

Available online on 15.08.2019 at <http://jddtonline.info>

# Journal of Drug Delivery and Therapeutics

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Research Article

## Anti-Osteoclastic Effect of Zinc Studied in MCF-7 Induced Osteoclastogenesis

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### ABSTRACT

Bone which a key structural support of the body, undergoes dynamic micro structural remodelling all over life to control automatic stress and calcium requirement in the body. A number of risk factors including oxidative stress, apoptosis and abnormal intracellular  $Ca^{2+}$  metabolism have been postulated to play a function in the inception and progress of bone osteolysis. Cancer cells establish a tight relationship with the host tissue, secreting factors that stimulate or inhibit bone cells, receiving signals generated from the bone remodelling activity, and displaying some features of bone cells. This interplay between tumour and bone cells alters the physiological bone remodelling, leading to the generation of a vicious cycle that promotes bone metastasis growth. Zinc is one of the most relevant minerals to human health, because of its antioxidant properties. The present study was aimed to investigate protective role of zinc against bone metastasis. In the present study, TRAP positive multinucleated cell count was low compared to CM treated cells. Zinc treatment suppressed MCF-7 induced mRNA levels of cytoplasmic 1 (Nfatc1), TRAP and Cathepsin-K. Hence, it can be concluded that zinc decreases osteoclastogenesis induced by MCF-7 cells.

**Keywords:** Oxidative stress; Metastasis; Bone remodelling; Zinc.

**Article Info:** Received 06 June 2019; Review Completed 18 July 2019; Accepted 24 July 2019; Available online 15 August 2019



### Cite this article as:

Nagalakshmi K, Shila S, Rasappan P, Anti-Osteoclastic Effect of Zinc Studied in MCF-7 Induced Osteoclastogenesis, Journal of Drug Delivery and Therapeutics. 2019; 9(4-s):94-97 <http://dx.doi.org/10.22270/jddt.v9i4-s.3258>

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### INTRODUCTION

Zinc is one of the essential minerals for human health because it serves as a co-factor for over 300 enzymes and 2000 transcription factors. Zinc is an important mediator of cellular signalling [1,2]. As an anti-inflammatory agent, zinc provides structural stability to cell membranes and it is also an important regulator of gene expression [3, 4, 5]. Zinc is a structural component of the enzyme superoxide dismutase present in the cytoplasm of cells [6]. Therefore, maintaining adequate concentrations of zinc in the cell compartments is essential for the proper functioning of the antioxidant defense system [7]. Zinc is bound to metallothionein under normal physiological conditions. In oxidative stress conditions, the micronutrient is released from its complex with metallothionein and is redistributed in the cells to exert antioxidant actions [8,9].

Oxidative stress is characterized by an imbalance between oxidants and antioxidants, due to the excessive production of reactive oxygen species (ROS) and the reduction in the rate of its removal by the antioxidant defence system. This metabolic disturbance favours the oxidation of biomolecules, contributing to the oxidative damage in the cells and tissues and consequently to the development of several chronic diseases such as obesity, diabetes and cancer [10,11,12].

Moreover, antioxidants and anti-carcinogenic mechanisms associated with zinc homeostasis appear to play an inhibitory role in the growth of neoplastic cells. Zinc acts in the protection against genomic instability and genetic mutations. In this sense, the superoxide dismutase is an anticarcinogenic enzyme. It inhibits the initiation, promotion, and progression phases in mammary carcinogenesis [13].

Bone is the third most frequent site of metastasis after lung and liver and typically indicates a short-term prognosis in cancer patients [14, 15]. Bone metastasis causes morbidity with severe pain, impaired mobility, fractures, spinal cord compression, bone marrow aplasia, and hypercalcemia [16]. Bone is a fertile soil for metastasis. Indeed, it supplies a unique environment for metastasis represented by the hematopoietic marrow in long bones and axial skeleton for which breast cancer cells have a preference [17,18]. Osteolysis is the most common manifestation of bone metastasis [19]. Once established in bone, the tumor cells secrete soluble factors promoting osteoclast differentiation and resorption. Growth factors are mobilized from the resorbed bone matrix, supporting tumor cells' survival and proliferation. In turn, the growing tumor releases pro-osteolytic factors, fostering further osteolysis [20]. The

mechanism of bone metastases and bone destruction found in cancer is also related to the direct activation of osteoclasts by RANKL, which is secreted by cancer cells [21,22]. In the present study an attempt has been made to evaluate the anti-osteoclastogenic effect of Zinc in MCF-7 induced osteoclastogenesis.

## MATERIALS AND METHODS

**Culture media:** (DMEM) dulbecco's modified eagle's medium with fetal bovine serum to a final concentration of 10%.

**Conditioned media (CM):** MCF-7 cell lines secrete the components necessary for osteoclastogenesis that is the components necessary for cell differentiation from precursor monocytes to osteoclasts.

**Cell lines :** The monocyte /macrophage-like cell line RAW 264.7 has been one of the most commonly used myeloid cell line for more than 40 years. RAW 264.7 cell line remains stable through passages: from passage no. 10 up to passage no. 30. MCF-7 is a breast cancer cell line isolated in 1970 from a 69-year old Caucasian woman[28]. MCF-7 is the acronym of Michigan Cancer Foundation-7, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers[29].

**MTT assay:** RAW264.7 cells were maintained in growth media with or without Zinc (10, 30, 60, 80,100, 200 Micro Molar) for 24 hours. Cell viability assays were performed using an EZcytox cell viability assay kit (Daeillab Service, South Korea) according to the manufacturer's instructions. Briefly, the cells were plated in 96-well plates at  $1 \times 10^4$  cells per well and cultured in growth media. At the indicated time points, cells were incubated for 4 h at 37°C. The number of viable cells in triplicate wells was measured at an absorbance wavelength of 450 nm.

### TRAP staining kit to detect osteoclasts

Measurement of TRAP activity and TRAP staining TRAP activity was measured from osteoclast culture supernatants using a TRAP Staining kit (Kamiya Biomedical Company). Supernatants (30  $\mu$ l) were incubated for 3 hours at 37°C with 170  $\mu$ l of the chromogenic substrates in a tartrate-containing buffer. TRAP activities were measured in terms of the absorbance at a wavelength of 540 nm. TRAP was stained using a similar method as described above. Cultured

cells were incubated with a fixative for 5 minutes at room temperature, washed with distilled water, and treated for 20 minutes at 37°C with the chromogenic substrate in tartrate-containing buffer. After staining, TRAP-positive multinucleated (nuclei  $\geq 3$ ) cells were counted using manual counting or a nuclei counter plug-in in the Image J program.

### Cell fractionation

RAW264.7 cells at 70–80% confluence were incubated in  $\alpha$ -MEM containing RANKL (35 ng/ml), with or without ZnSO<sub>4</sub>, for the indicated times, washed, and scraped in cold PBS. Cell pellets were fractionated into cytoplasmic and nuclear fractions using a NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Rockford, IL).

### Real-time reverse transcription-PCR

RNA was extracted from control and osteoclasts on the indicated days using TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNA was reverse transcribed using random hexamers and SuperScript-III reverse transcriptase (Invitrogen). The cDNA was used in real-time PCR with a KAPA SYBR FAST ABI Prism qPCR kit (Kapa Biosystems). The specific primer pairs are shown in Table 1. Nfatc1 and other mRNAs were measured using a StepOne (Applied Biosystems) Real-Time PCR System. The PCR program was initiated for 20 seconds at 95°C, followed by 40 thermal cycles of 3 seconds at 95°C and 30 seconds at 60°C, and terminated for 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C. Data were analyzed according to the comparative cycle threshold (Ct) method [30] and were normalized to GAPDH in each sample. We examined individual gene expression in triplicate and repeated each experiment more than three times. RT-PCR was performed using total RNA and gene specific primers. Elevated levels of transcript were analyzed using RNA gel stained with ethidium bromide.

### Statistical analysis:

The results are shown as the mean  $\pm$  standard deviation (S.D.) from at least three independent experiments. The differences between groups were analysed using Student's t-tests and  $p < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION:

The results of MTT assay (Fig 1) revealed the inhibitory concentration at 80  $\mu$  M Zinc Sulphate

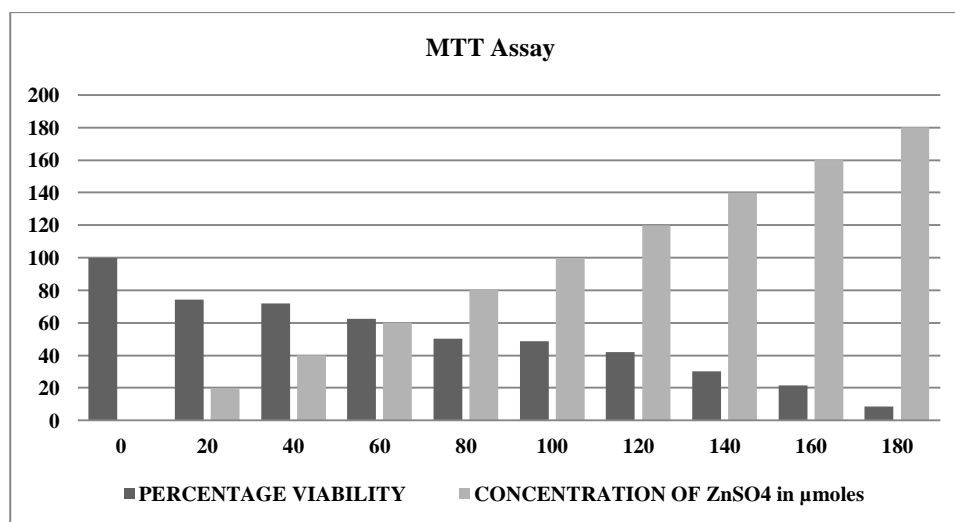
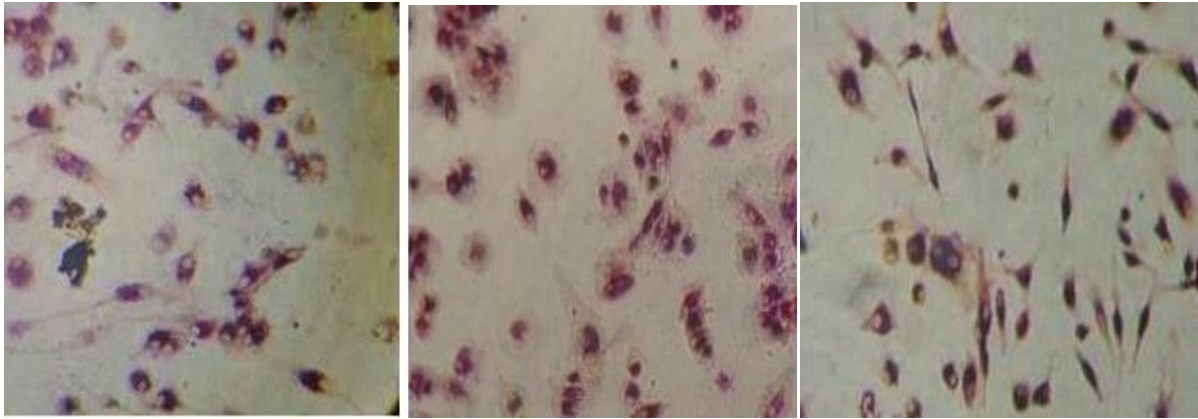


Figure 1: MTT assay for cytotoxicity of Zinc

Plate 1 depicts the TRAP staining for for Raw 264.7 (Group I ) *Osteoclasts* (Group-II) and Osteoclasts +Zinc (Group-III) cells



Group I: Raw 264.7

Group-II: Osteoclasts

Group-III: Osteoclasts +Zinc

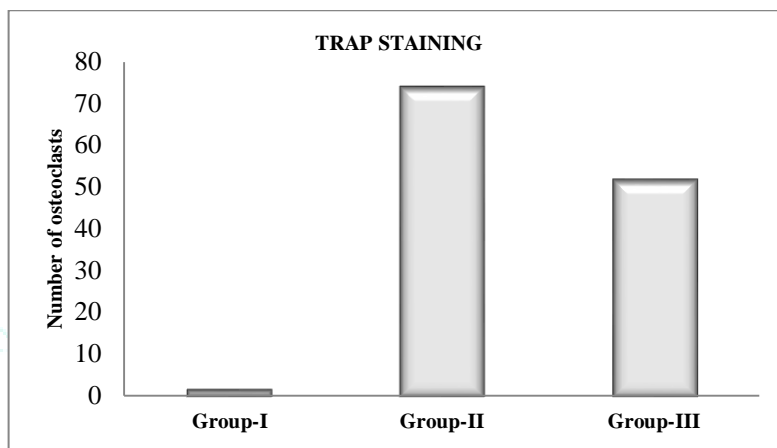


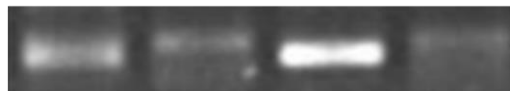
Figure 2 TRAP STAINING

Figure 2 depicts the TRAP staining for Raw 264.7 (Group I ) *Osteoclasts* (Group-II) and Osteoclasts +Zinc (Group-III). From figure 2, it is observed that osteoclast production was comparatively decreased in zinc treated cells.

Plate 2: mRNA expression patterns of osteoclastogenic gene by PCR analysis

CM - - + +

Zinc - + - +



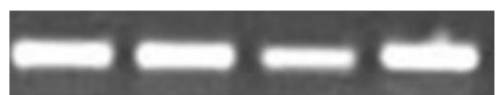
TRAP



Cathepsin K



NFATc1



GAPDH

Zinc treatment inhibited breast cancer-induced osteoclastogenic genes expressions. RAW264.7 cells were cultured in the presence or absence of CM along with or without Zinc for 48 h, and subsequently RT-PCR was performed using total RNA and gene specific primers. Elevated levels of transcript TRAP and Cathepsin K (Cath K) were found in the cases of CM-treated cells as compared with control and CM-induced transcript levels were diminished with Zinc treatment. CM-induced NFATc1 levels were diminished by Zinc treatment.

According to Denis RC and et al., *in vitro* analysis of the influence of breast cancer tumor cells on osteoclast formation or survival, or both, demonstrated that breast cancer cells induced a dramatic increase in the number of osteoclasts detected in culture [23]. In the present study, Zinc treatment inhibited MCF-7 induced osteoclastogenic genes expressions. RAW264.7 cells was cultured in the presence or absence of CM along with or without Zinc for 48 h, and subsequently RT-PCR was performed using total RNA and gene specific primers. Elevated levels of transcript TRAP and Cathepsin K were found in the cases of CM-treated cells as compared with control and CM-induced transcript levels were diminished with Zinc treatment. CM-induced NFATc1 levels were diminished by Zinc treatment. This ultimately inhibits osteoclast differentiation. In earlier studies Hie et al. demonstrated that Zn treatment could inhibit RANK expression during osteoclast differentiation [24]. Park et al have indicated that impairment of Nfatc1 activation is the cause of suppression during osteoclastogenesis. Zinc inhibits osteoclast differentiation by suppression of Ca<sup>2+</sup>-Calcineurin-NFATc1 signaling pathway [25]. Our results are in par with previous findings. Our studies reveal for the first time that MCF-7 induced metastatic osteoclastogenesis was suppressed in the presence of Zinc. It is noteworthy that the expression of NFATc1 the master regulator and the osteoclastogenic genes TRAP and Cathepsin-K is down regulated by Zinc.

## CONCLUSION

Bone metastases are serious complications which affects the quality of life, and performance state of cancer individuals. To overcome these complications the agents inhibiting osteolytic activity, and activity of RANK are in use. However, identification of the major molecules involved in this process could allow the discovery of new therapeutic targets for bone lesions. From the present study, it is observed that Zinc down regulates the osteoclastic genes of TRAP, Cathepsin -K AND NFATc1. Novel Zinc based agents can be developed to prevent, and treat bone metastasis, and further studies should be conducted investigating other molecules which play a role in the pathogenesis of bone metastasis.

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