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Abstract

A soft-tissue in vitro model of bisphosphonate induced localized non-traditional calciphylaxis

Background: Bisphosphonates (BPs) such as zoledronic acid (ZA) are widely used to treat complications of bony metastases in cancer patients. One of the serious adverse effects of ZA treatment is Bisphosphonates osteonecrosis (BON). The pathophysiologic mechanisms of BON are not fully elucidated. Scheper et al (2012) suggest than BON is correlated with a localized non-traditional calciphylaxis. In this project, we studied the effect of ZA on soft tissue by calcium deposition. Also, we hypothesized that albumin will reverse that effect.

Materials and Methods: Normal Oral Keratinocytes (NOK) and Human Gingival Fibroblats (HGF) were treated by different ZA concentration and Bovine Serum Albumin (BSA). Immunofluorescence and Western Blot were used to study the treatments effects.

Results: There is an increase in calcium concentration when cells were treated with ZA. However, there is a decrease when cells were treated with albumin.

Conclusion: Albumin can bind to the calcium released following ZA treatment. Our finding might suggest a method of BON prevention. Further studies are needed to determine if such effects are clinically relevant for in vivo models of BON.
A soft-tissue in vitro model of bisphosphonate induced localized non-traditional calciphylaxis

by

Weam Alfouzan

Dissertation submitted to the faculty of the Graduate School of the University of Maryland, Baltimore in partial fulfillment of the requirements for the degree of Master of Science 2013
Acknowledgement

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<td>American Dental Association</td>
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<td>AFCS</td>
<td>Alliance for Cellular Signaling</td>
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<td>ARONJ</td>
<td>Antiresorptive agent–induced osteonecrosis of the jaw</td>
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<td>BON</td>
<td>Bisphosphonates osteonecrosis</td>
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<td>BP</td>
<td>Bisphosphonate</td>
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<td>BSA</td>
<td>Bovine serum Albumin</td>
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<tr>
<td>DD</td>
<td>Dentine disc</td>
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<td>DDT</td>
<td>Dichloro-diphenyl-trichloroethane</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
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<td>GI</td>
<td>Gastrointestinal</td>
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<td>HGF</td>
<td>Human gingival fibroblast cell line</td>
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<td>Human serum albumin</td>
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<td>Intravenous</td>
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<td>Control cells</td>
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<td>NOK</td>
<td>Normal oral keratinocytes</td>
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<td>ONJ</td>
<td>Osteonecrosis of the jaw</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
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<td>SDS</td>
<td>Sodium dodecylsulfate</td>
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<td>SFM</td>
<td>Serum-free medium</td>
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<td>ZA</td>
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Chapter (1)

Introduction

In the United States, prostate cancer is the most common cancer in men (aside from skin cancer) and is the second leading cause of cancer-related deaths in the United States. About one in six men (192,280 in the US in 2009) will be diagnosed with prostate cancer during their lifetime, with 27,360 dying of their disease, thereby, comprising 10% of cancer-related deaths in men. [1] Similar to breast cancer, bone is the most common site for metastasis, and its involvement is a major cause of morbidity. [2] Breast cancer is the number one cancer in women. According to the National Institute of Health and the National Cancer Institute, one in every eight women has a risk of developing cancer over their lifetime (194,280 in the US in 2009). [1] Most of the deaths (40,610 in the US in 2009) associated with breast cancer are the result of metastasis and its physiologic effects on morbidity and mortality. [1] Bone is the most common metastatic site, with 65-75% of the women having metastatic lesions to the skeleton. [3]

There are many Antiresorptive medications used for the management of osteoporosis, osteopenia, metastatic bone cancer and hypercalcemia of malignancy. Bisphosphonate (BP) is currently the major drug in this class [4] and it is the drug of interest in this study.

In 2010 the clinical use of denosumab (Prolia™), a monoclonal antibody to RANKL (receptor activator for nuclear factor-kappa B ligand), was approved by US Food and Drug Administration for the treatment of postmenopausal osteoporosis and for the
prevention of skeletal-related events in patients with metastatic bone cancer. [5] Although BP and denosumab are different mechanistically in their antiresorptive actions, recent evidence indicates that both drugs are associated with the development of osteonecrosis of the jaw (ONJ). [6,7] There are some case reports of ONJ that was induced by anti-RANKL therapy. [8,9]

Bevacizumab or Avastin is a humanized monoclonal antibody that blocks vascular endothelial growth factor A, a key factor in angiogenesis. Avastin is indicated for metastatic colorectal, breast and renal cell carcinoma as well as glioblastoma and non-small cell lung cancer. Bevacizumab is estimated to have been given to more than 800,000 patients with cancer worldwide to date. [10] Its antineoplastic use has been related to a series of adverse effects such as hypertension, proteinuria, subconjunctival hemorrhage, thromboembolic events, and impaired wound healing. [11,12] Cases of ONJ related to chemotherapy using bevacizumab were first described in 2008. [8] To the best of our knowledge, there are only four reports published in the medical literature reporting ONJ related to bevacizumab. [12-14]

The article "Managing the Care of Patients Receiving Antiresorptive Therapy for Prevention and Treatment of Osteoporosis" is based on a literature review by an advisory committee of the American Dental Association (ADA) Council on Scientific Affairs and updates ADA's 2008 advisory statement. The authors concluded that the highest reliable estimate of antiresorptive agent–induced osteonecrosis of the jaw (ARONJ) prevalence is approximately 0.10 percent. Osteoporosis is responsible for considerable morbidity and mortality. Therefore, the benefit provided by antiresorptive therapy outweighs the low risk of developing osteonecrosis of the jaw. [15]
Bisphosphonates (BPs) such as zoledronic acid (ZA: Zometa) are widely used to treat complications of bony metastases in cancer patients, with an estimated 24 million BPs prescriptions are written in the U.S. alone [16]. Intravenous (IV) BPs have also shown significant efficacy in reducing bone pain, hypercalcemia and fractures secondary to bony metastases. [17] Indeed, monthly IV administration of BP is the standard of care, recommended by the American Society of Clinical Oncology clinical practice guidelines, for treatment of malignancy-associated hypercalcemia and metastatic osteolytic lesions. [18-20] Orally administered, and to a lesser extent IV, BPs are widely used for the management of osteoporosis and Paget’s disease. [21]

Bisphosphonates (BP) are synthetic analogues of the naturally occurring pyrophosphate [see figure (1)], which are able to chelate calcium ions [22-24].

![Figure 1](attachment:image.png)

**Figure (1):** The basic chemical structure for bisphosphonates shows two phosphate groups binding to a carbon atom. [89]

Zoledronic acid (ZA), a third-generation, nitrogen-containing BP, is one of the most potent available [25, 26]. Its high affinity for calcium crystals allows this pyrophosphate to bind to hydroxyapatite of the bone and inhibit osteoclast-mediated bone resorption, a property that has provided the rationale for their use as skeletal protectors of cancer-mediated, cytokine-induced hypercalcemia. [22-24,27,28]
One of the reported serious adverse effects of ZA treatment is Bisphosphonates osteonecrosis (BON), a condition first recognized in 2003 by Marx. [6] The author reported 36 cases of avascular osteonecrosis, 29 of which in the mandible, five in the maxilla, and two in both bones. Of the 36 patients, 24 were on IV pamidronate, 90 mg monthly; six used initially pamidronate at the same dosage, and then changed to IV zoledronate, 4 mg monthly; and six received only IV zoledronate, 4 mg monthly. The indications for the use of those drugs were multiple myeloma-related hypercalcemia (18 patients), metastatic breast carcinoma-related hypercalcemia (17 patients), and treatment of osteoporosis (one patient). [6]

The reported incidence of BON ranges from 1.3% to 19%, with a higher frequency in the mandible than in the maxilla [29-37]. BON occurs in a dose- and time-dependent manner, with cases being more prevalent in patients receiving intravenous (IV) dosing. [29-34] Median exposure to zoledronic acid prior to BON onset ranges from 9–30 months, but can be as brief as 3 months. [17] The cumulative hazard increases from 1% in 1 year of ZA treatment to 15% at 4 years of treatment. [17] The majority of cases occur in those who are taking therapeutic doses of high-potency intravenous (IV) bisphosphonates such as ZA and pamidronate disodium. [34]

BON is manifested clinically by poor oral wound healing, oral epithelium dehiscence, and exposure of the underlying intra-oral bone, culminating in necrosis of the exposed bone. A working definition of BON has been standardized by the American Association of Oral and Maxillofacial Surgeons and includes prior or current treatment with a BPs followed by persistent (>8 weeks) exposed necrotic bone in the maxillomandibular region, in the absence of radiation therapy to the jaws. [17] Early
stages of BON are difficult to note via conventional radiography, which will not
detect alterations until 30–50% of the bone is demineralized. [17, 38] BON patients
present with exposed necrotic bone, sites ranging in size from mm to cm, often with
surrounding inflammation and pain. Systemic antibiotic treatment and/or an oral
antimicrobial rinse are used in patients with asymptomatic exposed bone. [39] As
debridement is usually unsuccessful and carries the potential for further exposure of
healthy bone, a conservative palliative approach is often recommended. [39] Currently there is no highly effective clinical treatment for advanced BON, and
management may be limited to analgesia and control of disease progression, as
complete healing may never occur. [39]

The pathophysiologic mechanisms of BON are not fully elucidated. [40] In
approximately 60% of cases of BON, there is a reported previous dental procedure or
trauma; however, up to 40% of patients with BON have no such history. In fact,
many of the cases of BON occur at sites away from teeth on otherwise normal
undisturbed tissues. [29,41] The fact that BON occurs as both a wound-healing
phenomenon and a spontaneous occurrence supports our suggestion that the
pathogenesis is probably multifactorial.

Different mechanisms have been reported to account for the action of BPs, including
induction of apoptosis, [42,43] and disruption of the cell cycle. [44] They also have
anti-invasive, [45] antiangiogenic, [46] and antimigration effects. To date, there is still
no clear understanding of the molecular targets. One hypothesis suggested that the
microenvironment surrounding active osteoclasts is strongly acidic, inducing the
release of BP from the bone surface and creating high local BP concentrations. [33]
A growing body of evidence supports a notion that BON may be associated with soft-tissue toxicity. [20,47-50]

One of the major side effects of orally administered BP is cytotoxicity of the gastrointestinal (GI) tract, which is lined with stratified epithelium. Esophageal inflammation and ulceration are frequently reported in BP users; [51-53] and oral ulceration is also observed in individuals taking oral BP tablets. [54,55] These reports suggest that BP may have direct effects on soft tissues; however, the role of oral mucosal soft tissue in the pathophysiology of BON is poorly understood.

Reid et al. [47] initially suggested the possibility of soft-tissue toxicity from BP in a 2007 editorial. This concept conforms to the clinical condition most commonly known as BON, which is observed as a mucosal dehiscence leading to the formation of a superficial mucosal ulcer, which progresses and results in detectable bone exposure. The ulcerated area continues to extend with time, leading to bone necrosis and sequestration. [30]

Ravosa et al. reported that ZA treatment impedes oral wound healing by blocking the growth and migratory capacity of oral fibroblasts as well as down regulating the transcription of type-I collagen, functions necessary to deposit the granulation tissue needed for re-epithelization. Therefore, BP released from bone following tooth extraction may delay wound healing of the oral mucosal barrier and contribute to BON pathogenesis. [50]
Scheper et al. demonstrated that low concentrations of ZA rapidly and directly affected the oral mucosal tissues through the induction of a gene-regulated apoptotic process. These findings support the potential for soft tissue injury as an initiating and potentiating event for osteonecrosis. [20] Then by further study, they found that low concentrations of ZA released from bone can rapidly and directly affect the oral mucosal tissues, initially through the induction of apoptosis and long term through the inhibition of cell proliferation. [49]

Scheper et al. reviewed the tissue archives of the University of Maryland, Baltimore, Oral Pathology Consultants from 2003 to 2011 (with IRB approval) and found 10 cases of diagnosed BON which contained adjacent soft tissue. They demonstrated, Using von Kossa staining, the presence of calcium in 8 of the 10 BON cases within the connective tissue and surrounding blood vessels. They found that BONs clinical course and delayed wound healing is in part correlated to a localized non-traditional calciphylaxis. [56]

Calciphylaxis, is a rare condition, involving subcutaneous vascular calcification and cutaneous necrosis. It was first described in 1898 by Bryant and White, [57] but it was not until 1962 that the term “calciphylaxis” was coined by Hans Selye. [58,59] In humans, vascular calcification is an active process and is not sufficient to produce skin necrosis. Vascular calcification and thrombosis are both required to produce lesions of calciphylaxis.

Calciphylaxis most commonly occurs in patients with end-stage renal disease (ESRD) who are on hemodialysis or who have recently received a renal transplant. However,
it does not exclusively occur in ESRD patients. In certain hypercalcemic states, such as malignancy (Breast and Multiple Myeloma), hyperparathyroidism, cirrhosis, Crohns and collagen disease conditions a non-uremic non-traditional calciphylaxis is shown to induce vascular and tissue deposition of calcium in the submucosal tissues. [60-66]

The Diagnosis of calciphylaxis is made through a combination of clinical and histopathological features. Intravascular calcium deposition in the media of the dermal and subcutaneous arterioles [see figure (2)] is the most common histopathologic feature seen in calciphylaxis. [67] Cutaneous necrosis may be seen at different levels. Vascular thrombosis of pannicular or dermal arterioles was noted in the majority of patients. [67,68]

![Figure (2): The histological findings of calciphylaxis. [90]](image)

Although the pathogenesis of calciphylaxis is poorly understood, Liach et al. outlines three pathogenic factors: presence of high Ca × P product, elevated content of calcium salt in the skin and the presence of high parathyroid hormone (PTH) level. [69]
Calciphylaxis is a serious condition with significant morbidity and mortality, most commonly resulting from septicemia due to impaired integrity of the epidermis and dermis. [70] More than 50 percent of patients die (most commonly from sepsis) within one year of being diagnosed. [68] Wound care is of utmost importance in the management of calciphylaxis. Systemic antibiotics should be used, if indicated, and surgical interventions reserved only for severe cases. [71]

Calciphylaxis has a dismal prognosis with up to 80-percent mortality. [72] A two-fold increase in mortality is seen when cutaneous ulcers develop. [73] The key is to prevent patients with known risk factors from developing calciphylaxis. For example, controlling blood sugars in a diabetic patient and monitoring calcium-phosphate homeostasis is imperative. [71]

**In this project, we studied the effect of ZA on soft tissue by calcium deposition.**

**We hypothesized that albumin will reverse that effect by binding to calcium.**

Albumin is the most abundant plasma protein in the blood stream (35–50 g/l in human) that constitutes about 60% of the protein mass found in blood plasma. It is one of the major protein synthesis products of the liver (approximately 0.7 mg/h for every gram of liver tissue), characterized with an average half-life of 19 days. [74]

Albumins are proteins of relatively small size (ca. 67 kDa). It consists of a single polypeptide chain of 581 residues and exists in a multi-domain structure with complex ligand binding specificities. [75] The albumin molecule is highly organized with hydrophobic internal structure, stabilized by disulfide bonds, and a relatively
flexible hydrophilic surface. [76] Its structure and abundance defines its binding capabilities and the variety of physiological functions [see figure (3)], it binds long chain fatty acids, bilirubin, heme breakdown products, copper nickel and more. In addition it has a critical role in blood osmotic control and blood pressure regulation. [74] Therapeutically it is of high importance as it binds a variety of compounds, and therefore acts as a drug carrier for various drugs for different targets.

![Figure (3): Physiological effects of exogenous Albumin. [91]](image)

Bovine serum Albumin (BSA) is a serum albumin protein derived from cows. BSA has been one of the most extensively studied of this group of proteins, particularly because of its structural homology with human serum albumin (HSA). [77]

Pedersen et al, in his extensive work on the albumin binding of calcium, used the classical ultrafiltration technique and found that albumin binds calcium to one group of $12 \pm 1$ identical and independent binding sites, each having an apparent association constant of $K_A = 90$ to $100$ litre/mol at pH 7.40, ionic strength 0.15-0.16 mol/kg, and $37 \degree C$. [78-81]
Calcium binding by albumin was determined potentiometrically at physiological ionic strength and temperature as a function of pH. The binding data indicate at least 30 different binding sites with different association constants and different H+ interaction. [82]

Albumin causes a complex pattern of calcium signals in astrocytes, which are the most abundant cell of the human brain. A possible function for the calcium signals is suggested by the observation that the generation of calcium spikes by albumin is closely associated with the induction of mitosis. [83]
Chapter (2)

Materials and Methods

Cell Lines and Cell Cultures:

All experiments were performed using normal oral keratinocytes (NOK) and human gingival fibroblast cell line (HGF) (donated by Dr. Silvio Gutkind; National Institutes of Health and John Sauk; University of Maryland, respectively), selected as cells to represent the oral mucosa.

NOK were cultured in keratinocyte serum-free medium (SFM) (1X), Liquid with L-Glutamine, without Calcium Chloride (Gibco/Invitrogen; 10725-018). This medium was supplemented with BPE (25 ug/ml), epidermal growth factor (0.2 ug/ml and 1% penicillin-streptomycin. While HGF were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum, 100 units of penicillin, 100µg/ml streptomycin and 0.4 g/ml of hydrocortisone (Sigma Chemical Company, St. Louis, MO).

The cells were cultured at 37°C in a 5% CO2 air atmosphere until confluent and sub-cultured using a disaggregation assay with trypsin (0.1%) and Ethylenediaminetetraacetic acid (EDTA) (0.01%) in phosphate buffered saline (PBS) (pH 7.5).

For all experiments, cells were grown in 6 or 96 well plates at 5 x 10^4 cells/well and grown to 80% to 90% confluence. Control cells (NM) for all experiments were treated with the infusion solution alone in normal media (non-calcium containing infusion solution). All experiments were performed in triplicate and repeated on two separate occasions.
Drugs Treatments:

1- **Injectable ZA**: non-calcium containing infusion solution 0.36 Saline (Zometa; Novartis Pharmaceuticals Corp, East Hanover, NJ, US) was selected for these studies because it is the most widely reported BP associated with BON. ZA was used for all experiments at six different concentrations (0, 0.5, 1, 3, 5, 10 µM). These concentrations were selected because they are clinically relevant to patients receiving ZA, being representative of the lower limits of estimated plasma concentrations after a 15-minute infusion (baseline plasma concentration level is 1 µM). First, ZA was diluted to the appropriate concentration in NM. For each drug concentration, one dentine disc (DD), our mimic of bone, (IDS Ltd., Bolden, Tyne and Wear, UK) was used. The DDs were immersed in different ZA concentration (0, 0.5, 1, 3, 5, 10 µM) in 6-well plates for 24 hours. They were then removed from the normal or ZA-containing medium, washed three times in PBS and inserted into 6 or 96 well plates containing fresh medium without ZA added. Next, NOK or HGF cells were plated into the same wells as the DDs and allowed to grow onto and around the DDs to 90% confluence. The ZA (0.5 to 10 µM) treated and untreated (ZA 0 µM) DDs were then either left unchelated or were chelated using EDTA 0.001%. [see figure (4)]

The same experiment was done directly to both cell lines without using DDs. Cells were plated in 6 or 96 well plates containing fresh medium without ZA added. Cells were allowed to grow to 90% confluence. Then ZA was diluted to the appropriate concentration in NM and was added to both cell lines. The ZA (0.5 to 10 µM) treated and untreated (ZA 0 µM) cells were then either left unchelated or were chelated using EDTA 0.001%. The chelating agents were selected for their known activity and their
simulation of the oral environment, as various components of saliva are capable of chelation. Both cell lines were exposed to different duration of drug treatment (48, 24, 12, 6, 3, 1 hours). [see figure (4)]

2- **Albumin:** Hanks’ balanced salt solution-bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO) with pH 7.2, containing 2.5 mM probenecid, pH 7.45 (HBSS-BSA-probenecid, pH 7.45) was used to reverse the effect of ZA. The final concentration is 4 µM according to the Alliance for Cellular Signaling (AFCS) Protocol PP00000210. Cells were plated in the same manner. Then cells were treated with albumin for 6 hours. [see figure (4)]

3- **Calcium Chloride Hydrate (CaCl₂):** (Sigma Chemical Company, St. Louis, MO) was used to force express calcium effect on NOH and HGF. Both cell lines were plated in the same manner. Cells then were treated with 1 µM and 3µM of CaCl₂ for 24 hours. [see figure (4)], [84,85]

Figure (4): Cells were plated as shown in the figure to receive different drugs treatments
Methods:

A- Immunofluorescence:

First the FluoForte™ Dye solution was prepared by adding 100µl of dimethyl sulfoxide to the vial containing Reagent A (lyophilized GFP-Certified™ FluoForte™ dye) to get the reagent stock solution. Then 9ml of Reagent C (Hanks’ buffer with 20mM HEPES, 100ml) was mixed with the contents of 1 Vail (1 mL) of Reagent B (dye efflux inhibitor), 10ml to get the 1X Assay Buffer. After that, 10 µL of Reagent A stock solution added to 10 mL of 1X Assay Buffer and mixed well. This working solution was kept for 2 hours at room temperature.

Culture Medium was removed from the 96 well plates and 100µL of the FluoForte™ Dye solution was added to each well. The cell plates were incubated for 1 hour at room temperature. Then the calcium flux assay was run by monitoring the fluorescence at Ex=490nm/Em=525 nm with a fluorometric imaging plate reader (SpectraMax 340PC384 Absorbance Microplate Reader, Molecular Devices, LLC. CA, USA).

B- Western Blot Analysis:

Following the drugs treatments with ZA, BSA and CaCl2 as described above, cells were incubated and washed twice with ice-cold PBS, followed by lyses using Radioimmunoprecipitation Assay Buffer [50 µmol/L Tris (pH 7.4), 150 µmol/L NaCl, 1% triton X-100, 1% deoxycholic acid, sodium salt, 0.1% sodium dodecylsulfate (SDS), 100 µg/mL phenylmethysulfonyl flouride, 1 µg/mL aprotinin, 1 mmol/L
dichloro-diphenyl-trichloroethane (DDT), and 1 mmol/L sodium orthovanadate] for 10 minutes at 4°C. The wells were scraped and the recovered cell products centrifuged at 40,000g for 15 minutes at 4°C. The recovered proteins were measured and equalized using the Bio-Rad Protein Assay (Bio-Rad Labs, Richmond, CA) per the manufacturer's instructions. The Western blot was then performed using a Calmodulin Antibody (cell signaling, MA). Actin 1:500 dilution antibody (Sigma, St. Louis, MO) was used as a loading control. The blots were visualized using the Western Lightening Chemiluminescence Reagent Plus (Thermo scientific). All experiments were performed in triplicate and repeated on two separate occasions.
Chapter (3)

Results:

A- Immunofluorescence results:

When cells were treated with different ZA concentration, there is an increase in the calcium immunofluorescence expression. The maximum expression was seen at concentration of 1 µM. NOK cells with EDTA showed the maximum expression compared to NOK without EDTA and HGF with and without EDTA. [see table (1) and figure (5)]

<table>
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<th>ZA 3</th>
<th>ZA 5</th>
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Table (1): Immunofluorescence results when cells were treated with Zoledronic Acid.

Figure (5): Bar chart that represents Immunofluorescence results when cells were treated with Zoledronic Acid.
While when adding albumin, there is a decrease in the calcium immunofluorescence expression was seen. NOK cell line seems to have more reduction in their readings when compared to HGF. [see table (2) and figure (6)]

<table>
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</table>

**Table (2):** Immunofluoresence results when cells were treated with Zoledronic Acid and Albumin.

**Figure (6):** Bar chart that represents Immunofluoresence results when cells were treated with Zoledronic Acid and Albumin.
The two data were joined and a decrease in the calcium Immunofluorescence expression was seen in both cell lines. [see table (3) and figure (7)]

<table>
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<th>HGF+ E+alb</th>
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<th>HGF+ alb</th>
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<td>356.7</td>
<td>758.1</td>
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</tbody>
</table>

**Table (3):** Combined immunofluorescence results when cells were treated with Zoledronic Acid and Albumin.

**Figure (7):** Bar chart that represents combined immunofluorescence results when cells were treated with Zoledronic Acid and Albumin.
**B-Western Blot:**

In order to confirm the results of the immunofluorescence mentioned above, proteins were extracted from HGF and NOK cells. Western Blot analysis of proteins following probing with antibodies to Calmodulin demonstrated a time and dose dependent increase in both cell lines induced with different ZA concentration treatment. While when treating cell lines with albumin, a down regulation was seen. Then an up regulation was seen when cells were treated with calcium chloride as a force expression. [see figure (8)]

![Western Blot Results](image)

**Figure (8):** Western blot results for NOK and HGF with different drug treatments.
Chapter (4)

Discussion

Every year, an estimated 24 million bisphosphonate prescriptions are written in the U.S. alone. [16] One of the more recently reported serious adverse effects of BP treatment is BON. This condition was first recognized in 2003 and is predicted to occur in up to 28% of IV BP patients, with an estimated weighted prevalence of 13.3% in studies with accurate follow-up. [86]

Currently, BON lacks a defined pathophysiologic mechanism. Unfortunately, researchers initially ignored the cross talk between bone and oral mucosal soft tissue, as this condition is truly a chemonecrosis process. Scheper et al demonstrated the presence of calcium in 8 of the 10 BON cases within the connective tissue and surrounding blood vessels. They found that BON’s clinical course and delayed wound healing is in part correlated to a localized non-traditional calciphylaxis. [56]

Calciphylaxis is classically defined as a multifactorial renal phenomenon, whereby microcalcification of small blood vessels leads to soft tissue ischemia, necrosis and non-healing lesions. [60] This classic presentation is frequently fatal and is seen most often at end-stage renal failure. [62] However, in certain hypercalcemic states, such as malignancy (Breast and Multiple Myeloma), hyperparathyroidism, cirrhosis, Crohn’s and collagen disease conditions, a non-uremic non-traditional calciphylaxis is shown to induce vascular and tissue deposition of calcium in the submucosal tissues. [60-66] Since the mandible is a primary site for BP saturation and is commonly affected by trauma, infection and inflammation; an acidic microenvironment would release free
BP from bone and secondarily release calcium from its BP bound state to induce oral mucosal apoptosis/necrosis. [20,61,87,88]

Clinically, BON is observed as a mucosal dehiscence leading to the formation of a superficial mucosal ulcer, which continues to extend with time and results in detectable bone exposure, necrosis and sequestration. Moreover, like calciphylaxis, these lesions typically present as non-healing wounds treated empirically. Once a lesion occurs, there is tremendous difficulty in treating these lesions, which leads to delay or refusal of dental treatment and discontinuation of necessary cancer therapy.

In this project, we studied the effect of ZA on soft tissue by calcium deposition. Also, we hypothesized that albumin will reverse that effect by binding to calcium and probably prevent calciphylaxis. No similar research was conducted when searching in the literature.

Our Immunofluorescence examination demonstrated that ZA induced an increase in the levels of Calcium in HGF and NOK cell lines. The maximum expression was seen at concentration of 1 µM. [see table (1) and figure (5)] A possible explanation is that cells undergo lysis and apoptosis beyond the concentration of 1 µM of ZA.

The chelating agents EDTA were selected to mimic the effects that saliva may have on the release of BP from bone, so that we could prove that when bound to bone or calcium, BP is harmless, but when released by competitive chelation, the free BP acts directly on the mucosal cells. This explains our finding that cells treated with EDTA showed more expression compared to unchelated cells.
While when adding Albumin, a decrease in the calcium Immunofluorescence expression was seen. NOK cell line seems to have more reduction in their readings when compared to HGF. [see table (2) and figure (6)] Albumin constitutes about 60% of the protein mass found in blood plasma with complex legend binding specificities. Calcium binding by albumin indicates at least 30 different binding sites with different association constants and different H+ interaction. [82] Which explains the reduction in the calcium Immunofluorescence expression as albumin binds to calcium. NOK cell line has more expression to both ZA and albumin treatment. A possible explanation is the nature and the immunofluorescence signaling of these cell line when compared to HGF. In the Immunofluorescence methodology, our aim is to show a trend and in which direction that trend is running when both cell lines were treated with different drug treatments. Therefore, we did not use statistical analysis. Also, we supported our finding with the Western blot results.

The Western blot is a widely accepted nonquantitative technique used to detect specific proteins. However, It can be challenging to perform a western blot properly and get good results. Experience is perhaps the best tutor; even for an experienced technician it is time-consuming. Our result showed a dark background due to the use of a highly sensitive detection solution as the lower sensitive one did not show any results. No editing was done to the films to show the true bands and to have reliable results.

Western Blot examination demonstrated a time and dose dependent increase in both cell lines induced with different ZA concentration treatments. While when treating cell lines with albumin, a down regulation was seen due to albumin binding to
calcium. Then an up-regulation was seen when cells were treated with calcium chloride as a force expression. [see figure (8)]

This suggests that albumin can reverse the ZA effect on HGF and NOK by binding to calcium and prevent further calciphylaxis.

The sizable number of patients receiving BP therapy combined with the extremely long half-life of BP in bone (8–10 years) indicates that the prevalence of BON is likely to increase significantly. There is no present means of detecting which subpopulation of patients on IV BP therapy are at risk for development of BON. However, documented risk factors include use of nitrogen-containing BPs, particularly ZA, [17] dento-alveolar surgery, and relatively high cumulative dose. Median exposure to ZA prior to BON onset ranges from 9–30 months, but can be as brief as 3 months. [17] The cumulative hazard increases from 1% in 1 year of ZA treatment to 15% at 4 years of treatment. [17]

Currently, the only therapeutic regimen for BON is antimicrobial therapy, with surgical interventions reserved only for severe cases. [56] With the speculation of the pathogenesis of BON as a localized non-traditional calciphylaxis, will modify the management of BON. Part of the treatment regimen for calciphylaxis includes antibiotics and rebalancing calcium and phosphate, while avoiding local tissue trauma, similar to BON. [56] Our finding may help in the prevention and management of BON by balancing the calcium thus preventing calcium deposition in the oral soft tissues.
Further studies are needed to fully elucidate this model, including the biochemical mechanisms, the effect on dual cell lines of epithelial and fibroblast origin and to determine if such effects are clinically relevant for in vivo models of BON.

**Conclusion**

Currently, only preventive strategies of BON aimed at avoiding invasive oral interventions such as dental surgery and subsequent infection are the standard of care for patients with BON. Additionally, it is recommended that until healing from an invasive dental surgical treatment takes place, temporary discontinuation of BP therapy may be considered (up to 3 months) (Weitzman et al, 2007; Jeffcoat & Watts, 2008), however there is no evidence that this favourably affects outcomes and these recommendations flow from a clinical paradigm and fail to follow a causative mechanism. Our finding that albumin can bind to the released calcium following ZA treatment, might suggest a method of BON prevention. Further studies are needed to determine if such effects are clinically relevant for in vivo models of BON.
References:

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[52]. Watts NB, Becker P. Alendronate increases spine and hip bone mineral density in women with postmenopausal osteoporosis who failed to respond to intermittent


