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Effect of TRPV1 Agonist Capsaicin on Osteoclastogenesis in RAW 264.7 Derived Osteoclasts.

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Abstract: ***Osteoclasts are the cells uniquely responsible for dissolving both organic and inorganic components of bone during bone development and remodeling throughout life. The rate of bone remodeling and the number of remodeling sites are increased in a variety of pathologic conditions affecting the skeleton including post-menopausal osteoporosis, hyperparathyroidism, rheumatoid arthritis and metastatic bone cancer in which local and/or systemic alterations in the levels of hormones or pro-inflammatory cytokines stimulate bone resorption. Transient Receptor Potential Vanilloid 1 (TRPV1), calcium channels play a significant role in the control of pain, inflammation and other related processes. Hence in the current study, it is planned to explore the effects of TRPV1 agonist capsaicin on osteoclastogenesis in vitro on murine macrophage cell line RAW 264.7 induced to take up osteoclastic lineage by the addition of RANK (receptor activator of nuclear factor kappa B) ligand. The differentiation of cells of macrophage lineage into osteoclastic lineage upon the addition of RANK ligand was ascertained by TRAP (Tartarate Resistant Acid Phosphatase) staining, quantitative measurement of TRAP activity, light microscopy analysis and cell proliferation test. It was observed that capsaicin at a concentration of 250 µM/ml exhibited a statistically significant increase in TRAP activity, osteoclast number and in cell proliferation. The results of the study showed significant control of osteoclastogenesis through the pharmacological modulation of TRPV1 channels with agonist capsaicin.***
Keywords: Osteoclasts, TRPV1, RAW 264.7 cells, RANKL, Capsaicin.

I. INTRODUCTION

Bone in the adult skeleton is renewed continuously in response to a variety of stimuli by the process of bone remodeling. This involves removal of trenches or tunnels of bone from the surfaces of trabecular and cortical bone respectively by osteoclasts (Boyce et al., 2003). Osteoblasts subsequently fill in these trenches by laying down new bone matrix in them. Bone remodeling is a tightly regulated process in that formation follows resorption in a site specific manner and there are more than one million of these microscopic foci of remodeling at any time in the adult skeleton (Tatsumi et al., 2007). Osteoclasts are the cells uniquely responsible for dissolving both organic and inorganic components of bone during bone development and remodeling throughout life. Osteoclasts are bone resorbing cells arising from the osteoprogenitor cells derived from mononuclear precursors in the myeloid lineage of hematopoietic cells of the bone marrow. Bone is a storehouse of growth factors such as insulin like growth factors (IGF), transforming growth factor-β (TGF-β), fibroblast growth factors, platelet-derived growth factors and bone morphogenetic proteins (Hauschka et al., 1986). These growth factors are continually released into the bone marrow cavity via osteoclastic bone resorption during physiological bone remodelling. Thus bone is a fertile soil for metastatic cancer cells to colonize.

Bone resorption by osteoclasts is regulated by various molecular signals of which Receptor Activator for Nuclear Factor kappa B Ligand(RANKL) – a member of tumour necrosis factor superfamily and intracellular calcium signaling plays a decisive role (Boyce et al., 2007). RANKL is a membrane protein residing on osteoblasts and their precursors that activate osteoclast formation by stimulating its receptor RANK through interaction with an adaptor molecule TRAF6. Simultaneously, changes in the intracellular calcium signaling mediated through Transient Receptor Potential Vanilloid (TRPV) channels lead to calcium oscillations (Kajiya et al., 2010; Hwang and Putney., 2011). The resultant downstream intracellular signaling pathways lead to a cascade of biochemical reactions which upregulates the expression of genes regulating osteoclastogenesis and culminates in increased bone resorption and hence an imbalance in bone remodeling. The rate of bone remodeling and the number of remodeling sites are increased in a variety of pathologic conditions affecting the skeleton including post-menopausal osteoporosis, hyperparathyroidism, rheumatoid arthritis and metastatic bone cancer in which local and/or systemic alterations in the levels of hormones or pro-inflammatory cytokines stimulate bone resorption (Boyce et al., 2003).

TRPV1, originally named Vanilloid Receptor 1 (VR1) and commonly referred as the capsaicin receptor, was first described as a polymodal receptor activated by three pain-producing stimuli; vanilloid compounds (capsaicin, resiniferatoxin), moderate heat (≥43 °C) and low pH (<5.9) (Tominaga et al., 1998). TRPV1 is activated in response to various stimuli such as physical abrasion, heat, protons, and by a variety of pharmacological ligands such as capsaicin, the endocannabinoid anandamide and arachidonic acid metabolites. TRPV1 ion channels have been implicated in the regulation of pain perception, inflammation and cardiovascular homeostasis (Wong and Gavva, 2009). Several studies have demonstrated *in vitro* and *in vivo*, that inflammatory mediators (bradykinin, prostaglandin E2, extracellular ATP, glutamate and nerve growth factor) indirectly sensitize TRPV1 (Calixto et al., 2005). Following exposure of sensory neurons to inflammatory mediators, responses to capsaicin or heat are dramatically enhanced to the extent that body temperature can be sufficient to activate nociceptors (Liang et al., 2001). Inflammatory mediators sensitize TRPV1 function by various mechanisms; they may increase TRPV1 expression levels in the membrane (Zhang et al., 2005), induce TRPV1 phosphorylation by protein kinases (Mohapatra & Nau, 2003) or release the inhibition of TRPV1 by phosphatidyl inositol 4,5-bisphosphate, which render the channel more responsive to agonist stimulation (Bhave and Gereau, 2004).

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a highly potent active ingredient of hot red and chilli peppers that belong to the plant genus Capsicum (Solanaceae) (Holzer, 1991:Knotkov et al., 2008), is extensively used as a natural food additive for flavoring, seasoning, coloring, and antiseptic properties. It has been found to act on the capsaicin-sensitive afferent nerves and it has been extensively studied *in vivo* and *in vitro* for the treatment of migraine, chronic cough, overactive bladder and diabetes (Gunthorpe and Szallasi, 2008: Suri and Szallasi, 2008), as a potent analgesic (Roufogalisand Dedov: Sternerand Szallasi, 1999) and anti-cancer agent (Surh, 1999). Capsaicin has been well known to interact at primary sensory neurons exerting the characteristic actions, including excitation, desensitization and neurotoxicity (Holzer, 1991). These actions of capsaicin appear to be mediated by the stimulation of specific vanilloid receptors (Szallasi and Blumberg, 1999), which may have therapeutic value, particularly relieving pain (McCleane, 2000). Many of the capsaicin actions are mediated by the TRPV1 receptor, which is a non-selective membrane cation channel that is activated not only by capsaicin but also by noxious heat, acid and by recently described endogenous lipids (Tominaga, 2005). Many studies have reported that capsaicin exerts anti-proliferative effects by causing cell cycle arrest and inducing apoptosis in vitro in many different human cancers (Kimet al.,2007: Chow et al.,2007: Wu et al., 2006).

II. MATERIALS AND METHODS

A. *Culturing Of Macrophages*

Murine macrophages RAW 264.7 cells were procured from National Centre for Cell Sciences (NCCS), Pune, India. RAW 264.7 cells were cultured in DMEM, supplemented with 1X antibiotic antimycotic solution and 10 % fetal bovine serum (Himedia, India). Cells were grown under standard growth conditions (temperature 37°C, 5% CO_2 and 95% humidity) in a CO_2 incubator (Forma Scientific, USA). Confluent monolayer, cell cultures were detached using a cell scraper (Himedia, India) in Dulbecco's phosphate buffered saline (Himedia, India) and then subcultured at a split ratio of 1:3 in 12.5 cm^2 tissue culture flask (TCG2 – Himedia, India). The media was changed every alternative day. The cells were grown in growth medium containing 10 % FBS or maintained in maintenance medium containing 5 % FBS. After arriving confluency, the cells were seeded on to 96 well microtitre plates (Himedia, India) and were used for further assays.

B. *Differentiation Of Raw 264.7 Cells Into Osteoclasts*

Differentiation of osteoclasts from murine macrophages RAW 264.7 was carried out as per described by Collin-Osdoby et al. (2003). Confluent RAW cells were seeded in a 6-well plate (5 X 10^4 cells/well) in osteoclastogenic media (DMEM + 10% FBS + Soluble recombinant RANKL) were added to initiate osteoclast development.

Different concentrations of RANKL (5, 10, 20, 30, 40 and 50 ng/ml) were used to standardise the initiation of differentiation into osteoclast. RAW macrophages on treatment with RANKL were incubated for initial 3 days and were observed under microscope for the initiation of cell fusion and occurrence of multinucleated RAW-osteoclasts. Further fresh media along with RANKL were changed and continued to incubate (5-6 days) for obtaining the fully differentiated osteoclasts.

These osteoclast populations were used for further assays. OC cells used in the further assays made up at least 85% of the total treated cell population by counting the number of multinucleated cells.

The total number of cells in 10 fields of view, calculating the average of each and dividing the OC average number of cells by the total average number of cells.

C. Quantitation of TRAP Activity

Tartrate-resistant acid phosphatase (TRAP), a well characterized biochemical marker for osteoclast differentiation and activity. TRAP quantification was assayed by following the method of Collin-Osdoby et al. (1995). RAW cells (incubated with RANKL) were co-treated with different concentrations of capsaicin and capsazepine for 5- 6 days, were harvested by using cell lysis buffer (0.1% Triton X-100). Further, 100 µl of cell lysate (normalized with HBSS, 50 µg/ml protein) were plated in 6 well plate and placed on ice. To the above 100 µl of 10 mM p-nitrophenyl phosphate (PNPP) in 50 mM sodium acetate (pH 4.8) with 10 mM sodium tartrate was added and the plate was incubated at room temperature for 30 min. The reaction was stopped by adding 100 µl of 0.1 N NaOH and the absorbance of released p-nitrophenol was determined in a Bio-Rad ELISA reader at 405 nm.

D. Qualitative Analysis of osteoclast Differentiation - TRAP Staining

TRAP staining was performed according to Collin-Odsoby et al. (2003). Briefly, cells incubated with RANKL were treated with or without capsaicin and capsazepine. Then cells were washed with PBS and fixed in 1% formaldehyde for 15 min. Later the fixed cells were washed with PBS and incubated with methanol at -20° C for 5 min. Further cells were subjected to TRAP staining solution (mixture of naphthol AS-BI phosphoric acid, acetate buffer, tartrate solution and Fast Garnet GBC salt) at room temperature for 1 h in dark. Stained cells were washed with distilled water and visualized for multinucleated (3 or more nuclei) TRAP positive osteoclasts using light microscopy and photographed.

E. Studies on cell proliferation - BrdU incorporation test

Cell proliferation was measured by BrdU (5-bromo-20-deoxyuridine) incorporation test using a commercially available kit (Calbiochem/Merck, USA) following the manufacturer's instructions. Briefly, the harvested osteoclasts were seeded to a 96-well plate (600 osteoclasts /well/ 100 µl) and were treated with or without capsaicin for 24 h. Followed by incubation with BrdU label (1:2000 dilution) for 24 h, the plates were then washed and fixed with anti-BrdU antibody and peroxidase goat anti-mouse IgG HRP conjugate. The formed immune complex was measured using tetramethylbenzidine solution and the reaction was terminated using 2.5N sulphuric acid. Absorbance was measured at 450 nm in a microplate reader.

III. RESULTS

A. Effect of RANKL on Osteoclast Differentiation

Results indicated that 50 ng/ml of RANKL effectively induced the differentiation of RAW cells into multinucleated osteoclast lineage after 5 days in culture, which was observed by light microscopy (Fig. 1). As observed in 3rd day cells had membrane projections and appeared to fuse. More than 2 nuclei/cell was observed, it implicates the initiation of differentiation process. Whereas, on day 5, osteoclast cells, appeared larger (increased pseudopodial motility, associated with increased cell spreading) and were multinucleated, more number of cells with > 3 nuclei was observed as compared to undifferentiated cells which were small with single nucleus, and lightly adhered to the plate.

B. Effect of Capsaicin on Osteoclast Differentiation

The TRPV1 channel modulators capsaicin were treated with RAW cells (incubated with RANKL) to measure the role on osteoclastogenesis. After 5 days co-treatment with different concentrations of capsaicin and capsazepine on RANKL incubated RAW cells were confirming the differentiation by performing TRAP activity, counting osteoclast cell number and microscopic studies. The results of TRAP activity shows that, 0.5-6.25 µM/ml of capsaicin didn't show any effect on activity, but increasing the concentrations from 12.5-300 µM/ml showed significant increase in the TRAP activity (Fig. 2a). As observed, capsaicin strongly stimulated the formation of osteoclastogenesis in RANKL treated RAW 264.7 cells. The maximum activity was found at 250 µM/ml ($P < 0.001$).

The results of counting of osteoclast cell number in capsaicin treated cells were in agreement with TRAP activity (Fig. 2b). The significant increase in the number of osteoclast cells (> 3 nuclei) were found at cells treated with 250 µM/ml of capsaicin. The microscopic studies (Fig. 2c) shows that the more number of nuclei (8-9) are present in capsaicin treated cells as compared to control (RANKL treated RAW cells).

The TRAP staining is most commonly used stain to visualize osteoclasts is based on their high level of TRAP activity, which is upregulated in osteoclast development and is essential for their resorption of bone. The dark pink coloured cells indicate the differentiated osteoclasts. Fig. 2d shows the RANKL treated RAW cells were successfully differentiated into osteoclasts after 5 days treatment. Treatment with capsaicin significantly increased the number of TRAP positive osteoclast cells as compared to control cells (RANKL treated RAW cells).

C. Effect of Capsaicin on Osteoclast Proliferation

Results of BrdU incorporation assay show the effect of capsaicin and capsazepine treatment on osteoclast proliferation at 12 and 24 h period. Treatment of the cells at 12 and 24 h time periods with capsaicin increased the proliferation and statistically significant decrease in cell proliferation was observed in capsazepine ($P < 0.001$)as compared to untreated control cells.

IV. DISCUSSION

Bone remodeling is a physiological process in which old or damaged bone is removed by osteoclasts (bone-resorbing cells), then replaced by new bone formed by osteoblasts (bone-forming cells). Proper balance is controlled by the coupling of bone formation to bone resorption, an imbalance between these cells may occur under certain pathological conditions, which leads to abnormal bone remodeling and the development of bone disorders. Osteoclasts, multinucleated cells, are derived (or differentiated) from hematopoietic cells of the monocyte/macrophage family (Boyle et al., 2003). Mouse RAW264.7 macrophage cells have been shown to retain the capacity to differentiate into osteoclast-like cells in the presence of a receptor activator of NF-κB ligand (RANKL; Hsu et al., 1999), which is essential and sufficient to promote the maturation of osteoclasts. RANK is the receptor for RANK-Ligand (RANKL) and part of the RANK-RANKL signaling pathway that regulates osteoclast differentiation and activation.

The clinical relevance of RANKL-RANK relationship is underscored by the fact that many forms of osteoporosis are characterized by an increase in the ratio of circulating RANKL (Jabbar et al., 2011; Wasilewska et al., 2010; Xu et al., 2011). RANKL is a ~35 kDa type II transmembrane protein with a short N-terminal intracellular tail and a C-terminal extracellular region that contains a connecting stalk and receptor binding domain (Nelson et al., 2012). Membrane-bound RANKL activates RANK to generate osteoclasts through cell-cell contact. Results of the differentiation studies implicate, lower concentrations of RANKL were not sufficient to generate osteoclasts from the RAW macrophages. Lower RANKL concentrations delay the kinetics and final yield of osteoclasts formation from RAW cells (Yamamoto et al., 2002; Koseki et al., 2002; Shin et al., 2002).

When treated with RANKL, RAW264.7 cells that are committed to differentiate to osteoclasts, known as preosteoclasts, begin to express high levels of osteoclast-associated genes such as tartrate-resistant acid phosphatase (TRAP; Igarashi et al., 2002). TRAP is a binuclear iron protein that promotes the hydrolysis of nucleotides, aryl phosphates, and phosphoproteins (Hayman et al., 1989; Nash et al., 1993). The enzyme has been shown to partially dephosphorylate the bone matrix phosphoprotein osteopontin (Ek-Rylander et al., 1994). In addition to acting as a phosphatase, the enzyme is capable of generating oxygen radicals in the presence of hydrogen peroxide (Halleen et al., 1999; Hayman and Cox, 1994; Sibille et al., 1987).

Mice deficient in TRAP demonstrated that the enzyme is essential for a proper mineralization of cartilage in developing bones and for maintenance of the adult skeleton (Hayman et al., 1996). TRAP mainly involved in active bone resorption processes, since the localization of TRAP activity appears only at intracellular sites in lysosomes or bone-resorption area of osteoclasts. Transgenic mice overexpressing TRAP exhibit increased osteoclasticresorption of bone, which is largely compensated by an increase in the rate of bone formation (Angel et al., 2000).

TRPV1, originally named vanilloid receptor 1 and commonly referred as the capsaicin receptor, was first described as a polymodal receptor activated by three pain-producing stimuli; vanilloid compounds (capsaicin, resiniferatoxin), moderate heat (≥43°C) and low pH (<5.9) (Tominaga et al., 1998).TRPV1 activation rapidly evokes acute pain sensation and allows Ca^{2+} entry to initiate downstream signaling events.TRPV1 ion channels have been implicated in the regulation of pain perception, inflammation and cardiovascular homeostasis (Wong and Gavva, 2009).

Capsaicin, which is responsible for the piquancy of hot chilli peppers, is widely used in Asian diets as food additives. The noxious property of capsaicin is also exploited, as capsaicin injection has been serving as a standard animal model for pain study. The molecular basis for these actions has started to emerge since the cloning of its receptor, transient receptor potential vanilloid 1 ion channel (Caterina et al., 1997).Capsaicin is among the most powerful chemical agonists of TRPV1. Results of differentiation and proliferation assays indicates that capsaicin increased activity of TRAP and cell viability by activating TRPV1 channel. Being small and hydrophobic, capsaicin crosses the plasma membrane readily to reach its intracellular ligand-binding site on TRPV1 (Jordt and Julius, 2002), leading to channel activation and cation permeation. TRPV1 activation rapidly depolarizes nerves to evoke acute pain sensation and allows Ca^{2+} entry to initiate downstream signaling events such as neuropeptide release or production of other second messengers. Recent cryoelectron microscopy data, (Liao et al., 2013) together with computational modelling and mutagenesis studies, provide solid evidence of the molecular mechanisms of TRPV1 activation. Capsaicin sits in an intracellular binding site in a ‘‘tail-up, head-down’’ configuration. While the vanillyl and amide moieties establish directed interactions to anchor its binding position, the flexible hydrophobic tail may adopt several bioactive conformations and mediate hydrophobic contacts within the receptor (Yang et al., 2015).

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Legends

Fig. 1 :Effect of RANKL on osteoclastogenesis

RAW macrophages on treatment with RANKL (50 ng/ml) were incubated for 3rd and 5th days, observed under microscope.

Fig. 2a :Quantitation of TRAP activity

RAW cells (incubated with RANKL) were co-treated with different concentrations of capsazepine for 5-6 days. Values were expressed as mean ± SD (n=6). Comparison was made between control Vs. capsaicin groups. ***P < 0.001, **P <0.01, *P <0.05 and NS – non significant.

Fig. 2b :Determination of osteoclast numbers

The total number of cells in 10 fields of view, calculating the average of each and dividing the osteoclasts average number of cells by the total average number of cells was performed. Values were expressed as mean ± SD (n=6). Comparison was made between control Vs. capsaicin groups. ***P < 0.001, **P < 0.01, *P < 0.05 and NS – non significant

Fig. 2c :Morphological analysis of differentiated osteoclasts

Capsazepine treated and untreated cells were observed under Phase contrast microscopy.

Fig. 2d :Qualitative analysis of osteoclast differentiation

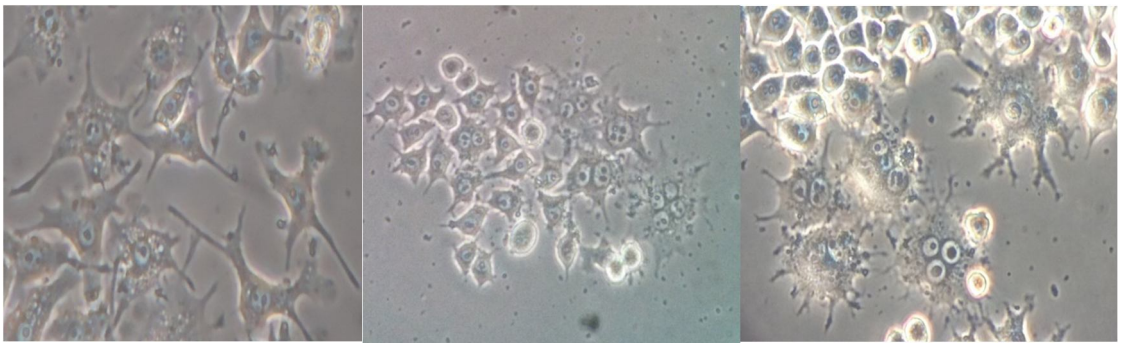
Capsazepine treated and untreated cells were fixed with formaldehyde and incubated with TRAP staining solution for 1 h in dark. TRAP positive osteoclasts were visualised in light microscopy and photographed.

Fig.3:Effect of capsaicin on osteoclast proliferation

Control and treated cells were treated with BrdU label, fixed with anti-BrdU antibody and peroxidase goat anti-mouse IgG conjugate. The amount of BrdU label incorporated into the cells which is a direct measure of cell proliferation was measured at 450 nm. Values were expressed as mean ± SD (n=6). Comparison was made between control Vs. capsaicin groups. **P < 0.01.

FIGURES

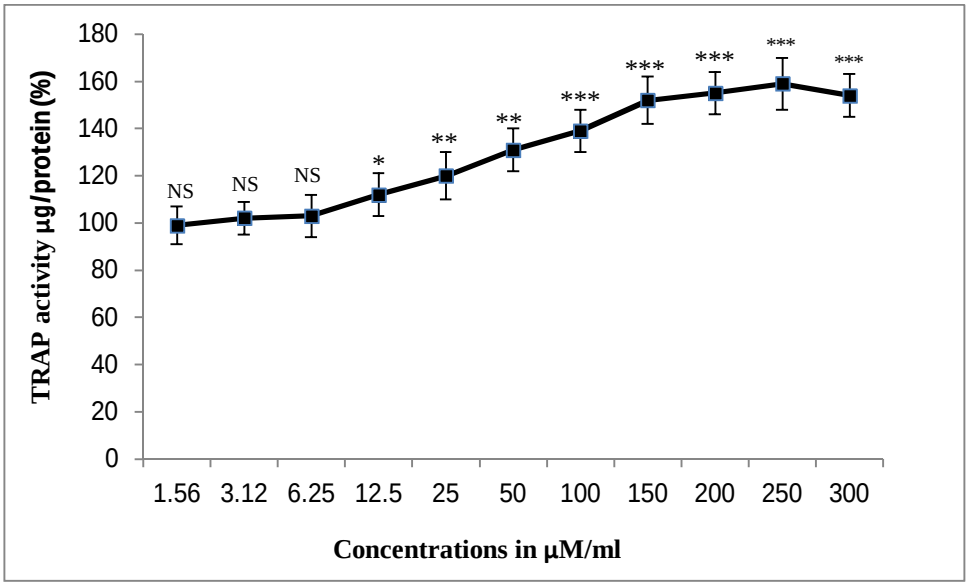
Fig. 1:Differentiation of RAW 264.7 cells into osteoclasts



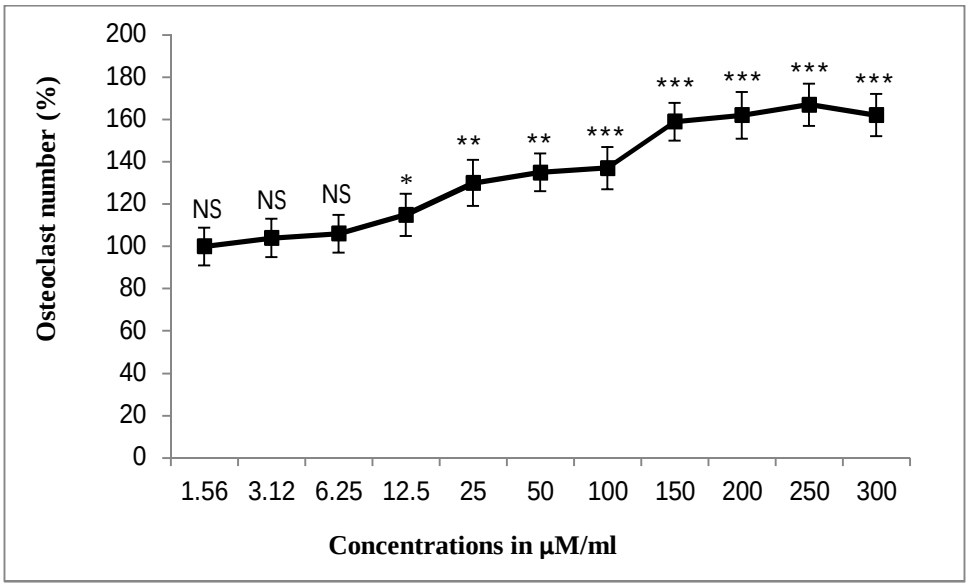
RAW 264.7 cells | RANKL treatedRAW cells - 3rd day | RANKL treatedRAW 264.7 cells - 5th day

Fig.2 :Effect of capsaicin on osteoclastogenesis

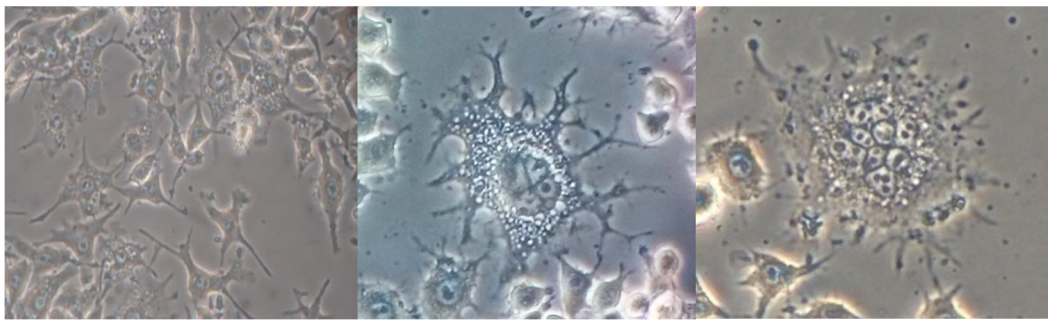
Quantitation of TRAP activity



Etermination of osteoclast numbers



Microscopic studies

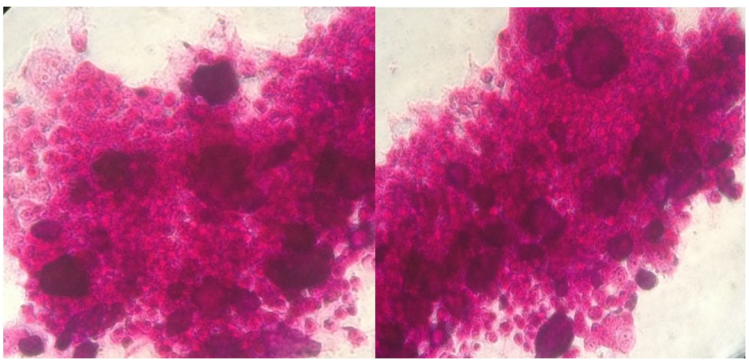


RAW 264.7 cells

RANKL treatedRAW 264.7 cells

RANKL treatedRAW 264.7 cells + capsaicin

a. TRAP activity



RANKL treatedRAW 264.7 cells

RANKL treatedRAW 264.7 cells + capsaicin

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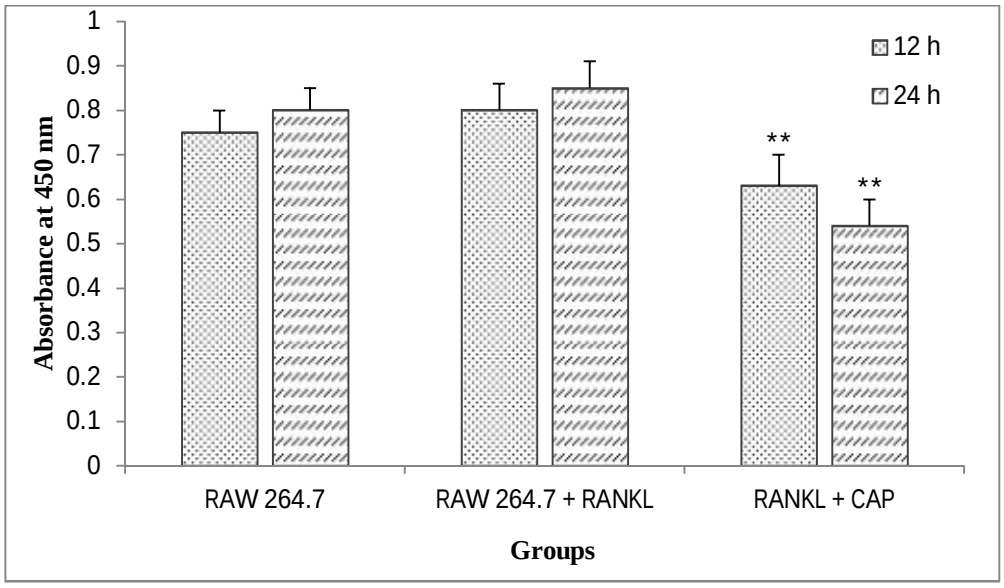
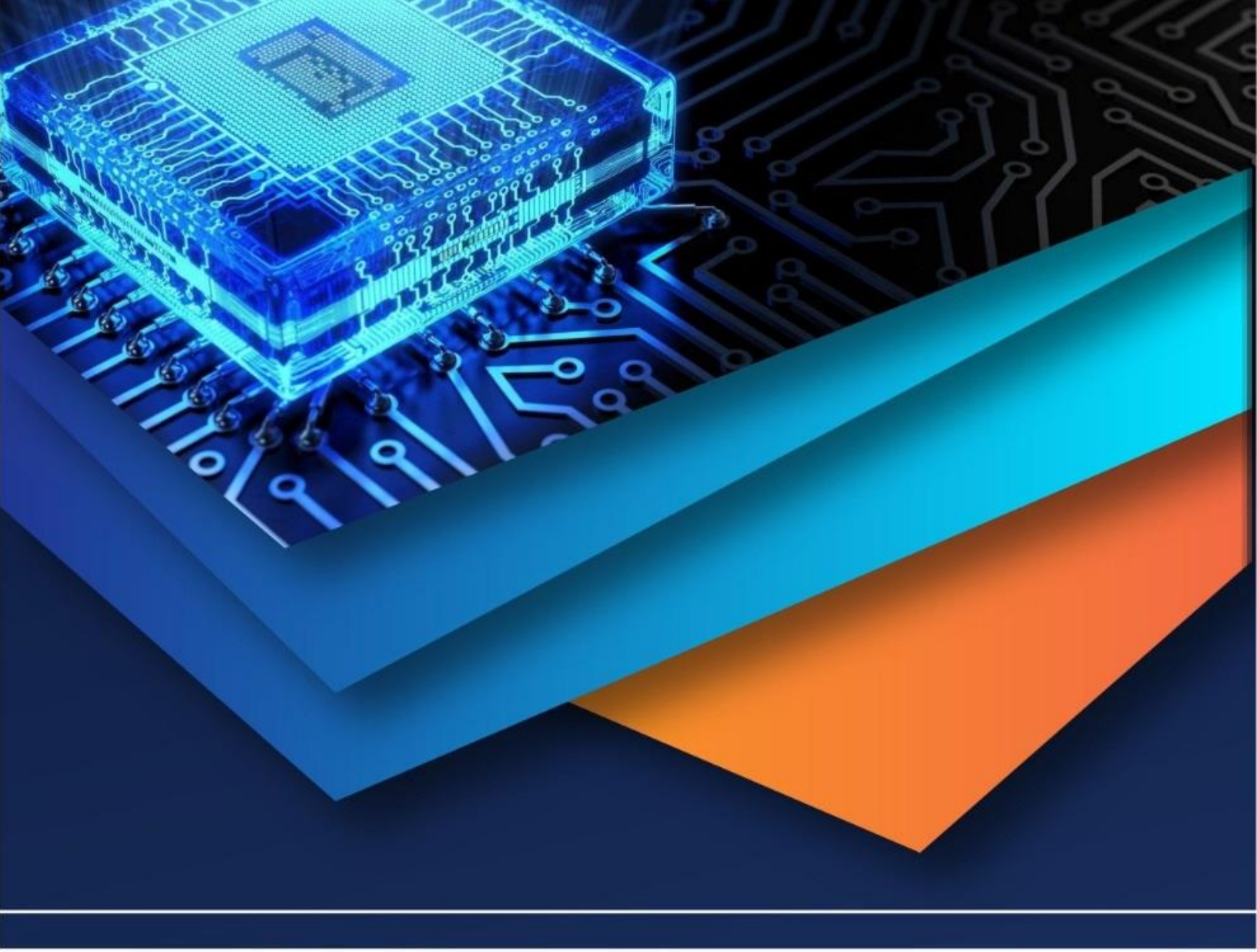
Fig.3 :Effect of capsaicin on cell proliferation – BrdU incorporation test





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45.98

IMPACT FACTOR: 7.129

IMPACT FACTOR: 7.429

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