Chapter – 2

MATERIALS
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MATERIALS AND METHODS

2.1 Materials

Anti-human IgG alkaline phosphate conjugate, bovine serum albumin (BSA), calcitonin (CT), chloroform (CHCl₃), Coomassie Brilliant Blue G-250 and R-250, dithiothreitol (DTT), epigallocatechin-3-gallate (EGCG), ethidium bromide (EtBr), isoamyl alcohol, monoclonal anti-TNF-α antibody, phenylmethylsulphonyl fluoride (PMSF), p-nitrophenyl phosphate (pNPP), recombinant human (rh) TNF-α, reduced glutathione (GSH) and glutathione reductase (GR) and SN50 (an inhibitor of NF-κB) and its analogue SN50/M, sodium azide (NaN₃), soluble N-acetyl-cysteine, Hydrogen peroxide (H₂O₂), standard protein markers and tween-20 were from Sigma Chemical Company, U.S.A. SN50 is a hybrid peptide containing nuclear localization sequence of p50 subunit of NF-κB heterodimer and has been shown to completely inhibit the translocation of NF-κB in human cell lines at 100 µg/ml [Lin et al., 1995]. Resveratrol and Curcumin were also of Sigma Chemical Company, U.S.A., whereas Allicin was obtained from LKT Laboratories, Inc. U.S.A. Ficoll-Paque was from Pharmacia, Piscataway, NJ, U.S.A. RPMI-1640 and α-MEM medium were from HiMedia, India. MTT cell viability assay kit and immunoassay kits for Osteopontin and TNF-α were from R & D Systems, U.S.A., and that for soluble RANKL was of Enzo life Sciences, U. S. A. Furthermore, 12-wells tissue culture plates were obtained from Techno Plastic Products (TPP), Switzerland. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were from NUNC, Denmark. All other chemicals were of the highest analytical grade available.

2.1.1 Study subjects:

Venous blood was obtained from healthy volunteers of both sexes (n=30) with no history of osteoporosis. Also, blood from patients with osteoporosis (n=50) was obtained from the patients attending J.N. Medical College Hospital of A.M.U. Serum was separated and stored at –20 ºC until required. The study had clearance from the Institutional Ethical Committee.
2.2 Methodology

2.2.1 Determination of protein concentration:

Protein was estimated by the methods of Lowry and Bradford [Lowry et al., 1951; Bradford, 1976].

2.2.1.1 Protein estimation by the Lowry (Folin-Ciocalteau) method:

Protein estimation by this method involves complexing of the protein’s peptide bonds with Cu$^{2+}$ under alkaline conditions [Lowry et al., 1951]. The resultant Cu$^+$ appears to catalyze the oxidation of tyrosine and tryptophan residues by reducing phosphomolybdate anions in the Folin reagent (a mixture of sodium tungstate, molybdate and phosphate), added subsequently. This reaction develops a blue color due to the formation of heteropolymolybdenum blue, which can be quantified by its absorbance at 660 nm.

2.2.1.1.1 Reagents:

2.2.1.1.1.1 Folin-Ciocalteau Reagent

The reagent was diluted 1:4 with distilled water before use.

2.2.1.1.1.2 Alkaline Copper Reagent

The components of alkaline copper reagent were prepared as follows:

(a) 2% sodium carbonate in 100 mM NaOH

(b) 0.5% copper sulphate in 1% sodium potassium tartrate

The working reagent was prepared fresh before use by mixing the two components in the ratio 50:1, respectively.

2.2.1.1.2 Procedure:

To 1.0 ml of protein sample was added 5.0 ml of freshly prepared alkaline copper reagent. After thorough mixing, the reaction mixture was allowed to stand at room
temperature for 10 minutes, followed by the addition of 1.0 ml of 1:4 times diluted Folin-Ciocalteau reagent. The contents were mixed immediately. The reaction was allowed to proceed for 30 minutes at room temperature and each tube was subsequently monitored at 660 nm. The protein content of the unknown sample was determined by using bovine serum albumin to construct a standard calibration curve.

2.2.1.2 Protein estimation by the Bradford method:

This method is based on strong binding of the dye Coomassie Brilliant Blue G-250, in acidic medium, to protein hydrophobically and at positively charged groups [Bradford, 1976]. In the environment of these positively charged groups, protonation is suppressed and a blue color develops ($\lambda_{\text{max}}$-595 nm).

2.2.1.2.1 Preparation of dye:

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1.0 liter and filtered through Whatman No. 1 filter paper to remove undissolved particles.

2.2.1.2.2 Procedure:

To 1.0 ml of solution containing 10–100 µg protein was added 5.0 ml of dye solution. The contents were mixed thoroughly by vortexing. The absorbance was read at 595 nm after 5 minutes against a reagent blank.

2.2 Polyacrylamide gel electrophoresis of proteins:

Polyacrylamide gel electrophoresis was performed under denaturing conditions as described by Laemmli [Laemmli, 1970].

2.2.2.1 Reagents:

2.2.2.1.1 Acrylamide-Bisacrylamide (30:0.8)

A stock solution of 30% acrylamide containing 0.8% bisacrylamide was prepared by dissolving 30 gm of acrylamide and 0.8 gm of bisacrylamide in a total volume of 100 ml. The solution was stored at 4°C in an amber colored bottle.
2.2.2.1.2 Resolving Gel Buffer

A stock solution was prepared by dissolving 36.3 gm Tris base in 48 ml of 1 N HCl. The contents were mixed, pH adjusted to 8.8 and the final volume brought to 100 ml with distilled water.

2.2.2.1.3 Stacking Gel Buffer

6.05 gm Tris was dissolved in 40 ml distilled water, pH adjusted to 6.8 with 1 N HCl and the final volume adjusted to 100 ml with distilled water.

2.2.2.1.4 Electrode Buffer

3.03 gm Tris, 14.4 gm glycine and 1.0 gm SDS were dissolved in distilled water, pH adjusted to 8.3 and the final volume made up to 1.0 liter with distilled water.

2.2.2.1.5 Sample Buffer

(a) 6.0 gm of Tris was dissolved in 80 ml distilled water and pH adjusted to 6.8 with phosphoric acid. The final volume was brought to 100 ml with distilled water.

(b) 1.0 mg of bromophenol blue and 12.5 ml of glycerol were added to 12.5 ml of the above solution. β-mercaptoethanol was added just before use.

2.2.2.2 Recipe for 10–20% Gradient Gel

Table 4: Resolving Gel (total volume: 30 ml).

<table>
<thead>
<tr>
<th>Reagents</th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide (30:0.8)</td>
<td>5.0 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>3.8 ml</td>
<td>3.8 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>150 µl</td>
<td>150 µl</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

The final volume was raised to 15 ml each with distilled water.
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Table 5: 2.5 % Stacking Gel (total volume: 10 ml).

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide (30:0.8)</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 μl</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>75 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>25 μl</td>
</tr>
</tbody>
</table>

The final volume was raised to 10 ml with distilled water.

Table 6: Recipe for 7.5% SDS-PAGE (total volume: 10 ml).

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide (30:0.8)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 μl</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>50 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

The final volume was raised to 10 ml with distilled water.

**2.2.2.2 Procedure:**

The glass plates (18 cm × 16 cm) were soaked in chromic acid and thoroughly washed with tap water followed by a final rinse with distilled water and ethanol. The plates were dried and sealed with 1% agarose and 1.5 mm thick spacers. The reagents were mixed and poured between the glass plates. The resolving gel was allowed to polymerize at room temperature, following which, the stacking gel was layered on top. A well-forming comb was inserted immediately and the gel was left to polymerize at room temperature. In case of gradient gels, a gradient of resolving gel was formed with the help of a gradient former (Bio-Rad, model 385). After ensuring complete polymerization, the protein samples (25–100 μg) in one-fourth volume of sample buffer were electrophoresed at 80 volts at room temperature. The gels were stained using 0.25 % Coomassie Brilliant Blue R-250 or with silver stain reagent.
2.2.2.4 Silver staining:

Silver staining was done by the method of Merril [Merril et al., 1983]. Briefly, the gel was incubated in 40% methanol and 12% acetic acid for 45 minutes followed by incubation in 50% ethanol for 30 minutes. Next the gel was treated with 0.02% hypo (sodium thiosulphate) for 1 minute. After washing with distilled water, the gel was placed in 0.2% silver nitrate (with 0.05% v/v formaldehyde), washed again with distilled water, and transferred to a 6% solution of sodium carbonate (with 0.05% v/v formaldehyde). After color development, the gel was washed with distilled water and the reaction was arrested by treating the gel with 3% v/v acetic acid and 5% v/v methanol. All the reagents used in this procedure were freshly prepared.

2.2.3 Preparation of RPMI-1640 medium:

Dehydrated RPMI-1640 medium of one unit vial (16.3 gm) was suspended in 950 ml of tissue culture-grade water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade water to remove all traces of powder and added to the above solution. 3.7 gm sodium bicarbonate was added to the medium and stirred until dissolved. The final volume was brought to 1000 ml with tissue culture grade water. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron using positive pressure rather than vacuum to minimize the loss of carbon dioxide, and stored at 4 °C till use.

2.2.4 Preparation of PBMC:

**Step 1:** In order to isolate peripheral blood mononuclear cells (PBMCs), 30 ml of blood was drawn from a healthy volunteer and patients into 60 cm³ syringes containing 3.8 units heparin/ml. Prior consent was taken from such donors. The heparinized blood, in 15 ml aliquots, was transferred to sterile 50 ml polypropylene centrifuge tubes and diluted 1:1 with sterile 10 mM, PBS, pH 7.4 at room temperature, followed by gentle mixing by inverting the tube a few times.
Step 2: Diluted blood was under layered with 15 ml of Ficoll-Paque at room temperature using an 18 gauge spinal needle. Care was taken to prevent mixing of the layers. The gradient was centrifuged at 1800 rpm for 30 minutes at room temperature with the centrifuge brake turned off.

Step 3: Using a sterile pipette, the upper clear layer containing plasma was removed. The PBMCs appeared as a dense white band (buffy layer) above the red blood cells and granulocytes layer. This was removed with another sterile pipette. The banded cells were combined in 10 ml aliquots.

Step 4: Ten milliliters of banded PBMCs were diluted with 25 ml of PBS in sterile 50 ml polypolyrene centrifuge tubes and centrifuged at 1100 rpm for 12 minutes at room temperature to remove platelets, which remain in the supernatant. The PBMC pellets were combined to four tubes, diluted in 30 ml PBS and centrifuged at 1100 rpm for 10 minutes at room temperature. This wash was repeated.

Step 5: The pellets were then combined and resuspended in 30 ml complete medium (CM) (RPMI-1640 medium containing 2 M L-glutamine, 25 mM HEPES, and no antibiotics). An aliquot was diluted 20-fold and counted using a hemocytometer under a light microscope using 10 x ocular and 40 x objectives.

2.2.5 Preparation of autologous serum for monocyte culture:

From the same donor, 30 ml of blood was drawn without anticoagulant and transferred to serum separator tubes. The blood was allowed to clot for at least 30 minutes, then centrifuged at 3000 rpm for 15 minutes at room temperature and the serum filtered through a sterile 0.22 µm filter unit. Autologous serum can be stored for a year or longer at –20°C.

2.2.6 Assay for Cellular Glutathione (GSH) Content

Glutathione was measured according to the method described by Anderson [Anderson, 1985]. Adherent monocytes obtained from PBMCs of healthy and
osteoporosis patients, treated with Allicin/Resveratrol/Curcumin, were collected (600 g, 7 min, 4°C) and washed with PBS. After protein precipitation with 5% metaphosphoric acid (0.2–0.3 ml/5×10^6 cells) by centrifugation (10,000g, for 30 min), the supernatant was used for GSH quantitation, and the pellet, dissolved in 0.2–0.3 ml of 0.5 M NaOH, was used for protein determination. Samples (40 µl) were neutralized with 2 M triethanolamine (10 µl) in a 96-well plate. The reaction was started by adding 200 µl per well of 0.4 U/ml Glutathione reductase enzyme in 143 mM phosphate buffer pH 7.5 containing 0.3 mM reduced nicotinamide adenine dinucleotide phosphate, 0.6 mM DTNB and 6.25 mM EDTA. The initial rate of 5-thio-2-nitrobenzoic acid formation was monitored. Similarly, effect of pretreatment of BSO (100 µM, 24 h) on the GSH content of monocytes from PBMCs, treated with or without Allicin/Resveratrol/Curcumin, was also determined.

**2.2.6.1 Assay for Glutathione Peroxidase (GPx) activity:**

The activity of GPx was measured as described elsewhere [Mohandas et al., 1984; Mates et al., 1999]. The oxidized glutathione (GSSG) produced during GPx reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was regarded as the rate of GSSG formation during the GPx reaction. Monocytes from healthy donors and patients with osteoporosis were co-cultured for 24 hours with or without 10 mM NAC, 100 µg/ml SN50, 100 µg/ml SN50/M and 0–500 ng/ml allicin. Thereafter, cells were gently scraped with lysis buffer containing protease inhibitors (50 mM Tris/HCl, pH 7.4; 1 mM EDTA; 500 mM PMSF). Protein concentrations of supernatants were determined by the method of Bradford with BSA as the standard, and were subjected to GPx activity determination. The reaction mixture (1.0 ml) containing 50 mM potassium phosphate (pH 7.0), 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 unit/ml glutathione reductase, 1.5 mM cumene hydroperoxide, and 20–100 µl of samples were incubated at 25 °C for 5 minutes. The reaction was initiated by the addition of cumene hydroperoxide. The kinetic change was spectrophotometrically recorded at 340 nm (37 °C) for 3 minutes.
GPx activity was calculated after subtraction of the blank value, as µmol of NADPH oxidized/minute/mg protein (U/mg protein).

2.2.7 Determination of Malondialdehyde (MDA) Levels:

Determination of MDA levels were carried out as described by Kaur et al. [Kaur et al., 2012]. The lipid peroxide levels in treated/untreated monocyte cultures in healthy subjects as well as patients were measured by precipitating the lipoproteins with trichloroacetic acid and boiling them with thiobarbituric acid, which reacted with malondialdehyde to form a pink color, as per the ‘Kei satoh’ method [Satoh, 1978]. The resulting chromogen was extracted with n-butyl alcohol and the absorbance of the organic phase was determined at the wavelength of 530nm. The determined values were expressed in terms of malondialdehyde in nmol/ml.

2.2.8 Cell culture:

PBMCs (5×10^6 cells/well) were added in 12-wells tissue culture plates in complete RPMI-1640 medium, and were subsequently incubated at 37°C, 5% CO₂ for 1–2 hours for adherence. Thereafter, non-adherent cells were removed by washing the plates extensively 4 times with RPMI-1640 medium. The adherent monocytes were cultured in RPMI-1640 supplemented with 2% autologous serum, followed by overnight resting at 37°C, 5% CO₂. This population of adherent cells is up to 95% monocytes, as observed by cytostaining and is 99% viable [Toossi et al., 1996]. Prior to treatment with supplements/compounds, the plates were washed twice with RPMI-1640 medium.

2.2.9 Treatment with Allicin / Resveratrol / Curcumin and monocytes viability assay:

The effect of allicin (0–500 ng/ml), Resveratrol (0-20 µg/ml) and Curcumin (0-20 µg/ml) on the viability of monocytes was assessed by using MTT Cell Viability Assay Kit (R & D Systems) according to the manufacturer’s instructions provided.
Table 7: Reagents supplied in the MTT cell viability assay kit.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT reagent</td>
<td>25 ml</td>
<td>2 – 8°C</td>
</tr>
<tr>
<td>Detergent reagent</td>
<td>250 ml</td>
<td>18 – 24°C</td>
</tr>
</tbody>
</table>

2.2.9.1 Assay Procedure:

Adherent monocytes from healthy donors and patients with osteoporosis were gently scraped with RPMI-1640 medium. After this, monocytes (3 × 10^4 cells/well in 100 µl) were added in 96-well tissue culture plates. Cells were incubated in RPMI-1640 with 2% autologous serum containing allicin (0-1000 ng/ml) resveratrol and curcumin (0-50 µg/ml) respectively for 24 hours at 37°C, 5% CO₂. After 24 hours, 10 µl of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well and incubation was continued for an additional two hours. When a purple precipitate was clearly visible under the microscope, 100 µl of detergent reagent was added to all wells, including control wells and incubated for two hours in the dark at 20°C. After incubation, the precipitate was solubilized and the absorbance of the resulting solution was measured at 570 nm using a micro plate reader. Control cells were treated exactly the same except that no allicin/resveratrol/curcumin was added to the wells. The percentage of viable cells was calculated by the formula as described by [Islam et al., 2000] and the results are expressed as “Viable cells (% of control cells)”.

\[
\text{Viable monocytes (% of control cells)} = \frac{\text{Absorbance of control cells}}{\text{Absorbance of treated cells}} \times 100
\]

2.2.10 Trypan Blue Exclusion Assay for Monocytes Viability:

Adherent monocytes were gently scraped with RPMI-1640 medium. Trypan blue suspension (1.6 mg/ml in saline solution) was added to the monocytes at a final concentration of 0.8 mg/ml. The cells were kept at 37°C for 7 minutes in a CO₂ chamber (5%), mounted on a hemocytometer and then observed under light microscope. The cells taking up Trypan blue (dead cells), and cells excluding the dye
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(viable cells) were counted. Percentage of viable cells was calculated by the following formula:

\[
\text{% cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained + unstained)}} \times 100
\]

2.2.11 Culture Conditions For Human Osteoclastogenesis Assays:

Whole population of freshly isolated PBMNC was plated in 96-well plates at \(6 \times 10^5\) cells per well in 0.2 ml of medium (\(\alpha\)-MEM, Gibco, pH 7.4, containing 10% FCS from Amimed, batch #S03485). Medium was also supplemented with the following cytokines, growth factors and hormones: 25 ng/ml human M-CSF (R&D Systems, Abingdon, UK), 50 ng/ml human RANKL (Insight Biotechnology, Wembley, UK), 5 ng/ml human TGF-\(\beta\)1 (R&D Systems, Abingdon, UK), and 1 \(\mu\)M dexamethasone (Sigma, Buchs, Switzerland). The cells were re-fed twice weekly by demi-depletion (half of the medium withdrawn and replaced with the fresh medium). The culture duration was 17 days for TRAP staining.

2.2.12 Effect of Natural antioxidants like Allicin from Garlic, Resveratrol, Curcumin and Epigallocatechin gallate (EGCG) on the generation of Osteoclasts from PBMCs:

To investigate the effect of natural antioxidants on osteoclast generation from PBMCs, varying doses of Allicin (0-500 ng/ml), Resveratrol (0-25 \(\mu\)g/ml), Curcumin (0-25 \(\mu\)g/ml) and EGCG from green tea (0-20 \(\mu\)g/ml) were added to the PBMC cultures seeded at a cell density of 2x10^5 cells/cm^2 in a 96-well plate in an osteoclastogenic medium as described above and incubated for 24 h (1 day), 72 h (3 day) and 120 h (5 days) at 37°C in a humidified atmosphere of 5% CO2. Thereafter, the cells were analysed with TRAP staining.

2.2.13 TRAP staining: quantification of TRAP-positive multinucleated cell.

TRAP staining of adherent cultures was done with a kit from Sigma (Buchs, Switzerland) exactly according to manufacturer's instruction. The stained cells developed red color of different intensity. The numbers of TRAP-positive
multinucleated cells were determined using the 1 × 1-mm grid placed in the ocular of the microscope. For TRAP assay, Zeiss Axiovert 100 microscope (Zeiss, Oberkochen, Germany) was used. The number of TRAP-positive multinucleated cells (>2 nuclei per cell) was measured at predetermined sites of the area of 1 × 1 mm. Five sites were measured in a well of a 96-well plate, and a mean value was calculated. Four wells were measured in total per one condition and these results were expressed as mean ± SEM.

2.2.14 Determination of soluble RANKL in Monocyte Culture Supernatants:

The concentration of sRANKL in various 3 days monocyte culture supernatants of osteoporosis patients was determined by use of a commercial ELISA Kit (Enzo Life Sciences). The reagents used were those supplied in the kit.

2.2.14.1 Reagents:

1. Prior to use, allow all reagents and samples to come to room temperature and mix well

2. One holder with precoated strips 12 x 8 wells

3. Wash buffer concentrate

4. SOL: OPG-Solution, ready-to-use 5.5 ml

5. STD: Standard, concentrate

6. CTRL:Control, ready-to-use

7. AB: Detection antibody, biotinylated

8. CONJ:Conjugate, streptavidin peroxidase-labeled

9. SUB: TMB substrate (Tetramethylbenzidine), ready-to-use

10. STOP: ELISA stop solution, ready-to-use
2.2.14.2 Assay procedure:

1. Prior to use, allow all reagents and samples to come to room temperature and mix well.
2. Mark the positions of STD/SAMPLE/CTRL (Standards/Sample/Control) in duplicate on a protocol sheet.
3. Take microtitre strips out of the kit. Store unused strips covered at 2-8°C. Strips are stable until the expiry date stated on the label.
4. Wash 5 times by dispensing 250 µl of dilute WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper.
5. Add 50 µl of STD/SAMPLE/CTRL (Standard/Sample/Control) in duplicate into respective well. Use the wash buffer as STD 0 pg/ml.
6. Add 50 µl SOL (OPG-solution) into each well.
7. Cover the plate tightly and incubate for 16 - 24 hours at 2 - 8°C.
8. Discard the contents of each well. Wash 5 times by dispensing 250 µl of dilute WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper.
9. Add 100 µl detection antibody into each well.
10. Cover the plate tightly and incubate for 2 hours at room temperature.
11. Discard the contents of each well. Wash 5 times by dispensing 250 µl of dilute WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper.
12. Add 100 µl CONJ (conjugate) into each well.
13. Cover the plate tightly and incubate for 1 hour at 2 - 8°C.
14. Discard the contents of each well. Wash 5 times by dispensing 250 µl of dilute WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper.
15. Add 100 µl of SUB (substrate) into each well.
16. Incubate for 20-30 minutes at room temperature (18-26°C) in the dark.
17. Add 50 µl of STOP (stop solution) into each well, mix thoroughly.
18. Determine absorption immediately with an ELISA reader at 450 nm against 620 nm (or 690 nm) as a reference. If no reference wavelength is available,
read only at 450 nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at 405 nm against 620 nm as a reference. The sensitivity is 1.56 pg/ml.

2.2.15 Determination of Osteopontin (OPN) in Monocyte Culture Supernatants:

The concentration of Osteopontin in various 3 days monocyte culture supernatants of osteoporosis patients was determined by use of a commercial ELISA Kit (R & D Systems).

2.2.15.1 Reagents supplied in the kit:

**Assay Diluent RD1-6:** 11 mL/vial of a buffered protein base with preservatives. May contain a precipitate. Mix well before and during use.

**Calibrator Diluent RD5-24:** 21 mL/vial of a buffered protein base with preservatives.

**Color Reagent A:** 12 mL/vial of stabilized hydrogen peroxide.

**Color Reagent B:** 12 mL/vial of stabilized chromogen (tetramethylbenzidine).

**OPN Conjugate:** 21 mL/vial of a polyclonal antibody against OPN conjugated to horseradish peroxidase with preservatives.

**OPN Micro plate:** 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against OPN.

**OPN Standard:** 200 ng/vial of recombinant human OPN in a buffered protein base with preservatives; lyophilized.

**Plate Sealers:** Adhesive strips.

**Stop Solution:** 6 mL/vial of 2 N sulfuric acid

**Wash Buffer Concentrate:** 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.
2.2.15.2 Reagent Preparation:

Bring all reagents to room temperature before use.

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

**OPN Standard** - Reconstitute the OPN Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 200 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 540 µL of Calibrator Diluent RD5-24 into the 20 ng/mL tube. Pipette 300 µL of Calibrator Diluent RD5-24 into the remaining tubes. Use the stock solution to produce a dilution series. Mix each tube thoroughly before the next transfer. The 20 ng/mL standard serves as the high standard. Calibrator Diluent RD5-24 serves as the zero standard (0 ng/mL).

**Sensitivity:** The minimum detectable dose of OPN ranged from 0.006-0.024 ng/mL. The mean minimum detectable dose was 0.011 ng/mL.

2.2.15.3 Assay Procedure:

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 100 μL of Assay Diluent RD1-6 to each well. Assay Diluent RD1-6 may contain a precipitate. Mix well before and during use.

4. Add 50 μL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 μL of OPN Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

9. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.
2.2.16 Enzyme linked immunosorbent assay (ELISA):

Antibodies were detected and quantified by ELISA using polystyrene flat bottom microtiter plates as solid phase [Alam and Ali, 1992; Islam and Ali, 1998]. The method described by [Islam and Ali, 1998; Arif et al., 1994] was followed for the assay.

2.2.16.1 Buffers and Reagents:

(a) Bicarbonate buffer: 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6

(b) Substrate buffer (for anti-human IgG alkaline phosphate conjugate): 15 mM sodium carbonate, 35 mM sodium bicarbonate and 2 mM MgCl₂, pH 9.6.

(c) Substrate: 0.5 mg/ml of \textit{p}-nitrophenyl phosphate (\textit{p}-NPP).

(d) Tris buffered saline (TBS): 10 M Tris, 150 mM NaCl, pH 7.4

(e) Tris buffered saline tween-20 (TBS-T): 20 M Tris, 144 mM NaCl, 2.68 mM KCl and 1.0 ml/litre Tween-20, pH 7.4

2.2.16.2 Direct binding ELISA:

Polystyrene micro titer plates were incubated with 100 µl of protein antigen (30 µg/ml in carbonate/bicarbonate buffer, pH 9.6) for two hours at room temperature followed by overnight incubation at 4 ºC. The plates were washed thrice with TBS-T and unoccupied sites blocked by 150 µl of BSA (1.5% in TBS, pH 7.4) for 4-6 hours at room temperature. Serially diluted sera in TBS were added to antigen-coated as well as control (antigen uncoated) wells. The antigen-antibody interaction was allowed to proceed for two hours at room temperature followed by overnight incubation at 4 ºC and subsequently the plates were washed four times with TBS-T in order to remove the unbound antibodies. Bound antibodies were assayed by means of appropriate anti-immunoglobulin alkaline phosphatase conjugate using \textit{p}-nitrophenyl phosphate as substrate. The reaction was stopped with 3.0 N NaOH and the absorbance of each well was monitored at 405 nm on an ELISA microplate reader. Each sample was
coated in duplicate and the results were expressed as a mean of $A_{\text{test}} - A_{\text{control}}$. For nucleic acid antigen, the plates were coated with 100 µl of calf thymus DNA at a concentration of 2.5 µg/ml in TBS, pH 7.4 and incubated for two hours at room temperature followed by overnight incubation at 4 °C. The rest of the steps were same as described above.

2.2.16.3 Inhibition ELISA:

The antigen binding specificity of antibody was determined by inhibition experiments [Hasan et al., 1991]. Varying concentration of inhibitors (0-20 µg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated for two hours at 37 °C followed by overnight incubation at 4 °C. The resulting immune complex was employed in the immunoassay instead of serum. The rest of the steps were as in direct binding ELISA. The results were expressed as percent inhibition.

$$\% \text{ inhibition} = \left(1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \right) \times 100$$

2.2.17 TNF-α Immunoassay:

The concentration of TNF-α in various culture supernatants as well as in serum of osteoporosis patients was determined by use of a commercial ELISA Kit (R & D Systems). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α was available pre-coated onto a micro plate. Standards and samples were pipetted into the wells and any soluble TNF-α present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of TNF-α bound in the initial step. The color development was stopped and the intensity of the color was measured.
2.2.17.1 Reagents supplied in the kit:

(a) **Assay diluent RD1F** - 6 ml of a buffered protein base with preservatives. It contained a precipitate and was mixed well before and during use.

(b) **Calibrator diluent RD6-35** - 21 ml of animal serum with preservatives.

(c) **Colour reagent A** - 12.5 ml of stabilized hydrogen peroxide.

(d) **Colour reagent B** - 12.5 ml of stabilized chromogen (tetramethylbenzidine).

(e) **Plate covers** - 4 adhesive strips.

(f) **Stop solution** - 6 ml of 2 N sulphuric acid (H₂SO₄).

(g) **TNF-α conjugate** - 21 ml of polyclonal antibody against TNF-α conjugated to horseradish peroxidase, with preservatives.

(h) **TNF-α microplate** - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against TNF-α.

(i) **TNF-α standard** - 10 ng of recombinant human TNF-α in a buffered protein base with preservatives, lyophilized.

(j) **Wash buffer concentrate** - 21 ml of a 25-fold concentrated solution of buffered surfactant with preservatives.

2.2.17.2 Working reagents:

(a) **Diluted calibrator diluent RD6-35** - 20 ml of calibrator diluent RD6-35 was mixed with 80 ml of deionized or distilled water to yield 100 ml of diluted calibrator diluent RD6-35.

(b) **Substrate solution** - Colour reagents A and B were mixed together in equal volumes within 15 minutes of use to form substrate solution. It was protected from light.
(c) **TNF-α standard** - TNF-α standard was reconstituted with 1.0 ml of distilled water. This reconstitution produced a stock solution of 10,000 pg/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

(d) **Wash buffer** - 20 ml of wash buffer concentrate was diluted into deionized or distilled water to prepare 500 ml of wash buffer.

**2.2.17.3 Assay Procedure:**

50 µl of assay diluent RD1F was added to each well of 96 well polystyrene microplate coated with a mouse monoclonal antibody against TNF-α. Thereafter, 200 µl of standards, samples, or control per well was added, covered with the adhesive strip provided and incubated for 2 hours at room temperature. The plate was washed four times by filling each well with wash buffer using a squirt bottle. After washing, 200 µl of TNF-α conjugate was added to each well, covered with a new adhesive strip and incubated for 1 hour for cell culture supernatants and 2 hours for serum samples at room temperature. After four washings with wash buffer, 200 µl of substrate solution was added to each well and incubated for 20 minutes at room temperature in the dark, a blue colour appeared. Thereafter, 50 µl of stop solution was added to each well to stop the reaction. Then the absorbance of each well was determined within 30 minutes, using a microplate reader set at 450 nm. The cut off or lower limit of sensitivity was 4.4 pg/ml.

**2.2.18 Interleukin-1β (IL-1β) Immunoassays**

The concentration of IL-1β in monocytic culture supernates were determined by Quantikine Human IL-1β Immunoassay Kits (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer’s instruction. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-1β has been pre-coated onto a microplate. Standards and culture supernatants are pipetted into the wells and any IL-1β present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-1β is added to the wells. Following a wash to remove any unbound
antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-1β bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.2.19 RNA extraction:

After lysis of monocytes in 0.5 ml TRIZOL reagent, 200 µl of chloroform was added to each sample, followed by vortexing for 2 minutes, and centrifugation at 3000 rpm for 5 minutes. Samples were then transferred to fresh eppendorf tubes and centrifuged at 14,000 rpm for 15 minutes at 4 °C. The aqueous layer was harvested and transferred to a fresh tube. The aqueous layer obtained was mixed with 500 µl of chloroform-isoamyl alcohol (24:1) and vortexed. RNA was precipitated using 50 µl of 1 M sodium acetate, and 475 µl of isopropanol at –20 °C for 3 hours in the presence of glycogen. This was followed by centrifugation at 14,000 rpm and the pellet obtained was washed two times with 75% ethanol, and resuspended in 87 µl DEPC-water. DNAase 1 digestion (10 µl of 10X DNAse 1 buffer in 0.5 M Tris pH 7.5, 0.1 M MgCl2, 1 mM DTT; and 50 µg/ml BSA, 2.0 µl RNAase inhibitor; 10U RNAase free DNAase 1) was employed to remove DNA. The reaction was stopped by the addition of 700 µl of 0.5 M Na2OAc and the RNA was re-extracted using 500 µl of acid phenol-chloroform (1:1). The aqueous layer was harvested, extracted again with chloroform-isoamyl alcohol and precipitated.

2.2.20 Reverse transcriptase polymerase chain reaction (RT-PCR):

The DNAase-treated RNA was subjected to reverse transcription using oligo (dT) primers with Superscript II reverse transcriptase (Invitrogen, Life Technologies, USA) according to the manufacturer’s instructions. RNA (2 µg) was transcribed into cDNA in a 20 µl reaction volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM of each deoxynucleotide triphosphate, 25 µg/ml oligo(dT)12-18 primers and 10 U/µl of Superscript II reverse transcriptase, at 42 °C for 50 minutes. The reaction was then stopped by heating at 70 °C for 15 minutes followed by rapid chilling on ice.
2.2.21 Polymerase Chain Reaction (PCR):

The primers used in the PCR are listed below. cDNA for β-actin was amplified with various primer sets supplied by (Stratagene, La Jolla, CA, USA). For PCR, 2 µl of each cDNA sample was used as template in the PCR amplification. The reactions were carried out in a 50 µl reaction volume containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM of each of the four dNTPs, 2U of Taq DNA polymerase (Invitrogen) and 0.2 µM of each forward and reverse primers. After initial denaturation for 2 minutes at 95 °C, 35 cycles at 95 °C for 15 seconds, 60 °C for 45 seconds were performed, followed by 72 °C for 1 minute. The reaction products were visualized by electrophoresis in 2% agarose after staining with 0.5 µg/ml EtBr.

2.2.21.1 Human primer sequences for osteoclast markers.

18S rRNA:

Forward: 5’-ACGGGGAATCAGGGTTCGA-3’,
Reverse: 5’-CTCGAAAGAGTCCTGTATT-3’

TNF-α

TNF-α primer RT: GGTTTTCTACAACA

TNF-α primer R: GTTCGAGAAGATGATCTGACTGCC

TNF-α primer F: AGGCGGTGCTTGGTCCCTCA

OPG

OPG primer R: 5’-GGGGACCACAATGAACAAGTTG-3’,
OPG primer F: 5’-AGCTTGCACCACCTCCCAAATCC-3’,

2.2.22 Quantitative real-time RT-PCR:

Real-time quantitative reverse transcriptase PCR (RT-PCR) provides a sensitive, reproducible, and accurate method for determining mRNA levels in tissues or cells.
The method is based on the detection of a fluorescent signal produced and monitored during the amplification process, without the need for post-PCR processing [Heid et al., 1996]. Two important findings led to the discovery of real-time PCR. First, the Taq polymerase has a 5′–3′ exonuclease activity [Holland et al., 1991], apart from its polymerase activity. Second, dual-labelled fluorogenic oligonucleotide probes have been created which emit a fluorescent signal only upon cleavage, based on the principle of fluorescence resonance energy transfer [Cardullo et al., 1988]. In the TaqMan assay (Applied Biosystems, Foster City, CA, USA), these two principles are combined. In this system a probe, the so-called TaqMan probe, is designed to anneal to the target sequence between the classical forward and reverse primers. The probe is dually labeled, with a reporter fluorochrome (e.g., 5-carbofluorescein, or FAM) at one end and a quencher dye (e.g., N,N,N′,N′-tetramethyl-6-carborhodamine, or TAMRA) at the other end. In the intact probe, the fluorescence emission of the reporter dye will be absorbed by the quencher dye. The probe will be degraded during the extension phase by the 5′–3′ exonuclease activity of the Taq polymerase, separating the reporter and quencher, thus resulting in an increase in reporter fluorescence emission. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation.

2.2.22.1 Procedure:

Internal fluorescent hybridization probes were used in ABI Prism 7700 Detection System (ABI/PerkinElmer (PE) Biosystems, Foster City, CA, USA), which allows the sensitive and specific quantification of individual host [Hartel et al., 1999], by quantitative real-time RT-PCR. TaqMan™ PCR primers and probes as well as target-specific RT primer for each assay were designed as described elsewhere [Islam et al., 2004]. The primer and probe sequences used have been previously reported [Islam et al., 2004]. All probes were dually labeled with FAM at the 5′ end and TAMRA at the 3′ end. The proximity of the dye (FAM) and the quencher tetramethylrhodamine (TAMRA) on the intact probe prevents detection of any fluorescence. However, degradation of the probe during the course of PCR allows the release and detection of FAM [Holland et al., 1991]. The PCRs for all amplifications were similar: 5 µl of
Materials and Methods

Each cDNA, 20 µl of Taqman Universal PCR Master Mix (PE Biosystems), which contains optimal amounts of AmpliTaq Gold DNA polymerase (which protects against amplicon carryover) and of dNTPs, and optimal amounts of probe and primers calibrated to allow measurement of the targets. First, cDNA was synthesized in the presence of 0.5 µl of murine leukemia virus enzyme (Invitrogen, USA)/reaction and 10 µM each RT primer, dNTPs, and other substrate. Conditions for PCR were similar for all products (1 cycle of 2 minutes at 50 °C and 1 cycle of 10 minutes at 95 °C and then 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C). The cycle threshold for each sample was compared with the cycle threshold values of known amounts of a standard DNA constructed for each target and amplified simultaneously. To assure lack of DNA contamination in the RNA samples, in some experiments, a duplicate tube of sample with no RT enzyme was included as control. DNA contamination remained negligible. In each sample, host 18S ribosomal RNA was used as the internal control. Expression of TNF-α mRNA was corrected to internal control (host 18S rRNA) in the same sample and was expressed as copies of TNF-α in 10^{10} copies of R18 (equivalent to 1 × 10^6 monocytes).

2.2.23 Statistical analysis: Results were analyzed by paired t-test. P<0.05 was considered statistically significant.

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