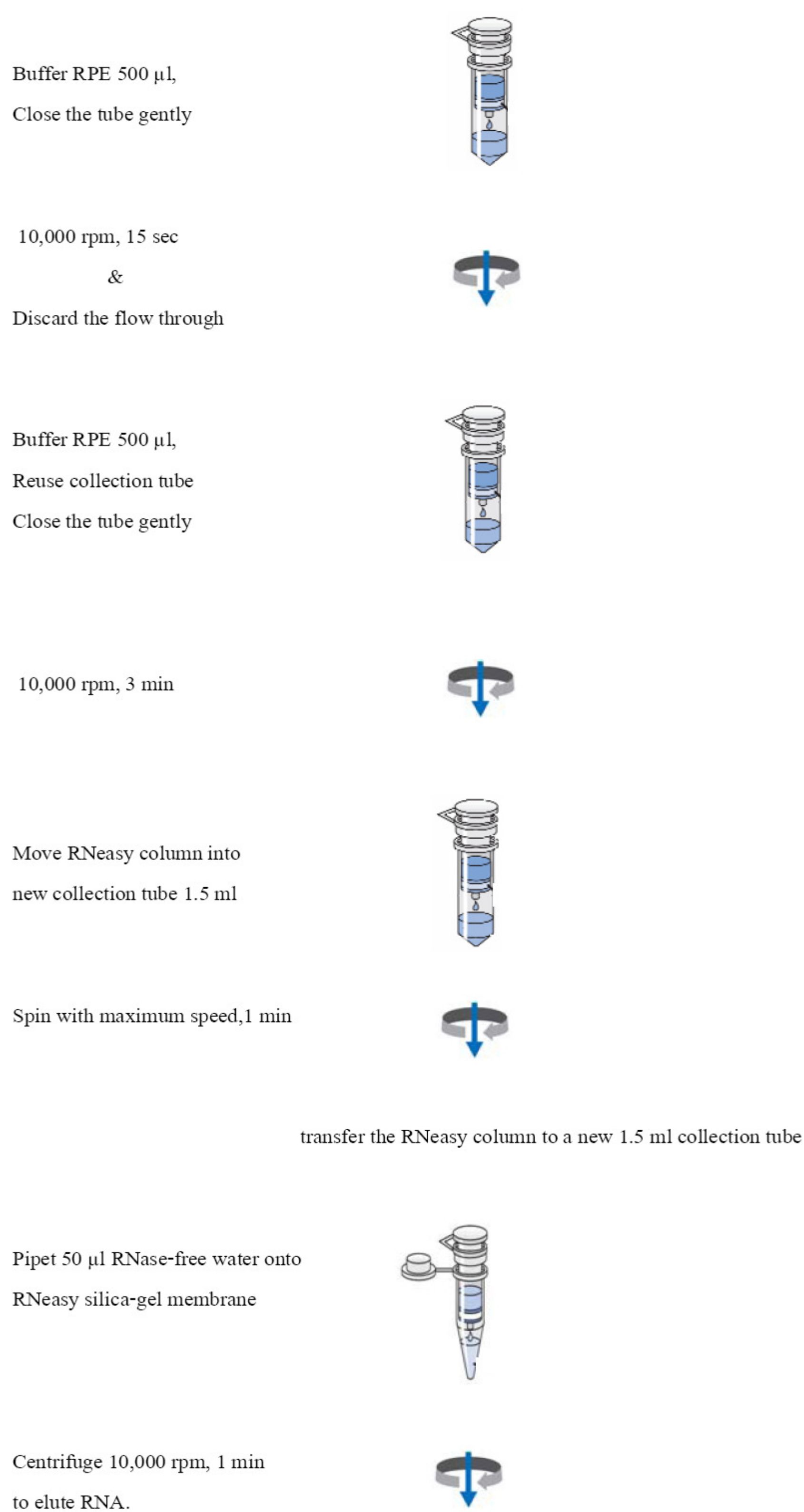

Figure 2.1 RNA extraction procedures (continued)

2.1.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed to assess the expression of OPG, RANKL. The house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control in this study. The sequence of the primer was taken from Maddi *et al.*[152] RT-PCR procedure was performed with the QIAGEN ® OneStep RT-PCR Kit (Qiagen, Valencia, CA); reverse transcription and PCR were carried out sequentially in the same tube. According to the manufacturer's instructions, 2 μg of total RNA was used per RT-PCR reaction in a total volume of 50 μl (Table 2.3). A typical thermal cycler program, including steps for both reverse transcription and PCR, was designed according to the OneStep RT-PCR Kit protocol. RT-PCR primers and conditions for PCR were optimized, and a condition suitable for all genes was used (Table 2.4 and 2.5). RT-PCR products were electrophoresed through 1% agarose gel, stained with ethidium bromide, and photographed by the Molecular Analyst®/PC analysis software (BioRad, Hercules, CA, USA). The signals were quantified by Scion Image Analysis Programme and expression ratios were normalized according to GAPDH density.

Table 2.2 Components of master mix preparation

Components	**Volume /reaction**
Rnase free water	variable
5x QIAGEN OneStep RT-PCR buffer	10 μl
dNTP Mix	2.0 μl
Forward Primer (0.6 μM)	3 μl
Reward Primer (0.6 μM)	3 μl
QIAGEN OneStep RT-PCR Enzyme Mix	2.0 μl
Rnase inhibitor	0.5 μl
RNA	2 μg
Total volume	**50 μl**

Table 2.3 Sequences of bases of forward and reward primers

Gene	Sequence	Product size (bp)
GAPDH	F: GAAGTGGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	313
OPG	F: AGACTTTCCAGCTGCTGA R: GGATCTCGCCAATTGTGA	469
RANKL	F: CAGGAGACCTAGCTACAGA R: CAAGGTCAAGAGCATGGA	381

Table 2.4 Program the thermal cycler in RT-PCR

Step	Time	Temperature (^{0}C)
1. Reverse transcription	30 min	50
2. Initial PCR activation	15 min	95
3. 3-step cycling Denaturation Annealing Extension	 30 sec 30 sec 1 min	 94 55 72
4. Number of cycles	Repeat step 3 - 35 cycles	
5. Final extension	10 min	72
6. Stop	∞	4

2.2 Effect of Emdogain on osteoclast formation

2.2.1 Osteoblast-like cell MG-63 cell culture

MG-63 human osteoblast-like cells from ATCC (Rocksville, MD) were used at passage 13. They were grown in DMEM supplemented with 10% FBS, 50 U/ml mycostatin, 100 μg/ml kanamycin, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mM L- glutamine in humidified atmosphere of 95% air/5%CO_2 at 37^0C. After 3 days in culture, the cells were passaged with Trypsin-EDTA (Gibco), counted and plated at a density of $2x10^4$ cells/cm^2 in 24 well-plates. The cells were maintained in 3 ml DMEM and condition as described above for 24 h for further cocultured with PBMCs.

2.2.2 Isolation of peripheral blood mononuclear cells (PBMCs)

To isolate the PBMCs, human blood (Buffy Coats) was diluted in PBS. On 15 ml of lymphoprep, 25 ml of diluted blood was carefully layered in 50 ml tubes. The tubes were then centrifuged in a Sorvall® RT 6000D centrifuged for 30 minutes at 1208 g. The white interphase, which contains the PBMCs, was collected and transferred to a 50 ml tube. Cells were washed twice with PBS and centrifuged at 400 g for 10 minutes. Cells were then resuspended in 1 ml DMEM with 10% FBS, 50 U/ml mycostatin, 100 μg/ml kanamycin, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mM L- glutamine. Before the cells went on to the further separation, they were counted and filled out in the proper amounts.

2.2.3 Cocultures of osteoblast-like cell MG-63 and peripheral blood mononuclear cells (PBMCs)

PBMCs, $1x10^6$cells/well was cocultured with osteoblast-like cell MG-63. The medium for the cocultures were DMEM supplemented with 10% FBS, 50 U/ml mycostatin, 100 μg/ml kanamycin, 100 μg/ml streptomycin, 100 U/ml penicillin, 10^{-8}M 1α, 25 dihydroxycholecalciferol (1α, $25(OH)_2D_3$: Sigma Chemical Co., St. Louis, MO) and10^{-7}M dexamethasone (Dex: Sigma Chemical Co., St. Louis, MO). EMD 100 μg/ml was added only to the experiment groups. The medium was changed every 2 days. After 14 days incubation, cell cultures were characterized histochemically for the expression of Tartrate Resistant Acid Phosphatase (TRAP) using a commercially available kit (Sigma Chemical Co., St. Louis, Mo). The cells were wash twice with PBS and were fixed in PBS buffered Paraformaldehyde 4% for 10 min. TRAP solution were

prepared following the manufacturer's instructions. 25 µl Fast Garnet GBC Base solution was mixed with 25 µl Sodium nitrate solution and kept for two minutes in a dark room. Mixed this solution with 4.5 ml of double distilled water at 37 ^{0}C, 50 µl Naphthol AS-BI solution, 200 µl Acetate solution and 250 µl of 1 M Tartrate solution. Then 500 µl of TRAP solution was added to the well and was incubated for 30 minutes at room temperature in a dark room. This is because Naphthol AS-BI solution is light sensitive. Finally washed the cells with double distilled water at 37 ^{0}C to stop the enzyme reaction. The cytoplasm of the TRAP^{+} cells was strained red. Stained cultures were examined under light microscopy at a magnification of x200. TRAP^{+} cells were considered as osteoclasts. We determined the number of TRAP^{+} cells; with three or more nuclei. The numbers of TRAP^{+} cells were expressed as the mean ± standard deviation [134] of duplicate experiments. Significant differences were determined using Student's *t*-test.