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Influence of TRPV1 Modulators Capsaicin and Capsazepine on Osteoclastogenesis *in vitro*

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Abstract—**The influence of TRPV1 modulators capsaicin and capsazepine to regulate the growth and proliferation of osteoclasts and thereby control osteoclastogenesis was studied 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 and subsequent light microscopy analysis. Crystal violet test, Sulphorhodamine B assay, NBT reduction test and Neutral red dye uptake test were taken as end point indicators to assess osteoclast growth and proliferation. It was observed that capsaicin at a concentration of 200µg/ml exhibited a statistically significant increase whereas capsazepine at a concentration of 94.2µg/ml (IC_{50} concentration of capsazepine) exhibited a statistically significant inhibition of osteoclast growth and proliferation. Results of the study indicate that both the compounds influence osteoclastogenesis with capsaicin showing a potentiating and capsazepine showing inhibitory effect thereby implicating their ability to influence bone resorption and hence bone remodeling. The compounds could be of therapeutic importance in musculoskeletal diseases and other inflammatory diseases of the bone to regulate the disruption in bone remodeling equilibrium.**

Index Terms— **Capsaicin, Capsazepine, Osteoclastogenesis, RAW 264.7, TRPV1 channels**

I. INTRODUCTION

Bone has multiple functions in vertebrates, including protection of vital organs and hematopoietic marrow, structural support for muscles, storage and release of vital ions, such as calcium and of growth factors stored in the matrix [1]. Osteoclasts, the body's principal bone-resorbing cells, are multinucleated giant cells of hematopoietic originthat not only play a critical role in skeletal development and maintenance but are also implicated in the pathogenesis of various pathogenic conditions including postmenopausal osteoporosis, cancer and other inflammatory diseases of the bone.

Calcium is crucial for bone homeostasis and has multiple roles in osteoclast formation, survival, and activity[2] as the skeleton is influenced both by external Ca^{2+} balance as well as by the intracellular Ca^{2+}signaling in bone cells. Normal bone homeostasis does not only depend on extracellular calcium, but also on intracellular Ca^{2+}signaling cascades that regulate the differentiation and functioning of multiple bone cells [3]. Indeed, 99% of the body's calcium is stored in bones where it contributes to its mechanical and structural properties. Consequently, bone requires a sufficient supply of calcium to maintain skeletal integrity [4]. The maintenance of extra and intracellular Ca^{2+} homeostasis is thus crucial for bone biology and depends for a large part on Ca^{2+}channels. Several types of Ca^{2+}channels exist including (i) Ryanodine receptors and inositol- 1,4,5-triphosphate receptors (IP3) which mediate release of calcium from the endoplasmic reticulum (ii) Store-operated calcium channels (SOCE), which include ORAI1 and STIM1 which mediate the flux of extracellular Ca^{2+}into the RER via ORAI1 upon intracellular store depletion sensed by STIM1 (iii) Voltage-gated Ca^{2+}channels(VGCC) that allow Ca^{2+}influx upon cell depolarization (vi) Stretch-activated Ca^{2+}channels that mediate Ca^{2+}influx after mechanical stimulation and (v) the transient receptor potential (TRP) family of cat ion channels [2].

TRPV families of ion channels are involved in the extracellular calcium homeostasis and intracellular Ca^{2+}signaling in bone cells. The TRPV family consists of six members, which are all membrane proteins composed of six transmembrane domains that form a cation permeable pore region. TRPV 1-4 are non-selective cation channels, whereas TRPV5 and 6 are highly Ca^{2+}selective. TRPV1 is activated by a variety of stimuli including heat, noxious chemicals and low pH [5]-[7]. TRPV1 is expressed in osteoblasts and osteoclasts and promotes differentiation of both cell types. Indeed pharmacological blockage of TRPV1 inhibits *in vitro* osteoclast and osteoblast differentiation [8]. Thus TRPV1 may directly affect and influence osteogenic cell differentiation. The TRPV1 channel functions as a pain and temperature sensor in mammalians [9].

Many pharmacological compounds which can have a profound influence on TRPV1 channels can therefore influence osteoclastic/osteoblastic activity and thereby can regulate the bone remodeling process which is crucial for maintaining the skeletal equilibrium. Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is an active component of chilli peppers, which is present in plants belonging to the genus Capsicum. Capsaicin belonging to the class of compounds called capsaicinoids is a potent TRPV1 channel agonist and is produced as asecondary metabolite by chilli peppers. It is a receptor ligand that regulates nerve-related pain-sensitive signals, inflammation and cancer growth [10]. Capsazepine is the potent antagonist of the compound capsaicin and hence is a TRPV1 antagonist

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[11]-[13]. It was obtained by modification of the chemical backbone of capsaicin [14].Capsazepine blocks the painful sensation of heat caused by capsaicin which activates the TRPV1 ion channel.

The objective of the current studyis explore the effects of TRPV1 channel modulators capsaicin (agonist) and capsazepine (antagonist) on the growth and development of osteoclasts*in vitro*.This is expected to provide leads about the influence of these compounds in controlling the TRPV1 channels and thereby the potential to exploit them in the therapy of many diseases involving dysregulation of bone remodeling in humans and higher animals.

II. MATERIALS AND METHODS

A. Procurement of chemicals: Capsaicin was procured from Hi media Laboratories Pvt.Ltd, Mumbai, India and Capsazepine was purchased from Sigma Aldrich,St.Louis, MO, USA.

B. Procurement and maintenance of RAW 264.7 cells: The murine macrophage cell line RAW 264.7 was procured from National centre for cell sciences (NCCS), Pune, India. The cells were cultured in Dulbecco's modified Eagle's medium with 200mM L-glutamine, Antibiotic and antimycotic solution (1X) and 10% foetal bovine serum. Cells were grown under standard growth conditions (Temperature-37^{o} C,5% CO_2 and 95% humidity) in a CO_2 incubator.When a confluent monolayer was formed cells were detached by using a cell scraper and then subcultured into various culture flasks (based on therequirement for assays). The media was changed every alternative day.

C. Osteoclast differentiation: RAW macrophage cells were subcultured in 6 well plate, with osteoclastogenic media (media that contains RANKL) at every alternative day. On the 6th day, multinucleated osteoclast cells were observed to emerge/differentiate from cells of macrophage lineage. To confirm the differentiation of RAW cells in to functional osteoclasts TRAP staining was performed and the differentiated osteoclasts used for various assays.

D. TRAP Staining: This was performed by following the method described by Takahashi *et al*., [15]. Briefly, the conditioned medium was removed from the plate and the cells were rinsed three times with PBS. Fixative solution was added and the cells were fixed 15 min at room temperature. Fixative was removed and the cells rinsed three times with PBS and once with deionized water. The cells were incubated at -20^{o} C in methanol for several minutes, followed by a rinse with water. Enough staining solution was added to cover the cells and the plates were incubated at 37^{o}C for 1 h in the dark. Stain was removed and the cells rinsed thoroughly with water. The sample was air dried and viewed under an inverted phase contrast microscope.

E. Preparation of stock solution of capsaicin and capsazepine: The stock solution of the test compounds was prepared by dissolving in DMSO (1mg/ml). From the stock solution, the required concentration of the compounds was diluted in incomplete media and used for various assays.

F. Crystal violet test: To determine the cell viability and growth, crystal violet assay was carried out as described earlier[16]. Briefly, the cells were plated and treated with different concentrations of the test compounds and allowed to proliferate for 72 h. After 72 h of proliferation the cells were fixed in 10% formalin saline (50µl/well) for 30 min. Then the cells were stained with crystal violet (0.05% w/v) for 30 min. The wells were then washed thoroughly with distilled water to remove any unbound dye and destained with Sorenson's buffer (0.1 M sodium citrate in 50% ethanol, pH 4.2). The absorbance of the extracted stain was measured at 540 nm.

G. Sulforhodamine B assay (SRB): Briefly, cell suspensions containing 1×10^4 viable cells/ml were plated onto 96-well plates and allowed to attach for 24 h at 37°C in a 5% CO_2 atmosphere. The cells were then exposed to the test compounds for 24 h. Cells were washed with PBS and fixed with trichloroacetic acid at 4°C for 1 h. After washing with water, cells were stained with SRB. Protein bound stain was solubilized with unbuffered Tris base [tris(hydroxymethyl)aminomethane]. The absorbance was then measured at 540 nm using a microplate reader. Results were expressed as relative absorbance of the treated cells compared with untreated controls.

H. NBT reduction test: NBT (Nitroblue tetrazolium) reduction test was performed following the method described earlier [17]. Briefly, after 72 h of proliferation, 10µl of nitro blue tetrazolium chloride (5 mg/ml in phosphate buffered saline – pH 7.4) was added to the cultured cells and incubated in a CO_2 incubator at 37ºC for 5 h. The cells were then washed three times with saline and the formazan crystals were solubilised by adding 100µl of isopropanol. The optical density was measured at 570nm in a microplate reader.

I. Neutral red dye uptake test: Neutral red dye uptake test was performed by the method of Parish and Mullbacher [18]. Briefly, after 48 h of incubation, the medium was removed and viable cells were stained for 45 minutes with 5 µl of neutral red (0.1%). The stain was then removed and the wells were washed three times with 0.9% saline. Neutral red was then released by the addition of 1:50 mixture of 100mM acetic acid and ethanol. The optical density was measured at 570nm in a microplate reader.

J. Statistical analysis: All the experiments were carried out in triplicate on atleast three different occasions and the mean of the replicate values were taken. Values were expressed as mean ± SD. Statistical analysis of the data was determined by Student's-t-test and comparisons were made between the untreated control groups and the treated groups.

III. RESULTS

A.Differentiation of osteoclast cells by TRAP assay:

Fig 1 illustrates the differentiation of cells of macrophage lineage into functional osteoclasts as determined by TRAP staining. Result shows that mononucleated macrophage cellsRAW 264.7 were differentiated into characteristic giant multinucleated cells implicating the differentiation of the cells of macrophage lineage into osteoclast cells. Unstained cells are indicative of undifferentiated cells. After confirming the differentiation of RAW 264.7 cells into osteoclast lineage cells, the differentiated osteoclasts were used for various cell growth and cytotoxicity evaluation assays.

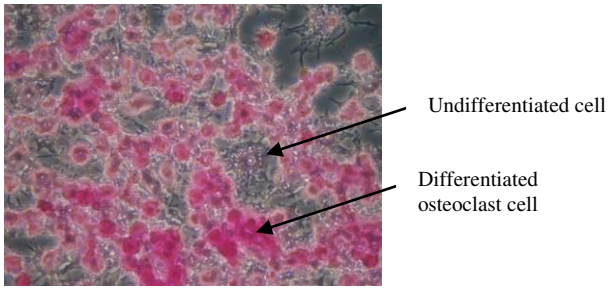

Fig 1: Differentiation of osteoclast cells from RAW 264.7 macrophage cells upon the addition of RANK ligand. Pink colour stained cells indicate differentiated multinucleated osteoclasts and unstained cell shows undifferentiated macrophage lineage cells. (Magnification- 10 X 4x)

B.Effect of capsaicin and capsazepine on osteoclast growth and proliferation in vitro (crystal violet test, Sulphorhodamine B assay, NBT and Neutral red dye uptake test):

Fig 2, 3 and 4shows the effect of capsaicin and capsazepine on the growth and proliferation of osteoclasts cells as determined by crystal violet test,SRB, NBT reduction test and Neutral red dye uptake test. Treatment with capsaicin (200µg/ml) induced a statistically significant increase in the growth of osteoclast cells ($P < 0.01$ for crystal violet and NBT reduction test; $P < 0.001$ for SRB and neutral red dye uptake test) as compared to untreated control. Results also indicate that the cells treated with capsazepine showed a marked decrease in growth, which was found to be statistically significant ($P<0.001$) as compared to untreated control in all the cell growth assays performed. IC_{50}value of capsazepine was found to be 94.2µg/ml.

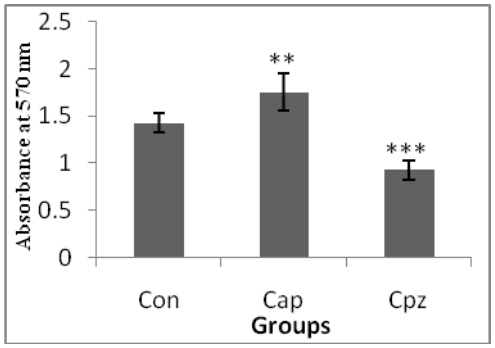

Fig 2: Effect of capsaicin and capsazepine on osteoclast growth (Crystal violet test). Values were expressed as mean ± SD. Student's-t-test analysis. Comparisons were made between untreated control and the treated groups. *** P< 0.001, ** P <0.01. Con- Control, Cap- Capsaicin and Cpz- Capsazepine

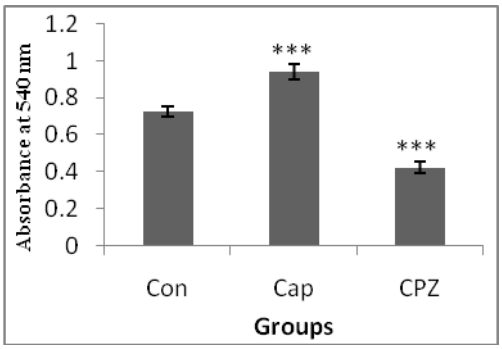

Fig 3: Effect of capsaicin and capsazepine on osteoclast growth (SRB assay). Values were expressed as mean ± SD. Comparisons were made between untreated control and the treated groups.*** P< 0.001. Con- Control, Cap- capsaicin and Cpz- Capsazepine.

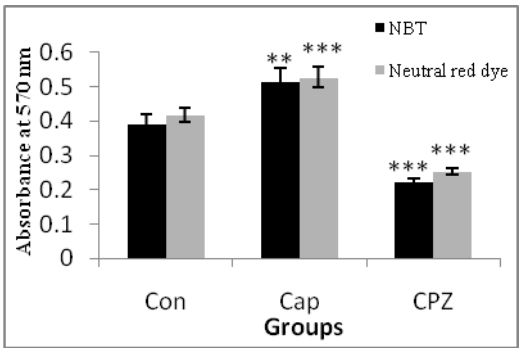

Fig 4: Effect of capsaicin and capsazepine on osteoclast growth (NBT reduction test and Neutral red dye uptake test). Values were expressed as mean ± SD. Comparisons were made between untreated control and the treated groups. *** P< 0.001, ** P <0.01. Con- Control, Cap- capsaicin and Cpz- Capsazepine.

IV. DISCUSSION

Osteoclasts are the cells uniquely responsible for dissolving both organic and inorganic components of bone during bone development and remodeling throughout life. These cells originate from hematopoitic precursors of the monocyte /macrophage lineage that are present both in the bone marrow and peripheral circulation and their numbers and /or activity are frequently increased in a wide array of clinical disorders that are associated with excessive bone loss. Bone resorption by osteoclasts is regulated by various molecular signals of which receptor activator for nuclear factor kappa B (RANK) ligand – a member of tumour necrosis factor superfamily and intracellular calcium signaling plays a decisive role [19].

Osteoclast differentiation is initiated by the simultaneous activation of RANK after binding to RANK-ligand (RANKL) and of the immunoreceptor tyrosine-based activation motif (ITAM)-associated immunoglobulin-like

receptor (IgLR)[20],[21]. These signaling pathways induce the activation of phospholipase C γ (PLC γ). PLC γ produces IP3, which evokes calcium release from the ER via IP3 receptors that will subsequently lead to typical Ca2 + oscillations [22]. The Ca^{2+}oscillations turn on a number of Ca^{2+}calmodulin activated proteins including calcineurin and calmodulin dependent protein kinases (CaMK). Upon activation of the phosphatase calcineurin, the NFATc1 (nuclear factor for activated T cells c1), the master transcription factor in osteoclast differentiation, becomes phosphorylated [23]. NFATc1 migrates into the nucleus and fuses to upstream tartrate-resistant acidphosphatase (TRAP), an osteoclast specific gene, cathepsin K, calcitonin receptor, and osteoclast-associated receptor (OSCAR), thus promoting transcription [20].

In the current study TRAP assay was performed to check the differentiation of RAW 264.7 cells in to functional osteoclast cells. TRAP staining is a very reliable and sensitive indicator of the differentiation of cells of macrophage lineage into functional osteoclasts and hence performed in the current study. The appearance of multinucleated osteoclasts are indicative of successful differentiation of mononucleated macrophages. To understand the influence of capsaicin and capsazepine on osteoclastogenesis various cell growth and viability assays were performed.

Crystal violet test has been employed for a wide number of applications including determination of cytotoxicity or cell death produced by chemicals, drugs, or toxins from pathogens [24] and to determine cell viability or the extent of cell proliferation [25] under different conditions. The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture [26].It is one of the most widely used cytotoxicity assay with many biomedical and environmental applications. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes [27]. NBT reduction test in combination with neutral red dye uptake test provide a good understanding of the influence of test compounds on growth of the cells *in vitro*.

The increased proliferation of osteoclasts following incubation with capsaicin implicate its positive influence on osteoclastogenesis and hence a tendency to increase bone resorption. On the contrary, the ability of capsazepine to inhibit the growth of osteoclasts implicates its inhibitory effects on osteoclastogenesis and thereby bone resorption. As most of the progressive inflammatory skeletal diseases including bone cancer in humans is characterized by a shift towards bone resorption and increased osteoclastogenic activity, the inhibitory effects exhibited by capsazepine gains importance as it opens up the possibility of it being exploited as a potential antiresorptive agent to control the progressive damage and deterioration of the bones in pathological conditions. Further advanced studies are ongoing in the laboratory to understand the mechanisms involved behind the antiresorptive functions of capsazepine by investigating apoptotic markers, genes controlling the expression of osteoclastic function and by studying the molecular targets involved in calcium and NF-kB signalling.

V. CONCLUSION

Results of the current study implicate the potential of TRPV1 modulators capsaicin and capsazepine to influence osteoclastic activity in vitro. Capsaicin potentiates osteoclastic activity and thereby activates bone resorption whereas capsazepine inhibits osteoclastogenesis, thereby inhibiting the bone resorption process. The compounds could be of therapeutic value to regulate osteoclastic activity and restore bone remodeling equilibrium that is disrupted in several musculoskeletal disorders and diseases.

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Conflict of Interest: The authors declare no conflict of interest.

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