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The Effects of Redox State on Osteoclast Differentiation

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1. Table of Contents

1. Table of Contents *2*

2. Abbreviations *4*

3. Abstract *6*

4. Introduction *7*

4.1 Bone remodeling **7**

4.2 Tartrate Resistant Acid Phosphatase **8**

4.3 Reactive Oxygen Species (ROS) **8**

4.4 Antioxidant systems **8**

4.5 Glutathione **9**

4.6 Cysteine **9**

4.7 X_c^- cystine/glutamate antiporter **9**

4.8 Thioredoxin system **10**

4.9 RAW 264.7 cell **11**

4.10 Aim of the project **11**

5. Materials and methods *12*

5.1 RAW 264.7 cell line **12**

5.2 Counting of RAW 264.7 cells **12**

5.3 Stimulation of RAW 264.7 cells **12**

5.4 TRAP Staining of RAW 264.7 cells **13**

5.5 Harvesting of cells for total RNA at day 5 ..... **13**

5.6 RNA purification **13**

5.7 Reverse Transcription (RT) reaction for cDNA synthesis **14**

5.8 PCR analyses of stimulated RAW 264.7 cells **14**

5.9 Glutathione and cysteine determination in RAW cells **15**

5. 9.1 Reduced form of glutathione and cysteine 15

5.9.2 Total form of glutathione and cysteine 15

5.10 Intracellular ROS production determination **15**

6. Results *16*

6.1 Optimization of RAW 264.7 cell culturing conditions during differentiation in the presence of redox modulators..... 16

6.2 Effects of redox modulators on RAW 264.7 cell differentiation to osteoclast and macrophage . 16

6.2.1 TRAP staining and morphological aspects 16

6.3 Gene expression study of RAW264.7 cells treated by redox modulator 18

6.3.1 Optimization of house keeping gene 18

6.3.2 Gene expression of RAW264.7 during osteoclast differentiation at presence of redox stimulators 22

6.4 Glutathione/cysteine measurement of treated RAW264.7 cells by redox stimulators 27

6.5 DCF experiment of RAW264.7 cell 30

7. Discussion 31

8. Acknowledgments 33

9. Bibliography 34

2. Abbreviations

PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
DTT	1, 4-Dithiothreitol
DTNB	5, 5'-Dithio-bis (2-nitrobenzoic acid)
TCEP	Tris (2-carboxyethyl) phosphine
MSG	Monosodium glutamate
RANKL	Receptor activator for nuclear factor κ B ligand
LPS	Lipopolysaccharide
RAW	Mouse leukaemic monocyte macrophage cell line
RNA	Ribonucleic acid
TRAP	Tartrate resistant acid phosphatase
Trx1	Thioredoxin-1
TrxR1	Thioredoxin reductase -1
CTR	Calcitonin receptor
Cat K	Cathepsin K
TbP	TATA box binding protein
4F2hc	4F2- heavy chain
PgK1	Phosphoglycerate kinase 1
Ppia	peptidylprolyl isomerase A
dNTP	Deoxyribonucleotide triphosphate
kDa	Kilo dalton
MEM	Minimum essential medium

DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
T75	Flask 75 cm^2 of surface
mRNA	Messenger ribonucleic acid
RNase	Ribonuclease
qPCR	Quantitative polymerase chain reaction
β-ME	β- Mercaptoethanol
RT	Reverse transcription
oligo dT	primer deoxythymidine
dH2O	Distilled water
HPLC	High performance liquid chromatography
GSH	Reduced glutathione
mBrB	Monobromo bimane

3. Abstract

Osteoclasts are derived from hematopoietic cells of the monocyte/macrophage lineage. They are responsible for the bone resorption process and they are non-dividing multinucleated cells. During the osteoclast differentiation process, cells lose their macrophage characteristics and express osteoclast-associated markers, such as calcitonin receptor and tartrate-resistant acid phosphatase (TRAP). Multinucleated cells are formed from mononuclear preosteoclasts that merge during the differentiation. During this process the redox state is altered and shifted towards a more oxidized state. Raw 264.7 cells differentiate to macrophages by addition of lipopolysaccaride (LPS) and differentiate to osteoclasts by addition of Receptor Activator for Nuclear Factor κ-B Ligand (RANKL). TRAP and NADPH oxidase (Nox) generate reactive oxygen species (ROS) during osteoclast differentiation. ROS play a central role in cell proliferation, activation, growth inhibition and apoptosis. ROS also has stimulating effects on bone resorption and differentiation of osteoclasts.

Differentiated osteoclasts are responsible for bone resorption. Balance of redox states inside and outside the cells plays a crucial role during cell differentiation. The aim of this project is to explore the importance of the redox environment and redox state during osteoclast differentiation by using Raw 264.7 cells. For this, we can develop an in vitro model system to study the effects of redox changes on osteoclasts and macrophages. The methods of cell culturing, TRAP staining, morphological evaluation, cell counting, RNA preparation, RNA quantification, cDNA synthesis and qPCR, measurement of glutathione/cysteine, and detection of ROS by DCF probes were used. Through understanding the changes of redox state during osteoclast differentiation we could understand how to control the redox balance in bone diseases.

The preliminary results indicate that alteration of the redox state in the extra- and intracellular environments affects osteoclast differentiation. This study shows the effects of some redox modulators and especially among them the effects of DTNB and MSG. MSG is a blocker of cystine uptake, which indicates an important role of cysteine for the differentiation of osteoclasts.

4. Introduction

The cellular redox state is crucial for the cell survival and it is essential for the cell to uphold a balance in the redox homeostasis (1). Reactive oxygen species (ROS) produced by various processes in the cell directly affects the redox state. The intra- and extra-cellular reactive oxygen species further have an essential effect and role in many cellular processes (2,3). During cell proliferation and differentiation, oxidoreduction of thiols (-SH) by thioredoxins (Trx), glutathione (GSH) and cysteine (Cys) regulates cell signaling due to functions of enzymes, receptors and transcription factors (2,4,5). Throughout osteoclast differentiation and bone resorption redox state is altered and shifted towards a more oxidative state (5,6,7).

4.1 Bone remodeling

Bone formation and bone resorption occur during bone homeostasis. These processes are associated with two types of bone cells: osteoclasts and osteoblasts. Osteoblasts, which are derived from osteoprogenitor cells, form and build the bone by secretion of bony matrix collagen fibers. The monocyte/macrophage lineage of hematopoietic cells in the bone marrow can differentiate into non-dividing multinucleated osteoclasts with numerous mitochondria, lysosomes, vacuoles and vesicles (8,9,10). The differentiated osteoclasts are capable of bone resorption (10). Tartrate resistant acid phosphatase (TRAP) and cathepsin K are the most important markers of the osteoclast (11,12). Factors that regulate osteoclastogenesis include colony stimulating factor-1, macrophage colony stimulating factor, transforming growth factor-β (TGF-β), receptor activator of NF-κB ligand (RANKL; or tumor necrosis factor-related activation-induced cytokine (TRANCE)), 1,25-ihydroxy-vitamin D_3, osteoprotegerin, parathyroid hormone, calcitonin and various pro-inflammatory cytokines. These are involved in osteoclast differentiation and bone resorption (9,11,13,14).

During bone resorption osteoclasts become polarized, dissolve crystalline hydroxyaptite and remove collagen fibers from the bone matrix. Several specific membrane domains emerge at the resorption site: ruffled border, sealing zone, functional secretory domain and basolateral domain. Actin cytoskeleton at sealing zone attaches to the bone matrix; intracellular acidic vesicle fusion occurs and builds the ruffled border which is the absorbing organelle. Hydrochloric acid and proteases in the vesicles dissolve crystalline hydroxyaptite and organic matrix at the area of resorption lacuna situated between ruffled border and bone surface. The osteoclast absorbs phosphate and calcium ions by the endocytotic process. Exocytosis of resorbed and transcytosed matrix-degradation products is probably performed by the secretory domain (13). ATP-dependent vacuolar proton pumps cause a drop in the pH level due to acidification at ruffled border and in intracellular vacuoles that occur during the osteoclast bone resorption process (13,15,16).

4.2 Tartrate Resistant Acid Phosphatase

Tartrate resistant acid phosphatase (TRAP) is an enzyme that belongs to the purple acid phosphatase family. Two forms of TRAP a 35 kDa monomer and 23 kDa dimer are known and the optimal pH for activity is between 4.9 and 6.0. TRAP is produced by various cells from monohistiocytic lineage including activated macrophages and dendritic cells, but it is mostly expressed in osteoclasts. TRAP is a metalloglycoprotein that contains two redox-active ferric ions center in its active site. This di-iron center and reduction of the disulfide bond are essential for regulation of TRAP activity. By the Fenton reaction in the TRAP redox active site ROS can be generated which participate in bone resorption of osteoclast. In this process a hydroxyl free radical is made by diferric site of TRAP reacting with hydrogen peroxide (7). ROS production by TRAP is crucial for bone metabolism. TRAP also plays an important role in immune clearance by increasing the level of superoxide (17,12).

4.3 Reactive Oxygen Species (ROS)

ROS molecules such as the superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) are generated from normal metabolism of molecular oxygen in aerobic organisms (3). These molecules are very unstable radicals that play an essential role in defense and redox signaling processes. They act as intracellular secondary messenger for cellular events such as cell differentiation and proliferation, activation or in other function such as growth inhibition and apoptosis. One of their functions is to mediate the oxidative stress response that can damage the cells (4). The oxygen-derived free radicals are associated with bone metabolism, osteoclastogenesis and bone resorption. On the other side it has been shown that hydrogen peroxide is responsible for signaling bone loss (18). In Fig.1 production and clearance of ROS is shown.

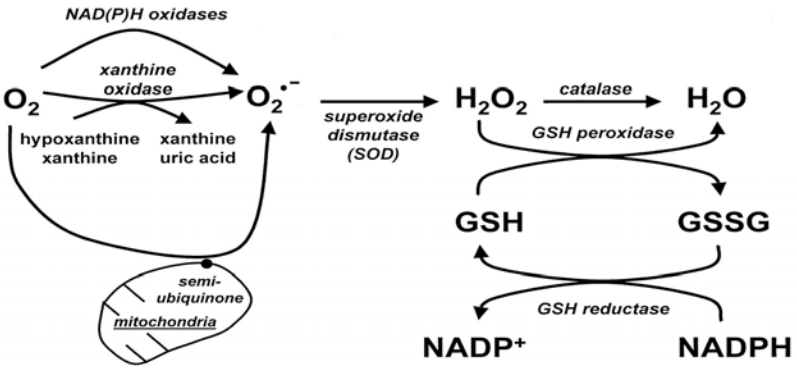


Figure 1. Reactive oxygen species pathways, production and clearance (19).

4.4 Antioxidant systems

The antioxidant systems prevent the production of oxidants. They act as a defense mechanism against free radicals, oxidative damages and the toxicity of ROS. The antioxidant enzyme superoxide dismutase (SOD) generates oxygen and hydrogen peroxide from superoxide, and

then hydrogen peroxide is converted to water and oxygen by catalase (18). Thiol antioxidants such as thioredoxins, glutathione and cysteine control the redox state of the thiol system. Thiol pathways are essential in cell signaling (2).

4.5 Glutathione

The glutathione concentration intracellularly and extracellularly is very important for many cellular events such as metabolism, differentiation, proliferation and apoptosis. The glutathione concentration depends on export from the cells and on the rate of glutathione production. Glutathione is biosynthesized from the amino acids glutamate (i.e. the ionic form of glutamic acid), cysteine and glycine. Reduced GSH is the most abundant form of glutathione in cells, but some oxidized GSSG and S-conjugate forms exist. Cellular GSH/GSSG export is important for keeping the balance of environmental redox states. GSH has an essential role to protect cells against ROS functions. The cysteine and glutathione transport system is dependent on endogenous and exogenous mechanisms in cells and causes changes in cellular redox status (2).

4.6 Cysteine

The active sites of many proteins contain cysteine. Protein function is related to oxidation or other modifications of the active sites. Many biological systems include redox sensitive cysteine, residues that have a role in cell signaling and macromolecular transport. The thiol element and cysteine have an important role in the redox circuit. Cysteine residues are found in many active sites with iron-sulfur groups. This part of the protein participates in electron transfer mechanisms. Cysteine is a rate-limiting precursor and therefore a regulatory factor for GSH synthesis (2).

4.7 X_c^- cystine/glutamate antiporter

The x_c^- cystine/glutamate antiporter is an anionic antiporter that is dependent on sodium ions. This system is responsible for transporting intracellular glutamate to the outside of the cell and for the uptake of cystine in to the cell. Inside the cells cystine is rapidly reduced to cysteine. The x_c^- transporter consists of two proteins, the 4F2hc heavy chain and the variable light chain xCT (20,21). This system is vital for reduction the oxidative stress in cells by increasing the intracellular glutathione levels (21,22). In cultured cells the x_c^- levels are affected by electrophilic agents such as diethyl maleate, oxygen and LPS (21). The x_c^- is down-regulated by RANKL (Fernandez et al., un published result). In Fig.2 the mechanism of the x_c^- system in trafficking of the cystine and glutamate inside and outside the cell related to oxidation and reduction of selenite and NADPH dependency is shown.

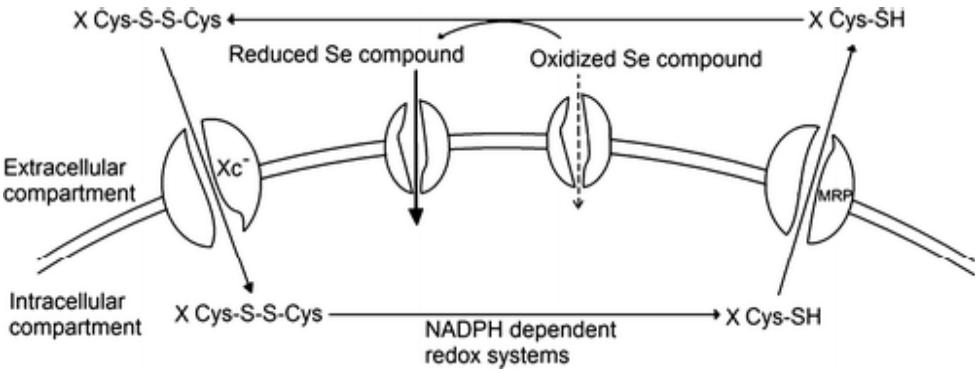


Figure 2. X_c^- GSH/Cys antiporter. Reduction of extracellular selenite is dependent on cysteine uptake (23).

4.8 Thioredoxin system

The thioredoxin system is the most important thiol regulator in cells. It uses a cysteine thiol-disulfide exchange system to reduce other proteins. They can regulate cellular redox statues in bone and other tissue. The thioredoxin system includes thioredoxin (Trx) and thioredoxin reductase (TrxR), which are dependent on NADPH. A -Cys-Gly-Pro-Cys (Cys at 32 and at 35 in Trx1) sequence is the conserved active site of Trx. Two cysteines in the Trx active site perform the redox activity of Trx through attack of the target disulfide bond and regulate the enzymes and activities of transcription factors. The cellular metabolic activity can be regulated by oxidized thioredoxin (Trx-S_2) that cuts the disulfide bonds. Thioredoxin reductase and NADPH are required for Trx activity by reducing its active site (24). The active site sequence of mammalian TrxR is Gly-Cys-seCys-Gly and it contains the unusual amino acid selenocysteine. This residue plays a main role to create the low substrate specificity of TrxR and causes catalytic activities (25). The thioredoxin system can control intracellular redox state and signal transduction. Stress signals modulate differentiation which is related to oxidative stress conditions. The Trx1 is more expressed in osteoclasts than in macrophages (26,25). Trx and/or Ref-1 (redox factor) enhance the DNA binding activity of AP-1, polyoma enhancer binding protein-2 (PEBP2), NF-κB, p53, and other transcription factors that are essential for osteoclast differentiation (7,26). In Fig.3 the reduction of Trx by TrxR and NADPH is shown.

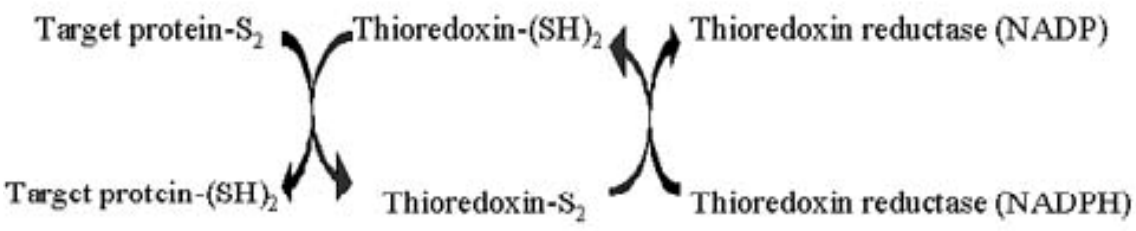


Figure 3. The reduction of Trx by TrxR and NADPH (27)

4.9 RAW 264.7 cell

The RAW 264.7 cell is a monocytic macrophage-like cell line derived from tumors in BALB/c male mice that was induced by Abelson murine leukemia virus (27). RAW cells are capable of differentiation into macrophages or osteoclasts in presence of different stimulating compounds. RAW cell differentiates into multinucleated TRAP positive osteoclast when exposed to the receptor activator of NF-κB ligand (RANKL). The RANKL is a member of the tumor necrosis factor family and plays regulatory role in osteoclast differentiation, activation, survival and apoptosis. RANKL cause the commitment of mononuclear osteoclast to form multinucleated resorbing cells. Binding of the RANKL to its receptor RANK induces increase in calcium concentration inside the cells and this is essential for differentiation of the osteoclast progenitor cell (28). Pro-inflammatory macrophages are generated from RAW 264.7 cell in the presence of LPS in the cell culture environment (29). LPS endotoxin is a membrane constituent of a majority of Gram-negative bacteria. They can bind to Toll-like receptor TLR4 and play an indispensable role in the inflammatory response (30). LPS is also one of the regulatory factors of osteoclastogenesis at different stages of differentiation. LPS stimulates secretion of nitric oxide (NO) free radical and IL-6 in the RAW 264.7 macrophage cell line.

4.10 Aim of the project

The aim of this project was to explore the importance of the redox environment and redox state during osteoclastogenesis and macrophage differentiation by using Raw 264.7 cells. For this we needed to develop an in vitro differentiation system and tools to manipulate and analyze the redox state. Cells were treated with redox modulators such as DTT, DTNB, TCEP, selenite and MSG compounds in presence of RANKL or LPS to study the effects of these agents during osteoclast and macrophage differentiation. TRAP was used as an osteoclast differentiation marker and the morphological changes during differentiation were evaluated by TRAP staining. The total and reduced GSH/Cys levels in both extra- and intracellular environments were measured. The expression of redox related genes such as Trx1, TrxR1, 4F2hc and xCT during osteoclastogenesis were determined on the mRNA level. TRAP, CTR and Cat K were analyzed as osteoclast genes and TbP as a reference gene were used.

5. Materials and methods

5.1 RAW 264.7 cell line

The RAW 264.7 cells were maintained in Minimum Essential Medium Eagle (MEMeagle) with 10 % fetal bovine serum (FBS). 1 % L-glutamine 10 X and 1 % Gentamicine were supplemented to medium. Cultured cells were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for three and five days. At day 2 the MEMeagle media were replaced with fresh media. Table 1 shows the time of cell culturing and treatments. Cells were thawed in T75 flasks. Cells were treated by stimulators of RANKL and LPS and redox modulators three days after thawing. This day is day zero. Cells were collected at day 5.

Table 1. RAW264.7 Cell culturing time table

Thu	Fri	Sat	Sun	Mon	Tue	Wed	Thu	Fri	Sat
				Day 0	Day 1	Day2	Day 3	Day 4	Day 5
Thawing the cells	Checking by microscope			Treating		Refreshing the medium			

5.2 Counting of RAW 264.7 cells

At day zero cells were scraped from T75 cell culture flask and were added to a plastic tube. The cells were counted in 4 squares of the Bürkner chamber. The total cell number for 24-well culture dish as well as 96-well culture dish was 12500 cells/cm^2 and 13000 cells/cm^2 for 6-well plate.

5.3 Stimulation of RAW 264.7 cells

At day zero the RAW cells were seeded for 3 to 4 hours in 24-well plates with cell density of 12500 cells/cm^2 in 24-well plate and 13000 cells/cm^2 in 6-well plate. After seeding the cells the pre treatments of DTT (50 µM), DTNB (100 µM), TCEP (50 µM), selenite (0.5 µM) and MSG (50 mM) were added. Thirty minutes later 2 ng/ml RANKL and 1 µg/ml LPS were added to each well. The negative control had only MEMeagle media in the wells and the positive control contained media plus either RANKL or LPS in the wells. Plates were incubated in CO_2 incubator at 37 °C for 3 and 5 days.

5.4 TRAP Staining of RAW 264.7 cells

After cell differentiation cells were fixed with 4 % formaldehyde in day 3 and day 5. The TRAP staining was performed using the Leukocyte Acid Phosphatase (TRAP) kit (Sigma-Aldrich). First dH2O with a temperature of 37 °C was mixed with the following solutions: Naphtol AS-BI Phosphoric Acid Solution (12.5 mg/ml), Acetate Solution (2.5 M pH 5.2) and Tartrate Solution. Fast Garnet GBC salt capsule was added to the mixture and filtered. Mixture was incubated at 37 °C for 10 minutes. The cells were washed with PBS and fixed in formaldehyde solution for 15 minutes at room temperature. TRAP-solution was added to each of the wells and left at 37 °C for approximately 1 hour. The plate was checked every 15 minutes in the microscope, until reasonable color had developed in the cells. The staining was aborted by adding dH_2O to each of the wells. The final step was to add formaldehyde (4 %) to the wells again, which keep the cells preserved for a long time.

5.5 Harvesting of cells for total RNA at day 5

RNeasy plus Mini Kit (Qiagen) was used for total RNA purification. Cells were first washed twice with PBS. Buffer RLT with the addition of 1 % β-ME was added to each well. Cells were scraped with a plastic cell scraper and pipetted into a QIAshredder column (Qiagen). The QIAshredder columns were centrifuged at maximum speed for 2 minutes in a table centrifuge and the flow through was saved and stored at -75 °C until RNA purification.

5.6 RNA purification

The RNA was purified by using RNeasy Plus Mini Kit (Qiagen). The homogenization of harvested cells was performed by lysing into the QIAshredder column as described above. This homogenized lysate was transferred to a gDNA Eliminator spin column and centrifuged for 30 s at 8000 x g. The flow-through was saved and the column discarded. 70 % ethanol was added to the flow-through and mixing was performed by pipetting up and down in the tube. The sample was transferred to an RNeasy spin column that was placed in a collection tube. The spin column was centrifuged for 15 s at 8000 x g. The column was saved and the flow through was discarded. The column was placed in the same collection tube as the previous step and buffer RW1 was added. Centrifugation for 15 s at 8000 x g was performed to wash the column membrane. The flow through was discarded and buffer RPE (with ethanol added) was added to the column and centrifuged for 15 s at 8000 x g, to wash the spin column membrane. The flow through was discarded and buffer RPE was added to the column. To ensure that the membrane was totally dry, centrifugation for 2 min at 8000 x g was needed. The RNeasy spin column was removed from the old collection tube and placed in a new one. To elute the RNA, RNase-free water was added to the column and centrifuged for 1 minute at 8000 x g. The small amount of each sample added to the Nanodrop Spectrophotometers machine and the absorbance at 260nm and the ratio 260/280 nm was measured. The machine was quantitated the RNA concentrations. According to this formula: $c = (A * e)/b$, c is RNA concentration in ng/microliter,

A is the absorbance in AU, e is the wavelength-dependent extinction coefficient in ng-cm/microliter and b is the path length in cm. The accepted extinction coefficient for RNA is 40 ng-cm per each microliter.

5.7 Reverse Transcription (RT) reaction for cDNA synthesis

Omniscript Reverse Transcriptase Kit was used for cDNA synthesis. A master mix containing 1*RT buffer, dNTP mix (0.5 mM), Omniscript (4U) and oligodT primer (1 µM) was prepared and mixed with 2 µg RNA and nuclease free water per reaction. The mixture was incubated for one hour at 37 °C.

5.8 PCR analyses of stimulated RAW 264.7 cells

The quantitive PCR was performed by using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) and screened with Bio-Rad CFX96 Real-Time system Thermal cycler. Ten ng/µl cDNA from each sample was used. The specific primer sequences are listed in Table 2. Primer concentrations were 300/300 nM for Trx1, 900/900 nM for TrxR1, for xCT 900/300nM, for TbP 900/900 nM and 300/300 nM for TRAP. The annealing temperatures for each primer are listed in Table 3. The cDNA concentrations (ng/µl) for each standard were 1.67 for STD1, 0.56 for STD1, 0.28 for STD3, 0.14 for STD4 and zero for STD5 (NTC).

Table 2. Primer sequences:

Primer name	Forward	Reverse
Trx1	*TTTCCATCTGGTTCTGCTGAGAC*	*CAGAGAAGTCCACCACGACAAG*
TrxR1	*CCATCCAGGCGGGGAGATTG*	*GAGTAAACACAGTCGTTGGGACAT*
xCT	*ACCTGCCTCTTCATGGTTGTC*	*TGGTTCAGACGATTATCAGACAGA*
4F2hc	*TCCAGGATCTTTCACATCCCAAGA*	*GCTCTCTGTTGCACGGTGAC*
TRAP	*TTCCAGGAGACCTTTGAGGA*	*GGTAGTAAGGGCTGGGGAG*
Cat K	*CTTTCTCGTTCCCCACAGGA*	*GTTGTATGTATAACGCCAGGGC*
CTR	*TCAGGAACCACGGAATCCTC*	*ACATTCAAGCGGATGCGTCT*
TbP	*AGAGAGCCACGGACAACTG*	*AAGGAGAACAATTCTGGGTTTG*

Table 3. Annealing temperature:

Primer name	Annealing temperature (°C)
Trx1	60 °C
TrxR1	60 °C
xCT	60 °C
4F2hc	60 °C
TRAP	60 °C
CTR	60 °C
Cat K	60 °C
TbP	61.4 °C

5.9 Glutathione and cysteine determination in RAW cells

5. 9.1 Reduced form of glutathione and cysteine

Cells were seeded in 6-well plates. At day 2 media was refreshed. For measuring the extracellular amounts of glutathione and cysteine the media at day 5 was used and for intracellular determination media of day 5 was removed and PBS was added to each well. Eight mM mBrB was added to media or PBS and samples were incubated at room temperature for 2 minutes in dark. The reaction was stopped by adding 80 % SSA to each well. The cells were scraped and the sample was collected in eppendorf tubes. Centrifugation of these tubes was done to get a pellet of any precipitated protein; where as the supernatants were measured with HPLC .The samples were stored at -70 °C.

5.9.2 Total form of glutathione and cysteine

Stimulated cells were seeded in 6-well plates. After 5 days the media was removed and PBS containing 50 mM DTT was added to each of the wells for the intracellular assay. For the extracellular assay media containing 50 mM DTT was used. The plates were incubated in room temperature for 30 minutes. Twenty mM mBrB was added to each well and the plates were incubated in the dark for 10 minutes at room temperature. The reaction was stopped by adding 80 % SSA. Cells were scraped from the wells and collected in eppendorf tubes. After centrifugation for 3 minutes at 3000 x g both the pellet and the supernatant were saved and stored at -70 °C.

5.10 Intracellular ROS production determination

RAW 264.7 cells were seeded in black 96-well plate for 5 days. A color-free medium with 10 % FBS was used. The medium was changed at day 2. Cells were washed two times with PBS after removing out the media at day 5. The PBS with added non-fluorescent CM-H2DCFDA probe (Invitrogen Corporation) diluted by DMSO was used in each well. The probe was taken out and cells were washed with PBS. The dilution of redox modulator treatments in PBS was added to the wells and the plate was incubated in dark. Florescence production of CM-DCF was measured in 485 nm and 527 nm at several time points (30 min, 1h, 2h, 4h, 8h and 24h) for detection of ROS production.

6. Results

6.1 Optimization of RAW 264.7 cell culturing conditions during differentiation in the presence of redox modulators

To investigate how redox modulation affects cells during osteoclast and macrophage differentiation the RAW 264.7 cells were cultured in the presence of RANKL, LPS and redox modulators.

DMEM (Dulbecco's Modified Eagle Medium) is recommended by ATCC (American Type Culture Collection), in addition of fetal bovine serum to a final concentration of 10 %. Due to glutamine reactions in this medium, MSG (monosodium glutamate) redox modulation RAW 264.7 cells did not grow and differentiate well. To solve this problem MEMEagle (Minimum Essential Medium (MEM, developed by Harry Eagle) cell culture medium without L-glutamine was used. 10 % concentration of inactivated FBS, 10x 1 % of L-glutamine and 1 % of gentamicine as antibiotic were added to medium. L-glutamine was added to medium each week in the same environmental conditions as DMEM cell culturing. By using MEMEagle medium all the redox modulators worked well and less cell death was observed.

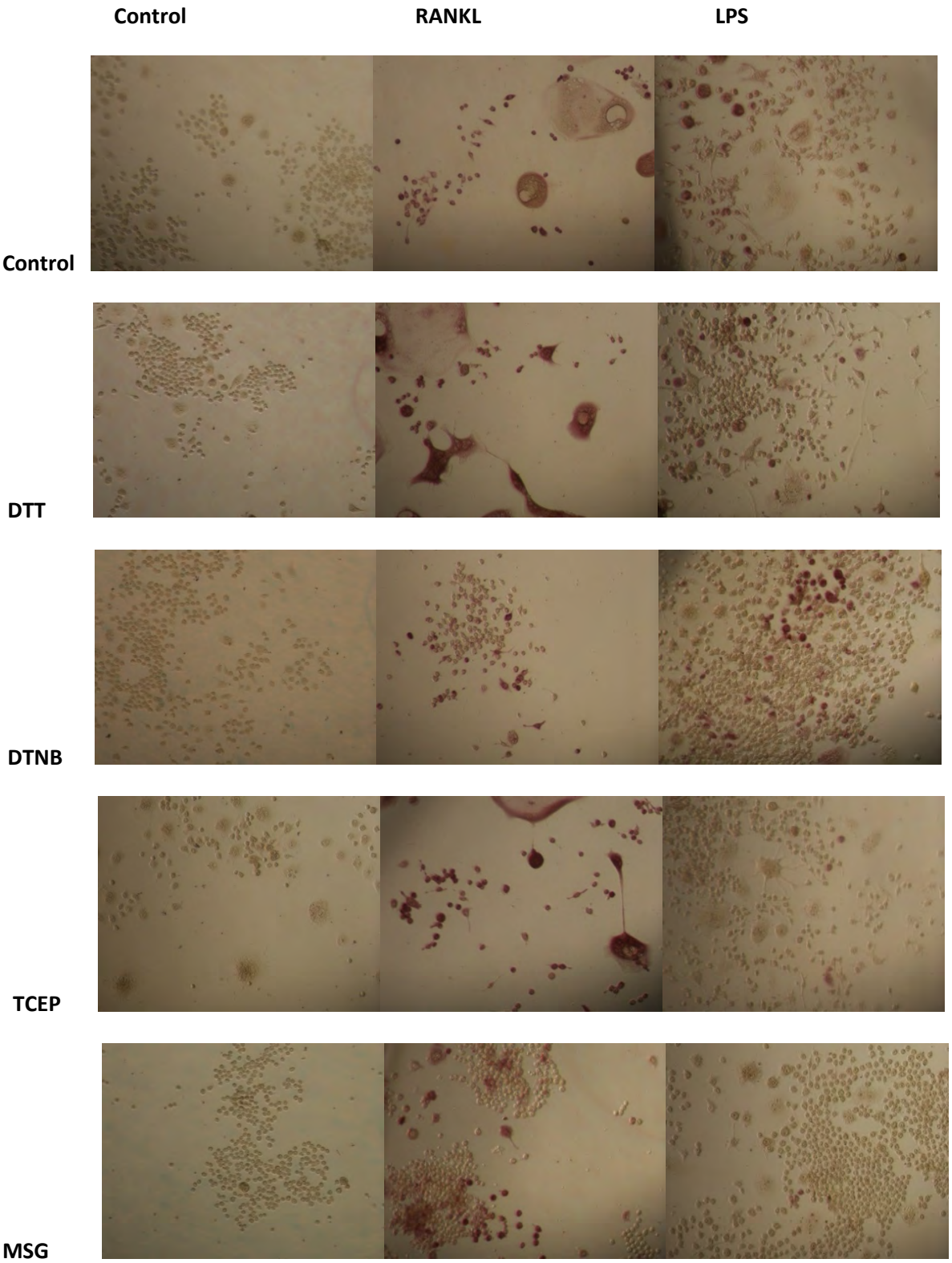
To define the best cell density for culturing, 6250 cells/cm^2 was tested. At this density cells were grown at the edges of wells and the amount of spread cells at the middle of wells was too little. Secondly 12500 and 25000 cells/cm^2 were tested. At the level of 25000 cells/cm^2 cell differentiation was too low and cell death had occurred. It appeared that 12500 cells/cm^2 in 24-well plate and 13000 cells/cm^2 in 6–well plate was the optimal density. At these conditions more cells have more space to grow and cell differentiation was observed at the whole wells.

6.2 Effects of redox modulators on RAW 264.7 cell differentiation to osteoclast and macrophage

6.2.1 TRAP staining and morphological aspects

Tartrate resistant acid phosphatase (TRAP) was selected as a marker of osteoclast differentiation. To investigate the effects of the redox environment and morphological changes due to redox modulator functions in the presence of RANKL and LPS, TRAP positive activity of multinucleated cells during osteoclast or macrophage differentiation were evaluated.

The morphological changes of RAW264.7 during differentiation to osteoclast (RANKL stimulation) and macrophage (LPS stimulation) in the presence of non-toxic concentration of DTT, DTNB, TCEP, MSG and selenite can be seen in Fig.4. TRAP activity can be distinguished by the purple color of stained cells. DTT is a reducing compound that can enter the cells. TCEP is also a reductant but cannot enter the cells. DTNB can oxidize all thiols only extracellularly. Selenite has an oxidizing effect both inside and outside the cells and MSG is a compound that blocks the xCT transporter and inhibits cystine transfer into the cells.


Control
RANKL
LPS
Control
DTT
DTNB
TCEP
MSG

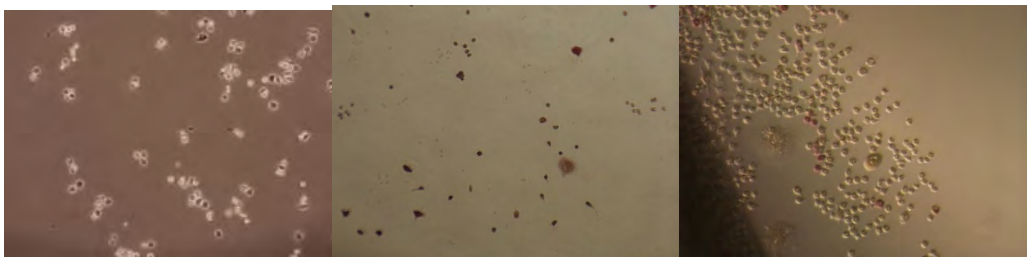
Se 

Figure 4. TRAP staining of RAW 264.7 cells at day 5, stimulated with RANKL and LPS. Cells were treated by redox stimulators: DTT (50 µM), DTNB (100 µM), TCEP (50 µM), MSG (50 mM) and selenite (0.5 µM)

In the first column TRAP staining of control cell without stimulators of RANKL and LPS shows that there is no TRAP activity after any of the redox treatments. The MSG and DTNB redox treatments show similar results with no multinuclear TRAP positive cells. Compared to control the cells grown in the presence of DTT are smaller. In the first row controle cells stimulated by RANKL are TRAP positive and large moltinuclear osteoclasts. LPS stimulation shows weak TRAP staining of some mononoclear cells and most mono- and multinuclear cells are TRAP negative.

In the second column in the presence of RANKL all cells show positive TRAP staining in all treatments. The MSG and DTNB show similar results with no multinuclear TRAP positive cells and most of the cells are TRAP negative mononuclear. The DTT and TCEP show similar results with multinuclear TRAP positive cells and mononuclear TRAP positive cells. Selenite inhibite cell formation of multinuclear TRAP positive .

In the tird column exposure to LPS and redox modulators TRAP staining show the multinuclear TRAP negative and no multinuclear TRAP positive cell. Very few mononuclear TRAP positive cells are seen in control, DTT and DTNB. Mononuclear TRAP positive cells in TCEP, MSG and selenite shows few TRAP positivit activity. In all treatments the large multinuclear TRAP negative macrophages can be seen but they are very few in MSG and DTNB and selenite treatments. TCEP and DTT have same effects and multinuclear TRAP negative macrophages are obvious .

6.3 Gene expression study of RAW264.7 cells treated by redox modulator

6.3.1 Optimization of house keeping gene

To study the gene expression of RAW264.7 cells treated by redox modulators and investigate the effects of these treatments during osteoclast differentiation we have to use the most stably expressed reference gene. The primers of actin (900/900 nM), TATA box binding protein (TbP) (900/900 nM) and Phosphoglycerate kinase 1 (PgK1) (900/900 nM) with annealing temperature of 62 °C, 61.4 °C and 61.4 °C were chosen as candidate reference or house keeping genes.

The melting curve of the actin primer shows a peak at 83 ºC (Fig.5 A). The standard curve shows an efficiency of 50.2 % (Fig.5 B). The melting curve of TbP shows a peak at 84 ºC (Fig.5 C). Standard curve shows an efficiency of 80.2 % (Fig.5 D). The melting curve for PgK1 shows a peak at 82 ºC (Fig.5 E). Standard curve shows an efficiency of 98.9% (Fig.5 F)

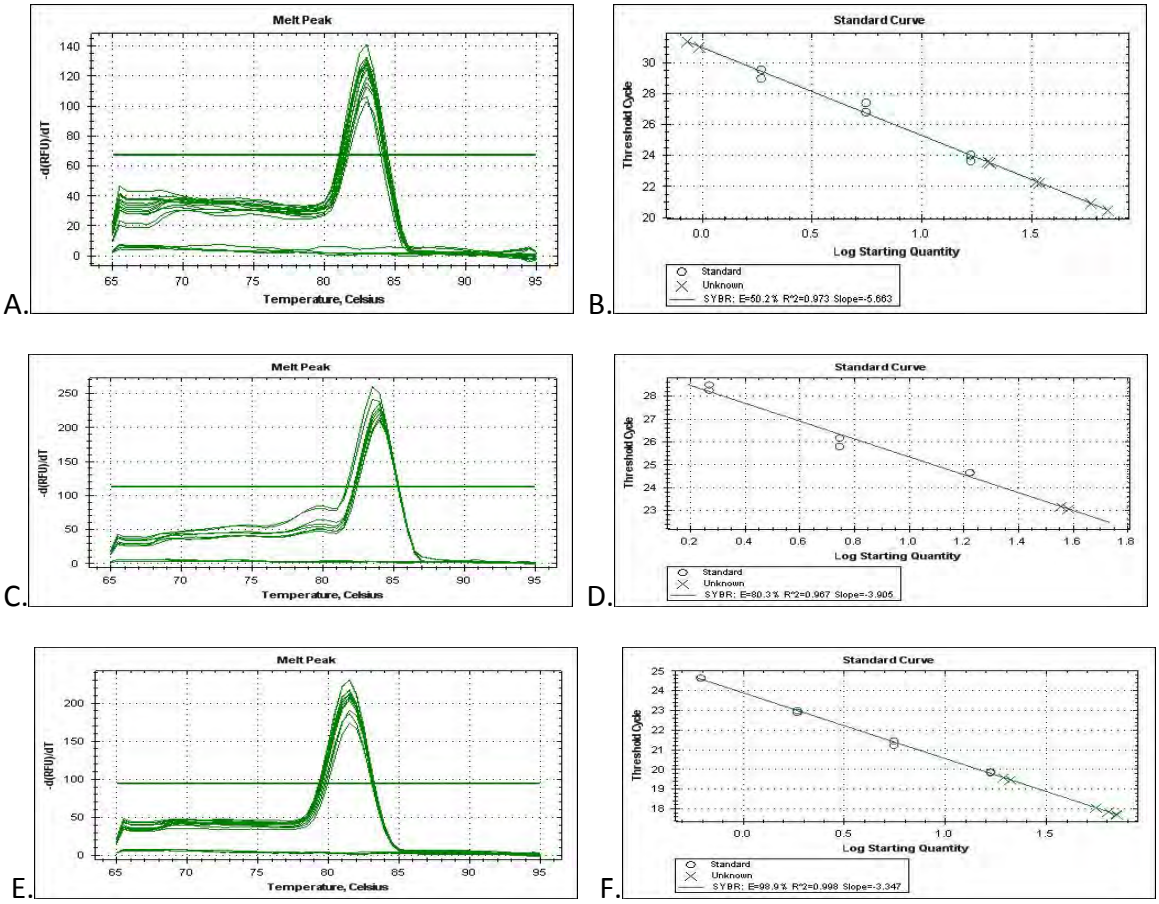


Figure 5. Optimization data for the housekeeping genes of actin (A-B), TbP (C-D), PgK1 (E-F) primers pair. Concentrations of all forward and reverse primers were 900nm.

The experiment was repeated for actin, TbP, PgK1 and peptidylprolyl isomerase A (Ppia), which were also included. The primer concentrations for both forward and reverse were 900 nM and annealing temperature of 61.4 ºC for all primer pairs.

The melting curve of actin primer shows a peak at 83 ºC (Fig.6 A). The efficiency of actin standard curve shows in Fig 6 B. The melting curve of Pgk1 shows a peak at 82 ºC (Fig.6 C). The efficiency of PgK1 standard curve shows at Fig.6 D. The melting curve for Ppia shows a peak at 82 ºC (Fig.6 E). Standard curve efficiency of Ppia shows in Fig.6 F. The melting curve for TbP shows a peak at 84 ºC (Fig.6 G). The efficiency of TbP standard curve shows in Fig.6 H.

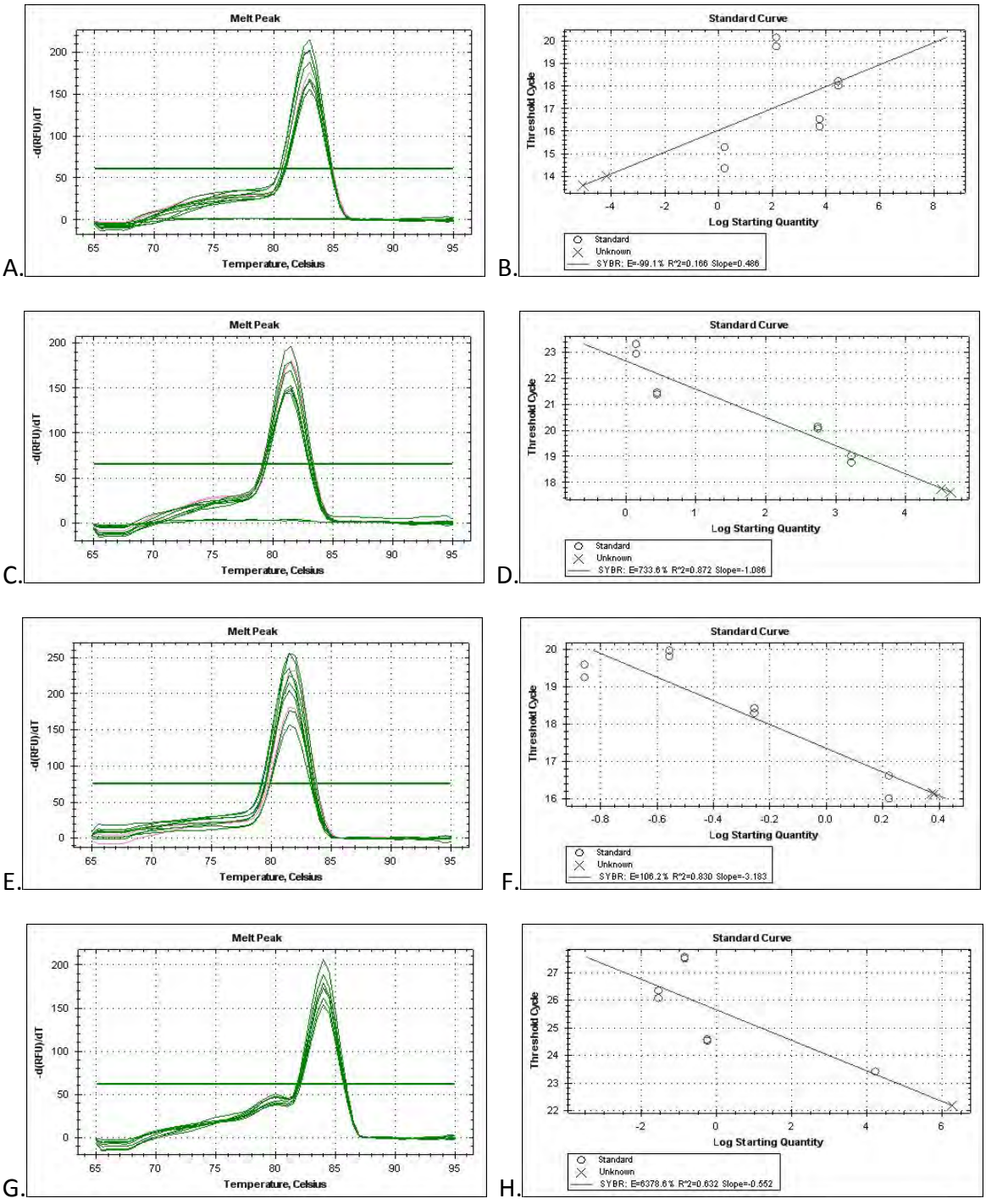


Figure 6. Optimization data for the housekeeping gene of Actin (A-B), PgK1 (C-D), Ppia (E-F), TbP (G-H) primers pair. Concentrations of all forward and reverse primers were 900nm.

The results from both experiments were used in the bestkeeper analyzing software. Both experiments results are shown in Table 4 and 5. The results of standard deviation, which are

shown by analyzing program for actin and PgK1, were not good enough. The Ppia was skipped from second experiment because the standard curve was not available at that time. Therefore TbP was chosen as the house keeping gene for the following experiments.

Table.4 first experiment analyzing by using bestkeeper software

CP data of housekeeping Genes:	**Actin**	**PgK1**	**TbP**
	HKG 1	**HKG 2**	**HKG 3**
n	**8**	**8**	**8**
geo Mean [CP]	**24.13**	**19.38**	**22.89**
ar Mean [CP]	**24.43**	**19.48**	**22.89**
min [CP]	**20.49**	**17.71**	**22.49**
max [CP]	**31.33**	**22.86**	**23.27**
std dev [± CP]	**3.37**	**1.65**	**0.29**
CV [% CP]	**13.81**	**8.45**	**1.26**
min [x-fold]	**-4.39**	**-3.15**	**-1.27**
max [x-fold]	**18.70**	**10.97**	**1.25**
std dev [± x-fold]	**3.94**	**1.95**	**1.12**

Table 5. Gene study results of second experiment by bestkeeper software

CP data of housekeeping Genes:	**actin**	**PgK1**	**TbP**
	HKG 1	**HKG 2**	**HKG 3**
n	**8**	**8**	**8**
geo Mean [CP]	**19.68**	**18.21**	**22.76**
ar Mean [CP]	**20.09**	**18.22**	**22.77**
min [CP]	**13.59**	**17.63**	**22.18**
max [CP]	**23.58**	**19.59**	**23.27**
std dev [± CP]	**3.14**	**0.65**	**0.42**
CV [% CP]	**15.64**	**3.59**	**1.82**
min [x-fold]	**-11.90**	**-1.49**	**-1.41**
max [x-fold]	**4.88**	**2.59**	**1.35**
std dev [± x-fold]	**3.59**	**1.30**	**1.18**

6.3.2 Gene expression of RAW264.7 during osteoclast differentiation at presence of redox stimulators

The expression levels of the TRAP, CTR, Cat k, xCT, 4F2hc, Trx1 and TrxR1 genes during differentiation of RAW 264.7 to osteoclast (by RANKL stimulation) and to macrophage (by LPS stimulation) in the presence of redox modulators DTT, DTNB, TCEP, MSG and selenite was performed and the results are shown below.

The gene expression results of the osteoclast genes of TRAP, CTR and Cat k and target genes of xCT, 4F2hc, Trx1 and TrxR1 is shown in Fig.7. In this figure the RAW 264.7 cells are treated only by redox stimulators of DTT, DTNB, TCEP, selenite and MSG. Cells were at day 5, without RANKL and LPS. The data is shown as mean value +/- standard deviation of intra assay replicates.

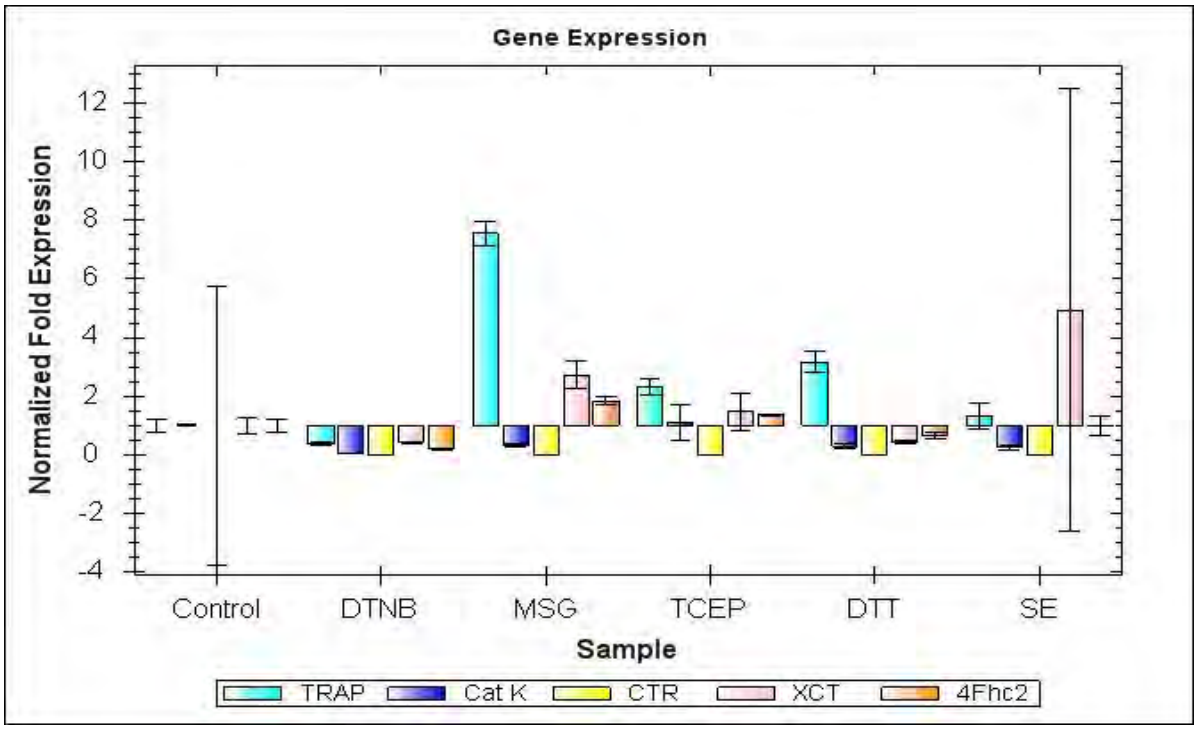


Figure 7. Control plate of gene expression study

Cells treated with redox modulators show that the expression of the TRAP gene is increased by redox modulator treatments in order of MSG, DTT, TCEP, and selenite. Expressions of all transcripts are decreased by DTNB. Cat K expression is decreased by all redox stimulators in the same way. It decreased more by DTNB treatment. Expression of CTR is similar to Cat K and mostly the same in all redox treatments.

The results shows that the xCT expression the most in Se treatment and follow by MSG and TCEP treatments. The xCT expression compared to control is decreased by DTNB and DTT. 4F2hc is another sub type of $\mathbf{x_c^-}$ system and it expressed more by MSG also by TCEP treatment. Compared to control it does not change by Se treatment and it is increased by DTT and DTNB treatments.

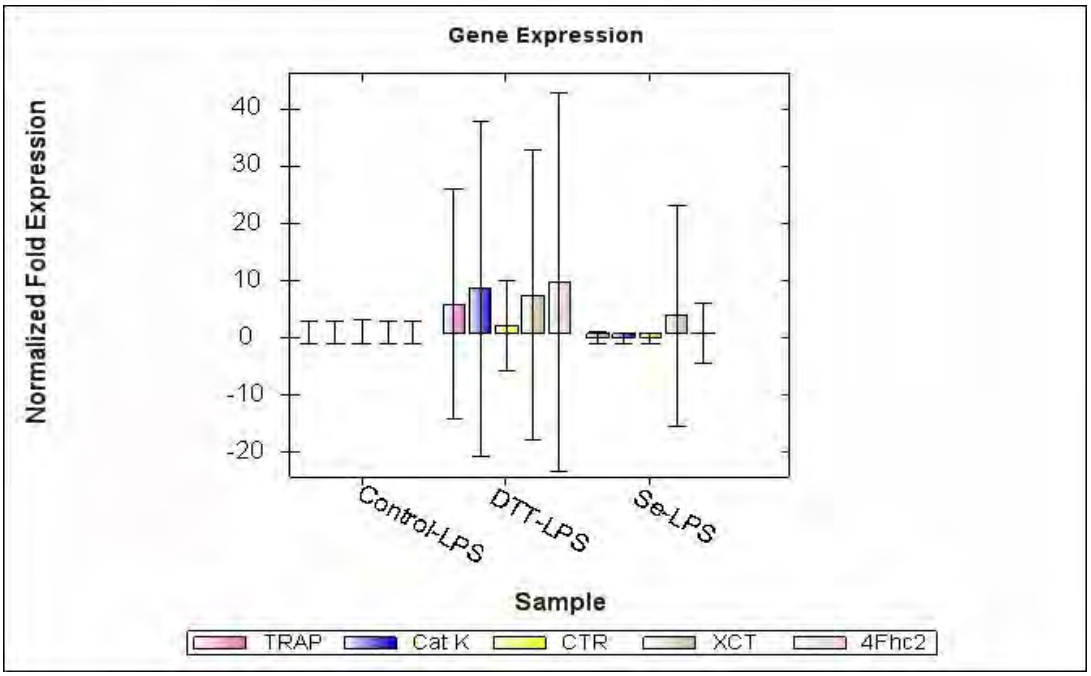


Figure 8 A. mRNA levels after stimulation with LPS by using DTT and selenite

The results from LPS treatment with addition of redox stimulators are shown in Fig.8 A and Fig.8 B. In presence of LPS the expression of TRAP is down regulated by most of the redox modulators, especially by MSG treatment. Only in addition of DTT treatment, the gene shows upregulation.

The Cat K expression is mostly increased by DTT and DTNB. It decreased by MSG and TCEP. Se shows down regulation of Cat K expression as well. CTR expression increases by DTT and decreases by other redox treatments of MSG, TCEP, DTNB and Se. XCT expression increased by DTT, Se and DTNB and decreased by TCEP and MSG treatment.

4F2hc was more expressed by DTT and DTNB redox stimulators but less expressed by TCEP and MSG. Compared to control selenite treatment does not show difference in expression of 4F2hc gene. This could be because of the large intra assay variation in this sample. Fig.8 B shows the level of gene expression of DTNB, TCEP and MSG redox modulators in the presence of LPS.

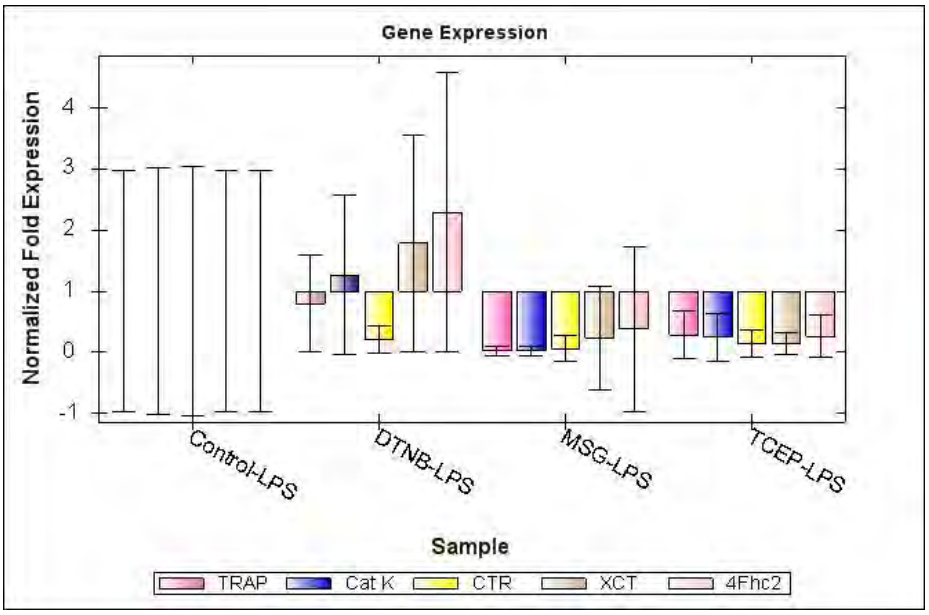


Figure 8 B. mRNA levels of DTNB, MSG, TCEP treatments in the presence of LPS

In the presence of RANKL gene expression of Cat K is very high. In Fig.9 A and Fig.9 B gene expression levels of other genes from RANKL stimulated cells can be seen.

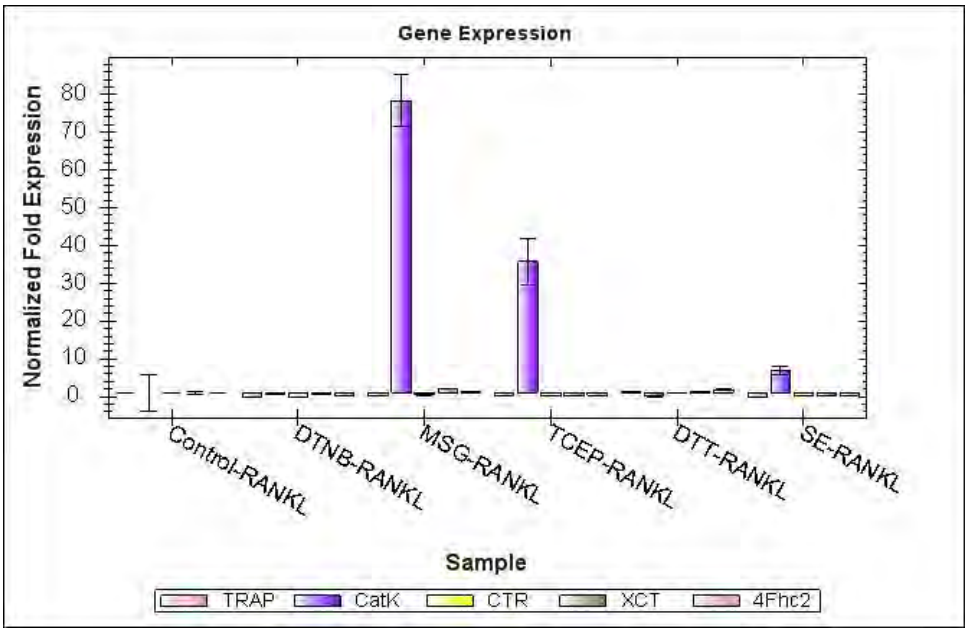


Figure 9 A. mRNA levels after treatment with RANKL

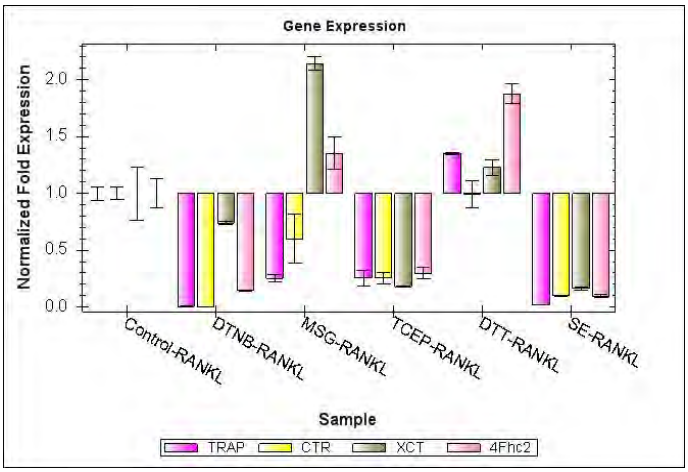


Figure 9 B. RANKL exposure. TRAP, CTR, xCT and 4F2hc expression

In the presence of RANKL, the TRAP expression is decreased by DTT, Se, TCEP and MSG redox stimulators. TRAP less expressed by DTT treatment. CTR gene expression is down regulated by all redox stimulators treatments. DTNB treatment shows the largest variation fallowed by Se, TCEP and MSG. It does not show any changes by DTT treatment. The xCT more expressed especially by MSG and DTT. It less expressed by TCEP, Se and DTNB. 4F2hc show the same results as xCT but it is more expressed by DTT and less expressed in selenite treatment. Expression of Trx1 and TrxR1 primers in the presence of RANKL and LPS is shown in Fig.10 and Fig.11.

In the presence of LPS, expression of Trx1 and TrxR1 in treated cells by DTT and Se redox modulator show intra assay variation. Fig.10 shows the expression of these two genes by addition of DTNB, MSG and TCEP redox modulators.

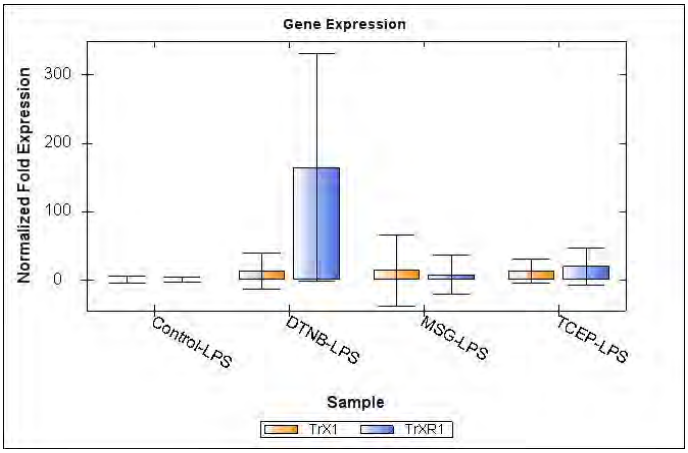


Figure 10. mRNA level of Trx1 and TrxR1 by using LPS and addition of DTNB, MSG and TCEP redox modulators.

In the presence of LPS, gene expression of Trx1 increased by using DTNB, MSG and TCEP. TrxR1 show most expression especially by DTNB treatment and followed by TCEP and MSG redox stimulators.

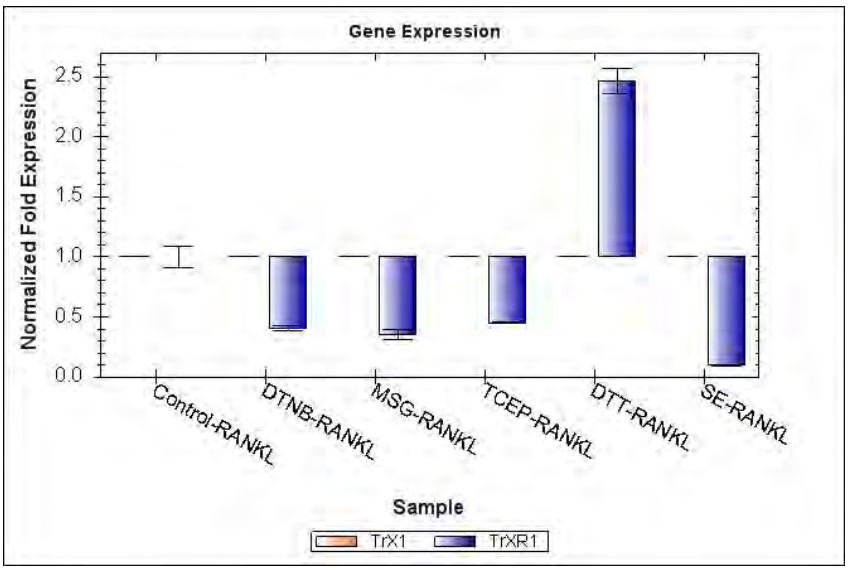


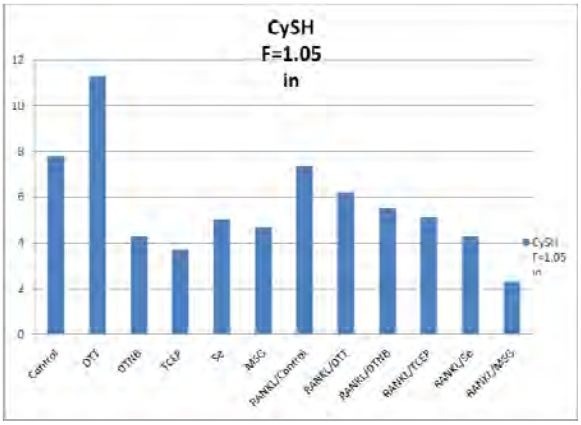
Figure 11. RANKL; Trx1 andTrxR1 expression

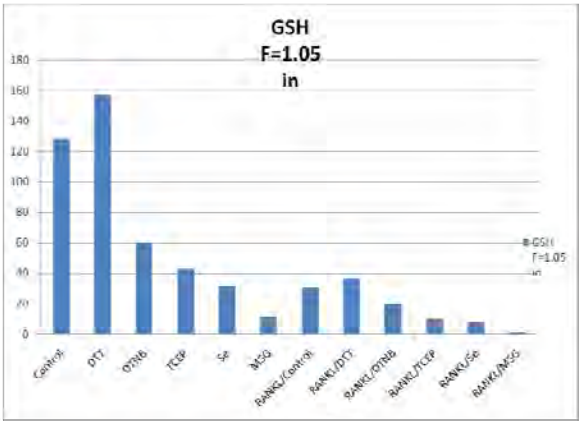
Trx1 expression in the presence of RANKL does not show any expression in this time loading. TrxR1 expression is increased by DTT redox stimulator. It is down regulated by Se, MSG, DTNB and TCEP.

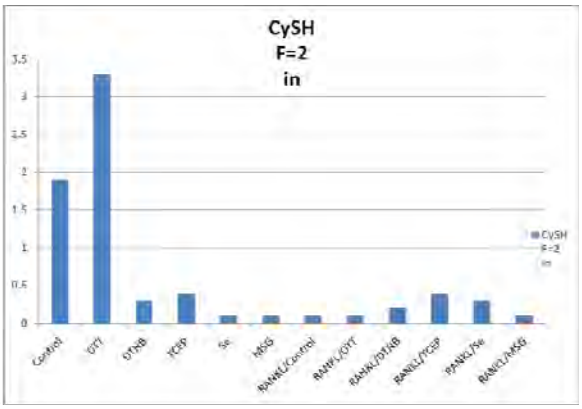
6.4 Glutathione/cysteine measurement of treated RAW264.7 cells by redox stimulators

The glutathione/cysteine level is a good indicator of changes in the redox state. Both extra- and intracellular GSH/Cys levels in total and reduced forms were measured to investigate the effects of different redox modulators during osteoclast differentiation.

The total and reduced forms of extra- and intracellular glutathione and cysteine of RAW264.7 cell treated by DTT, DTNB, TCEP, Se and MSG in the presence of RANKL and LPS was measured by HPLC analysis. The results are shown in Fig.12 and Fig.13.

A. 

B. 

C. 

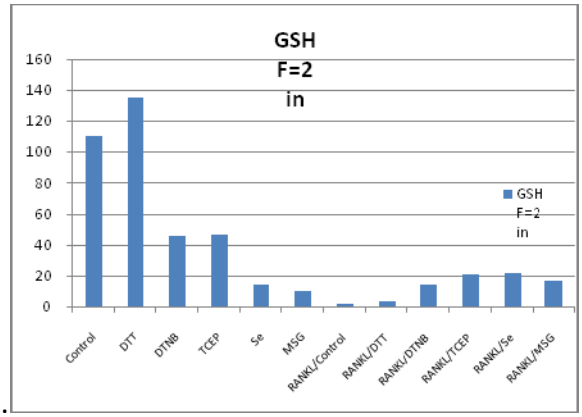
D. 

Figure 12. Intracellular GSH and Cys amounts of total forms (A-B) and reduced forms (C-D)

The total amount of cysteine in side the cell is not affected by RANKL and differentiation. However, DTT increased the amount of total form of cysteine but this is not apparent when RANKL was added. Additional oxidizing modulators decrease the total amount of cysteine inside the cells as well.

The total amount of glutathione intracellularly is decreased by addition of RANKL. The lowest quantity shows when MSG was added. DTT cause a rise in the total amount of glutathione inside the cells stimulated by RANKL.

The amount of reduced form of cysteine inside the cells shows a big drop even by TCEP and MSG treatments. Addition of oxidizing modulators leads to a decrease of the cysteine level inside the cells. Cells stimulated by RANKL shows a decrease of the reduced form of glutathione inside the cells as well.

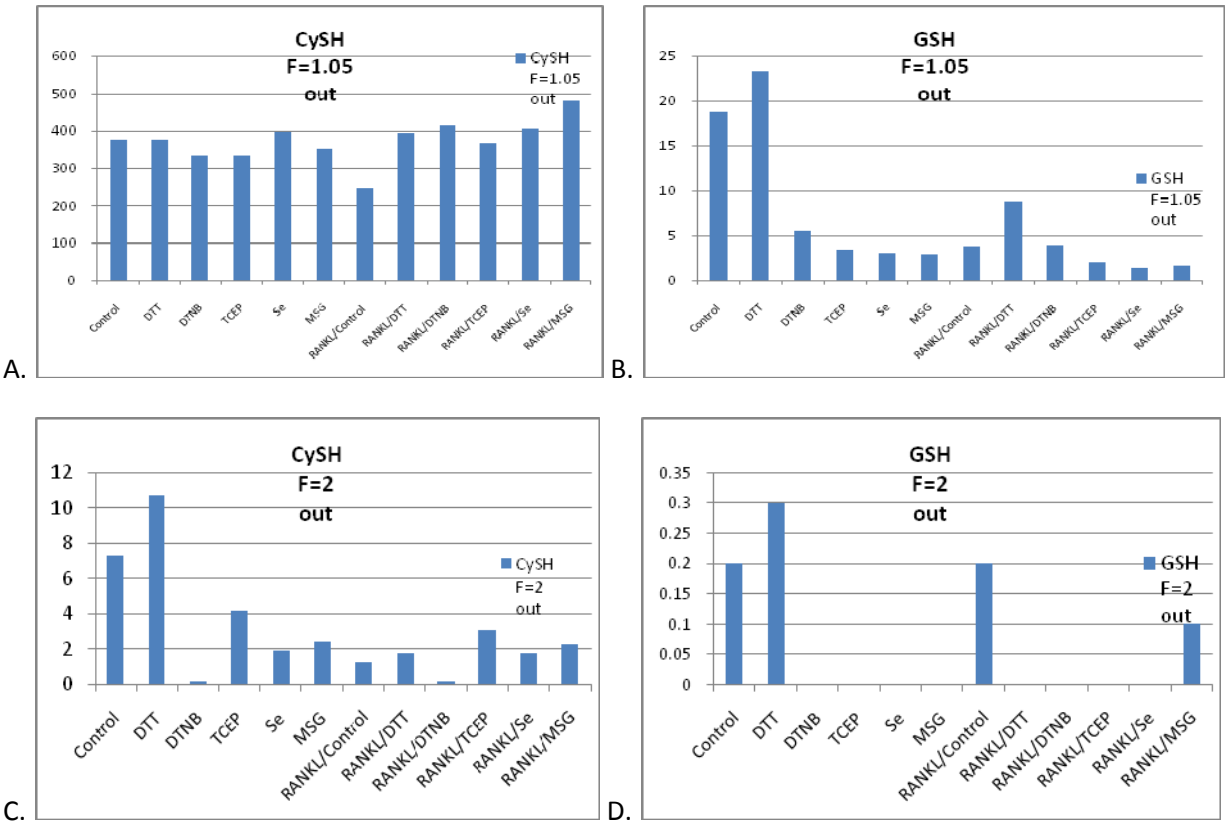


Figure 13. Extracellular GSH and Cys amounts of total forms (A-B) and reduced forms (C-D)

Fig.13 shows the extracellular measurements of glutathione and cysteine. The total amount of cysteine outside the treated cells by redox modulators is raised. This pattern is the same in cells stimulated by RANKL. The reduced form of cysteine in additional of oxidizing modulators did not show changes after RANKL stimulation. Additional of DTT shows drop of cysteine outside the cells.

Total amount of glutathione was decreased in the present of RANKL. This results show that there is no reduced form of glutathione outside the cells even in additional of DTT and RANKL also MSG redox modulator.

To show how many percent of cysteine and glutathione is intra- and extracellular the quantity of actual ratio from the HPLC results is shown in Table 6. The actual ratio comes from the amount of reduced form divided by total amount of cysteine and glutathione, intracellularly or extracellularly.

Table 6. Actual ratio of cysteine intracellular (A) cysteine extracellular (B) glutathione intracellular (C) glutathione extracellular (D)

A.

Reduced/Total	%
Control	24.3 %
DTT	29.2 %
DTNB	6.9 %
TCEP	10.8 %
Se	2 %
MSG	2.1 %
RANKL/Control	1.3 %
RANKL/DTT	1.6 %
RANKL/DTNB	3.6 %
RANKL/TCEP	7.8 %
RANKL/Se	6.9 %
RANKL/MSG	4.3 %

B.

Reduced/Total	%
Control	1.9 %
DTT	2.8 %
DTNB	0.05 %
TCEP	1.24 %
Se	0.47 %
MSG	0.68 %
RANKL/Control	0.52 %
RANKL/DTT	0.45 %
RANKL/DTNB	0.04 %
RANKL/TCEP	0.84 %
RANKL/Se	0.44 %
RANKL/MSG	0.47 %

C.

Reduced/Total	%
Control	86 %
DTT	85 %
DTNB	76 %
TCEP	(110) %
Se	46 %
MSG	95 %
RANKL/Control	7.1 %
RANKL/DTT	11.4 %
RANKL/DTNB	72 %
RANKL/TCEP	(199) %
RANKL/Se	(280) %
RANKL/MSG	

D.

Reduced/Total	%
Control	1.05 %
DTT	1.28 %
DTNB	0
TCEP	0
Se	0
MSG	0
RANKL/Control	5.26 %
RANKL/DTT	0
RANKL/DTNB	0
RANKL/TCEP	0
RANKL/Se	0
RANKL/MSG	6.25 %

6.5 DCF experiment of RAW264.7 cell

To determine the Intracellular ROS production, the DCF experiment was applied.

The experiment was only performed once and the signals were too weak in order to draw any conclusion. The experiment needs further optimization.

7. Discussion

To explore the intra- or extracellular redox environment effects during differentiation of osteoclasts and/or macrophages the RAW 264.7 cells in the presence of some redox modulators such as DTT, TCEP, DTNB, selenite and monosodium glutamate (MSG) were used. TRAP staining assay was performed to study the morphological changes of RAW264.7 cells. Stimulation by RANKL causes osteoclast differentiation, while the giants of macrophage can be seen by LPS stimulation. These are morphologically very different from the multinucleated and TRAP positive osteoclasts. Treatments with LPS do not yield many multinucleated TRAP positive cells but rather mononuclear TRAP negative cells.

To optimize the cell number for all experiments (5 days culturing), different cell densities were tested. The recommended cell number of 5000 cells/cm^2 was shown to be too low with cells only growing in the edges of wells. In the concentration of 25000 cells per cm^2 cells were dead because cells number was too high and there were not enough space and nutrition for cells survival. Finally 12500 cells/cm^2 in 24-well plate and 13000 cells/cm^2 in 6-well plate were applied. Cells adhered to surface of cell culture plate very fast. Therefore, half amount of cells treated by modulators and stimulators were applied at the middle of well. After three to four hours the rest of material were added to achieve an even distribution of cells in the wells. The conclusion from this data is that the cell concentration is important for growth and differentiation of cells.

In order to study the morphological changes caused by different treatments of redox modulators in the presence of RANKL and LPS; cells were evaluated by microscopy after TRAP staining. In the presence of RANKL, experiments with additional exposure to DTNB, MSG and selenite show many small mononuclear cells that were TRAP negative. The mononuclear TRAP negative cells from DTNB treatment show the inhibitory effect of thiol oxidation outside the cells during osteoclast differentiation. By using MSG to block the glutamate/cysteine transporter and thus prohibiting cysteine to enter the cell, we can show the role of cysteine during osteoclast differentiation. MSG in the presence of LPS had the same effect by inhibiting the formation of TRAP positive cells and shows the importance of xCT transporter. Selenite treatment, which results in the oxidation of thiol inside and outside the cells, had the same effect as MSG.

All results from PCR and gene studies show high intra assay variation. Therefore, we cannot draw any conclusion from them. The mRNA level of TRAP was highly up regulated after treatment with MSG; perhaps this is compensation by the progenitor cells. The expression of xCT and 4F2hc after MSG treatment also resulted in an up regulation, which is a possible correlation to the inhibition of cysteine uptake. When the cysteine glutamate transporter gets

blocked cells seems to try and compensate this by up regulations of xCT and 4F2hc gene expression. This further proves the importance of cysteine in osteoclast differentiation Cat k expression is down regulated in unstimulated cells but the reason still is not known. Cat K is an expressed gene in osteoclast. Cat K is cysteine protease and have important role in differentiation (31). Up regulation of Cat K in the present of RANKL and MSG is interconnected to protease function of Cat K. The osteoclast marker of CTR gene is down regulated by all redox treatment in the presence of RANKL and LPS. By addition of MSG, selenite and DTNB redox modulators CTR is expressed at lower levels and it could explain the undifferentiated mononuclear results from morphological studies. Unstimulated cells show that TRAP expression is up regulated after incubation with TCEP and DTT.

Due to indications the role of cysteine and glutathione in osteoclast differentiation the intra- and extracellular levels of cysteine and glutathione were measured. The ratio which is calculated from results of GSH/Cys measurement shows how many percent of cysteine and glutathione in- or outside the cells that is reduced. The cell's extracellular environment is more oxidized, especially after the addition of oxidizing agents. 80-85 % is the normal range of reduced GSH intracellularly. A highly interesting finding is that treatment and differentiation with RANKL results is in a very oxidizing environment, since oxidation of thiols prior to treatment with RANKL inhibits differentiation. The importance and availability of reduced cysteine seems to be essential for osteoclast differentiation. It also indicates that differentiation to osteoclasts requires reduced cysteine and that this is oxidized during the differentiation process.

Three independent experiments were performed but only one of them was analyzed. Therefore these results are very preliminary and no major conclusion can be drawn. It Is however worth pointing out that since three different methods (morphological, mRNA expression and GSH/Cys level measurement) show similar results and all indicate that cysteine play a crucial role in osteoclast differentiation, it will be worth exploring further.

8. Acknowledgments

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