Original Article

Zoledr0nic Acid May Enhance the Doxorubicin Cytotoxicity Effect in Osteosarcoma Cell Line

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Abstract

Background and Objectives: osteosarcoma is primary malignancy of the skeleton, commonly developing in children and adolescents. Zoledronic acid is heterocyclic nitrogen containing bisphosphonate,have potent antiresorptive effective in bone disease such osteoporosis, Paget disease and in malignant tumor metastasis to bone. Its antitumor properties arise from the suppression of the mevalonate pathway of tumor cells.

Method and Material: osteosarcoma cell line "MG". These cells were exposed to serial concentrations of zoledronic acid (500,250,125,62,31,15µg/ml) alone or in combination with doxorubicin (5µg/ml). After 24h incubation, cytotoxicity was assessed using an MTT assay, ElIZA method, are used to evaluated anti-inflamatory effect of zoledronic acid on via measurement of IL-6,and TNF-α level.

Result: zoledronic acid has demonstrated a significant decrease in cell number at all serial concentration (500,250,125,62,31,15µg/m), While zoledronic acid -doxorubicin combination was applied to the cells, highly significant cytotoxic synergism was shown at all concentrations comparison control group. After evaluated by MTT assay. ELIZA method was showed zoledronic acid at high concentration decrease IL-6, TNF-α levels.

Conclusion: zoledronic acid has an antiproliferation effect on MG osteosarcoma cell line at serial concentration, while zol –doxorubicin was showed significant cytotoxic synergism above 62µg/ml concentrations, and has showed zoledronic acid at high concentration have anti-inflamatory properties decreasing forIL-6, TNF-α.

Keyword: MG Cell Line, Zoledronic Acid, Doxorubicin, Osteosarcoma.

Received date: 07 August 2022 **Accepted: 10 September, 2022** **Published: 07 October, 2022**

DOI: 10.47750/pnr.2022.13.04.037

INTRODUCTION

Osteosarcoma is a primary malignant tumor, which occur in the metaphysis of long bones, mostly was occur in children and adolescents. The common a ways for treatment of osteosarcoma are involve a surgical excision, adjuvant chemotherapy, and radiotherapy. [1]. Osteosarcoma can be classified, into two stages: localized and metastatic; the stage that was affecting in the bone and the tissues in which it progressed only, based on the viability of surgically removing the tumor, this stage called localized osteosarcoma. While the metastatic stage of osteosarcoma was demonstrated that the cancer has diffusion from the original site to other organ sites, making it more hard to treat.[2].

According to several of studies that bisphosphonates may suppress cancer cell-bone matrix binding, invasion, of cancer into the bone. [3]. The mechanism of nitrogen containing bisphosphonate, zoledronic acid, involving(geranylgeranyl pyrophosphate)inhibition, reduction of Ras prenylation, and intracellular calcium chelating; Zol showed cytostatic effects on "MG-63" cells whereas cytotoxic efficacy has reveal following increasing of the dosage and period of the treatment.[4] according to several research; such Wu *et al* reports, in animal model with osteosarcoma he was found when treating him with zol, a primary tumor growth is reducing, reduce lung metastases and prolong survival.[5] Through inhibition of essential enzymes, farnesyl diphosphonate synthase in the mevalonate pathway; Whereas blocked the FPPS has been toxic effective on osteoclasts. Either by suppressing of the prenylation of signalling GTPases, such as Ras, Rho and Rac, correlated with cells proliferation and viability, or accumulation of isopentenyl pyrophosphate, may be converted to "ApppI" via aminoacylt RNA-synthetases; By "ApppI" will be suppressed the mitochondrial adenine nucleotide transl

ocase and induction of apoptosis. [6] in addition isopentenyl pyrophosphate that was accumulated in monocyte will activated of "γδTcell" to proliferate, leading to releasing proinflamatory cytokines.[7]**;** Another pathway to zol can enhance apoptotic for tumor cells alone, and colony formation blocking ; associated by rise in reactive oxygen species, the hypothesis, which ″ROS″ are involving in apoptosis and colony formation blocked with zol treatment of SACC-83 cells.[8]**.** zoledronic acid had showing synergetic effect when combination with anticancer agents such doxorubicin, cisplatin, and etoposide on various types of cancer cells. [9] Another study combining zol with antitumor drugs may be get augmentation "antitumor" and "anti-metastatic" efficacy.[10]

Zol is a potent antiresorpative agent in bone cancer, and solid tumor with spread to bone, in this study we will explain beneficial activity when combination with anticancer agent, it is acting through synergism mechanism, to inducing additional antitumor effect.

Method and Material

MG cells line is "osteosarcoma cells" were gained by a cancer research laboratory/ college of medicine/ university of Babylon; and ripe in RPMI-1640 medium with penicillin (100 U/ml), streptomycin (100 g/ml), and 5% "fetal bovine serum" at 37°C in 5% CO2. Tissue culture 96-well plates are using to seeding MG cells, at a density of (5×10^5 cells/ml) before 24hr of drugs utilizing. Zoledronic acid monohydrate powder (4 mg/5ml) (Cipla, India) were utilized and dissolved and diluted by growth medium to get the final concentrations (500,250,125,62,31,15µg/ml). Doxorubicin (Pfizer, US) was diluted using complete growth medium to get final concentration (100,50,25,12.30,6.25,3µg/ml). MTT assay was used to assessed drugs cytotoxicity.[11]. By ELIZA method will evaluated anti-inflamatory effect of zoledronic acid by measured IL-6, and IFN-α, after 24 hours of incubation period, and 68 optical density is measured at wavelength of 450 nm. [12].

Statistical analysis

Microsoft Office Excel 2016 and Sigma plot version 12.5 software were used to analyze the data. The ANOVA one-way test was employed to determine whether there were significant differences between the data means. The p-values ($p\leq 0.05$) was declared statistically significant.

Results

Effect zoledronic acid on MG osteosarcoma cell line

The result showed there are a highly significant decrease ($p<0.001$) in cell viability of the concentrations (500, 250, 125µg/ml) but at concentration(62, 31, 15 µg/ml) showed significant decrease($p<0.050$)comparison with control group after incubation for 24 hr.

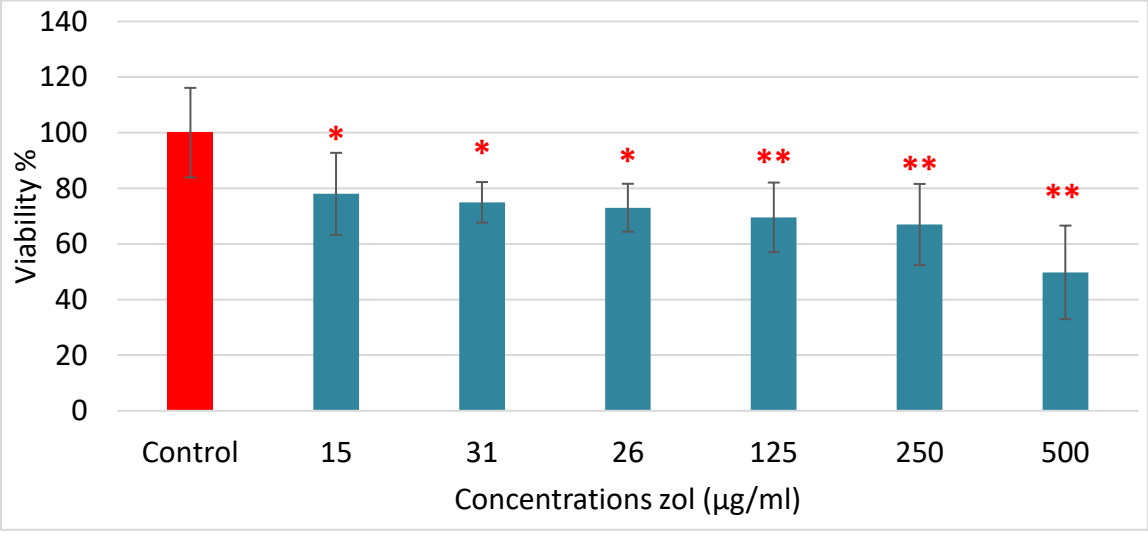

Figure 1: Effect of zoledronic acid on cell viability Percentage of MG osteosarcoma cell line after incubation for 24hr measured by MTT assay.

** indicates highly significant (P<0.001) effect.

Synergetic effect of zoledronic acid and doxorubicin on osteosarcoma cells

When cancer cells treated with serial concentrations of ZA in the presence of constant doxorubicin concentration (5µg/ml), highly significant decrease $p<0.001$ has observed in cell viability, in all zol conc. comparison with control group.

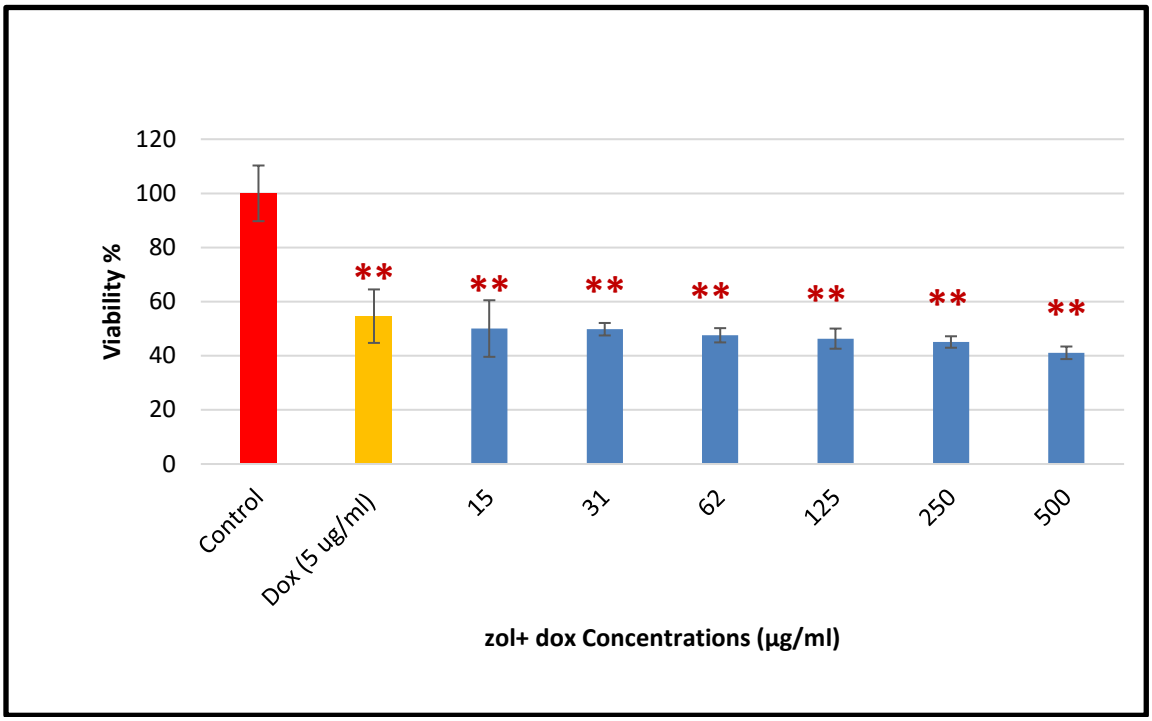

Figure 2: Represent Cell viability Percentage of MG cell line at serial conc. zoledronic acid with doxorubicin at constant conc. after incubation for 24hr by MTT assay.

** high significant p<0.001

While a synergism effect was observed. ZA concentrations above 62 µg/ml induce significant cell viability reduction compared to positive control (Dox) and negative control.

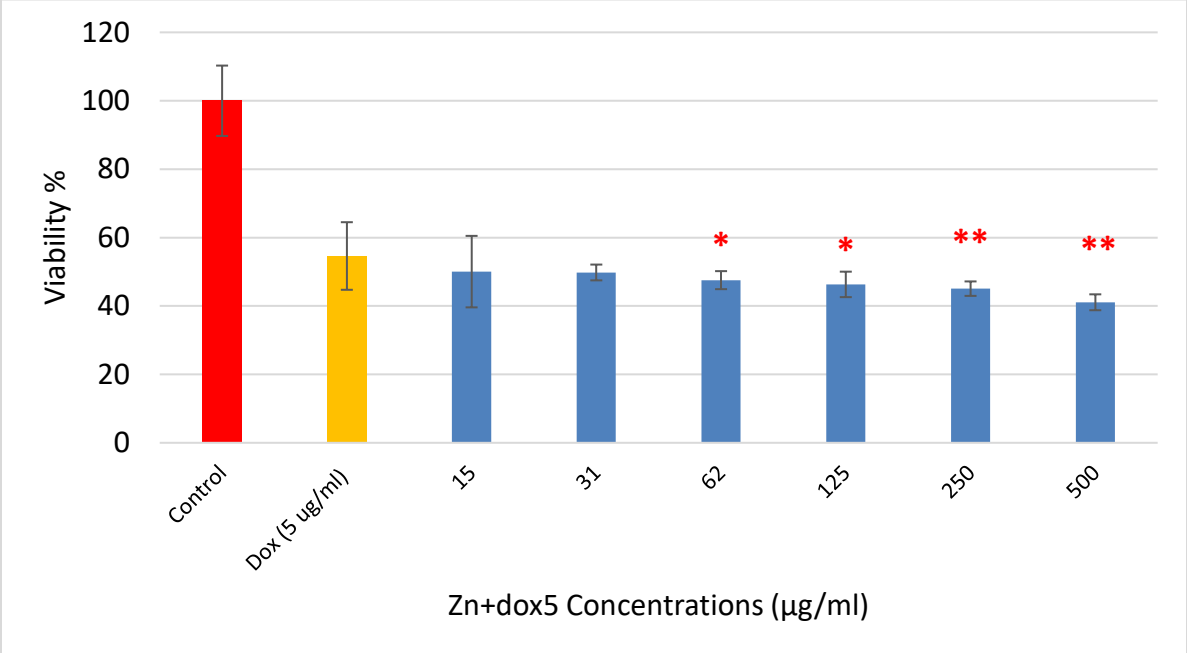

Figure 3: synergism between zoledronic acid and doxorubicin causing sever cytotoxic effect on MG cell line. Dox: doxorubicin, *: significant P<0.050

**: highly significant P<0.001 effect.

Effect of zoledronic acid at serial concentration on IL-6 level on Osteosarcoma cell

The result showed there were a highly significant decrease ($p<0.001$) of IL-6 level at concentration of zoledronic acid (500µg/ml) compression with control group while no significant difference showed below of (250µg/ml).

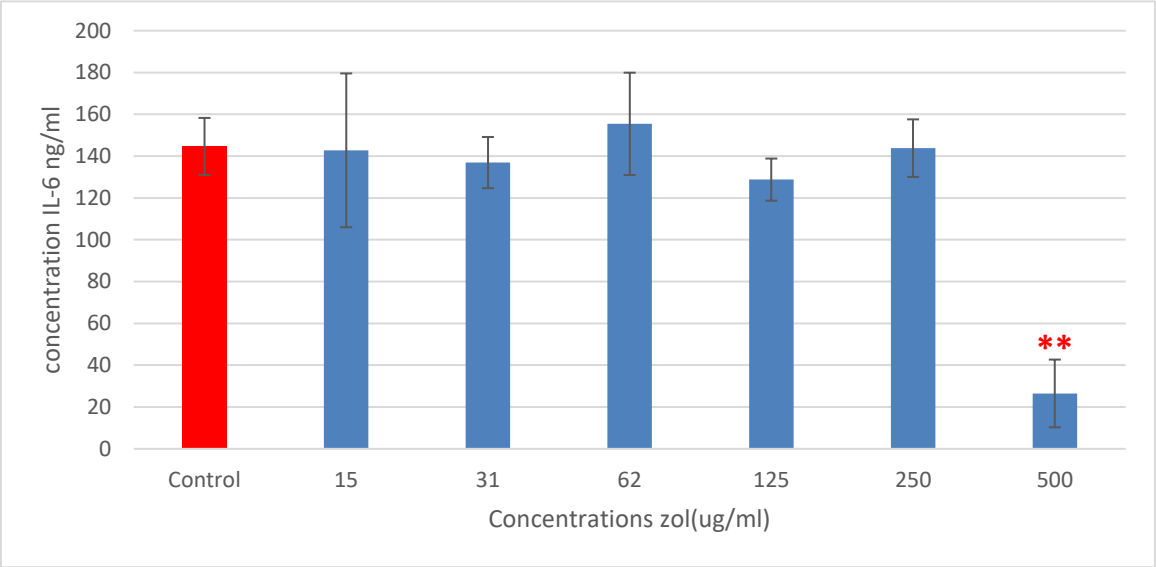

Figure 4: Effect of zoledronic acid in serial concentration on IL-6 level in osteosarcoma cell line.

** high significant Effect of zoledronic acid at serial concentration on TNF-α level for osteosarcoma cell line

The result showed there were a significant decrease $p<0.050$ at zol concentration (500µg/ml) comparison with control group, while at zol concentration (15µg/ml) showed a significant increase $p<0.050$ comparison with control group at 24hr incubation period.

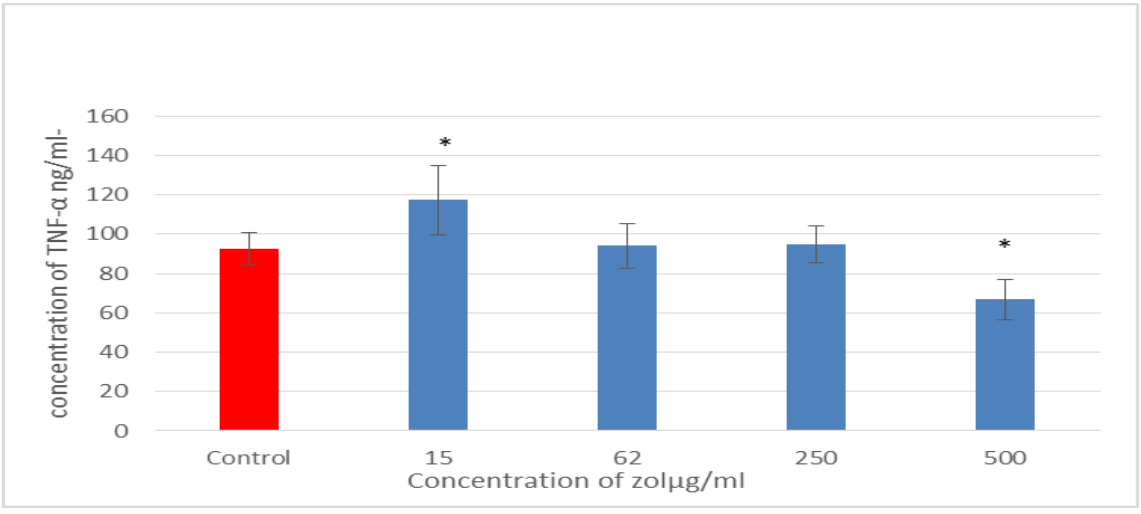

Figure 5: effect of zoledronic acid at serial concentration on TNF-α level in osteosarcoma cell line.

*significant $p<0.050$

Discussion

Osteosarcoma (OS) is a malignancy produced osteoid that was originated from mesenchymal, its consider primary malignancy of bone and its fatal in both children and adults predominatel.[13], metastasize locally (within the same extremity) or systemically (to other organs, such as the lung).[14] several studies have demonstrated that BPs have ability to inhibit proliferation and induce apoptosis in tumor cells by dose dependently in vitro, also has been showed

reduction in tumor cell adhesion, invasion, and angiogenesis overall confirm that BPs are magical agents potential, in reduce related skeletal event, especially zoledronic acid consider the highest potency of its classes in the treatment of metastatic cancers. [15].

The results in our studies have demonstrate efficacy of zoledronic acid when it was treated alone with osteosarcoma cells line, in (figure1) showed that zol have highly significant decrease in viable cell number when used alone at concentration (500, 250, 125µg/ml) and significant decrease in viable cell number at concentration (62,31,15µg/ml) comparisons with control when evaluated by MTT assay.

This agree with. Conry, Rodriguez and Pressey reports was found that zoledronic acid exert direct anti-proliferative effect in osteosarcoma, and induces apoptosis in vitro, also have immune activation and anti-angiogenic activity.[16]

In this study Bosch-Barrera *et al* that he was explain zol have antiproliferation effect is mediated, vital enzyme blocking, farnesyl diphosphonate (FPP) synthase in the "mevalonate pathway" and induction of apoptosis. [6]. According. To Wang *et al* that showed zoledronic acid have been direct effect on osteoclast by block macrophage differentiation into osteoclasts and induce apoptosis by inhibition of mevalonate pathway and ugmentation ROS-mediated apoptosis. (Wtang *et al*., 2020b)

also agreement with Ouyang *et al.* report that was demonestrated zoledronic acid can been blocking for cancer cell grouth through cell cycl arrest in the S phase inducing blocking of OS cell proliferation, and enhancig of apoptosis, this inhibitory effect for zoledronic acid is mediated in high doses. [18]. The standard therapeutic for OS according to The European Osteosarcoma Intergroup (EOI), The doxorubicin and cisplatin regimen since 1983.[19],and they are consider the first choice of treatment for osteosarcoma that commonly used, either alone or in combination,and approved for treatment primary tumor bone ; but the patients with osteolytic bone or metastases to bone have required to addition therapy such nBP acting as antiosteolytic effect to prevent metastasis. [4] Doxorubicin has demonstrate greatest activity in inhibition rapidly dividing cells and delaying the progression for solid and liquid tumors used mainly in breast cancer, sarcoma and multiple myeloma. [20], by intercalation into DNA and inhibition topoisomerase type II [21]

also this results confirmed with Goldsby *et al*,report that zol can be safely added to the backbone of chemotherapy used to treatment metastatic osteosarcoma.[22]

In another side when combination zoledronic acid in serial concentration (500, 250, 125, 62, 31, 15.25µg/ml) with doxorubicin at concentration 5µg/ml as showed in (figure 2, 3) has demonstrated highly significant decrease in viable cell number $p<0.001$comparison with control after incubation 24 hr. belonged to mechanism of Zol as antitumor agent, and have ability to augments the effects of anticancer agents in various cancer cell lines synergistically.[23]. According to[24] report that has been shown zoledronic acid increasing the anticancer effects synergistically when combining with cytotoxic drugs in vivo, comparison when it's using alone. [24]. Beneficial of combination zoledronic acid with another anticancer drugs to decrease toxic dose, increase respond and decrease side effect.

In these study, the results have showed zoledronic acid in serial concentration effected on viable cell number of osteosarcoma cell line by decreasing viability percentage comparing with another species cells line may be belong to the pharmacokinetic of zol is class of nPB have high affinity for bone, was prefered to bone as result of their structural similarity to pyrophosphate, make its potent as antiresorptive agent rather than their propensity to affect mineral dissolution in vitro.[25]. So its concentration in bone is higher than 100-time that in serum and another organ after administration of zoledronic acid, and thus will supply by adequate concentration to exerting direct antitumor effect. [26]. In osteosarcoma, osteoid and immature bone is offspring, that they have containing focal calcium hydroxyapatite crystals, whereas zoledronic acid is BP analog of endogenous, pyrophosphate that potent calcium-containing hydroxyapatite binding of bone mineral make its attractive concentration in primary osteosarcoma [27]..

Another mechanism may interpreted zoledronic acid manner against osteosarcoma. zol was found to inhibit mevalonate pathway through blocking of farnesyl pyrophosphate synthase in tumor cells leading to increase isopentenyl pyrophosphate level, this intermediates which led to the activation of "γδTcells" enhancing cytotoxicity against tumors.[28] according to Li *et al.*, report that have been shown "γδ Tcells" was play role in tumor immunity, they have antitumor effect.[29]. in addition isopentenyl pyrophosphate that was accumulated in monocyte will activated of" γδT cell" to proliferate. [7] in this study, in (figure 4) showed a significant decrease $p<0.050$ at zol concentration (500µg/ml)in TNF-α level,whereas at zol concentration (15µg/ml) showed a significant increase $p<0.050$ in TNF-α comparison with control group. While (figure 5) was shown level of IL-6 high significant decrease $p<0.001$ at concentration of zol. (500µg/ml). so zol in high conc. have anti-inflammatory effect by reducing of cytokines release. TNF-α,IL-6.

According to clinical study that was found decrease in serum IL-6 for patient with prostate cancer metastasis to bone when added zoledronic acid to chemotherapy (docetaxel). [27].

In bone tumor lesions, osteolytic are predominantly, as in primary osteosarcoma and metastatic bone tumors that its release of osteoclast-energizing cytokines; that enhancing bone resorption by osteoclasts. In another side, through osteolysis, the bone matrix- releasing factors can be support for tumor growth, all these hasten events frame the so-called

‘‘vicious cycle”.[30], Maurizi and N. Rucci report; found that TNF-α is one of factors that releasing through osteolysis, whereas play role in induces osteoclast differentiation directly and independent manner by stimulating (NF-κB) and(JNK) in a RANKL; and indirectly by activation the osteoblasts to express RANKL also IL-6 and is potent osteoclastogenic factor in the same way [31],. The results has agreement with study of Kimachi *et al* suggest that zoledronic acid has inhibited the mevalonat pathway for several types of cells, such monocytes/macrophages, osteoblasts, and various cancer cells which they are responsible for production of proinflammatory cytokines, through inflammatory tissue destruction, bone resorption, and this in final leading to inhibition of TNF- α and IL-6 releasing.[32] so zol according to results have anti-inflammatory properties.

Zoledronic acid is antiostealysis by inhibition mevalonat pathway of osteoclast cells leading to antiproliferation and induce apoptosis. zol have different mechanism to exert antitumor effect but cannot explain which mechanism of action zol in this study.

Conclusion

Zoledronic acid has an antiproliferation effect on MG osteosarcoma cell line at serial concentration, while zol – doxorubicin was showed highly significant cytotoxic synergism above 62µg/ml conc, and have anti-inflammatory properties in high concentration by decreasing cytokines releasing.

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