EFFECT OF BISPHOSPHONATE / GRAPHENE OXIDE COMPLEX ON PROLIFERATION AND DIFFERENTIATION OF MESENCHYMAL STEM CELLS AND BREAST CANCER CELLS

by

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ABSTRACT

EFFECT OF BISPHOSPHONATE / GRAPHENE OXIDE COMPLEX ON PROLIFERATION AND DIFFERENTIATION OF MESENCHYMAL STEM CELLS AND BREAST CANCER CELLS

Bisphosphonates have strong healing impact on postmenopausal osteoporosis and also apoptotic effect on breast cancer cells (MCF-7). Zoledronic Acid (ZOL) is a third generation (nitrogen contained) bisphosphonate. In this study, the effects of ZOL, Graphene Oxide (GO), and conjugation of these two samples (ZOL-GO) were observed on both cell lines of human bone marrow mesenchymal stem cells (MSC) and of breast cancer cells (MCF-7). The conjugation of ZOL (Sigma, 10 mg powder) and GO (Sigma, 2mg/mL) was obtained via mixing them with magnetic stirrer and sonicator. During the preparation of samples; ZOL stock solution was obtained by mixing ultra pure water (5mg) with ZOL powder (10 mg) and GO stock solution was obtained by mixing ultra pure water (4500 μL) with GO (500 μL). The final concentrations that used in this study were for ZOL were 200 μM, 50 μM, 12.5 μM and for GO were 11.7 ng/mL, 2.91 ng/mL, 0.73 ng/mL and for ZOL-GO complexes were 200 μM ZOL - 11.7 ng/mL GO, 50 μM ZOL - 2.91 ng/mL GO, 12.5 μM ZOL - 0.73 ng/mL GO. Fourier Transform Infrared Spectroscopy (FTIR) results was obtained for the characterization of the samples. For cell proliferation, Alamar Blue viability test and Acridine Orange (AO) dying was conducted. The viability of MSC was not affected significantly in the presence of ZOL-GO complex however the viability of MCF-7 cells remarkably decreased on day 3. For observing cell differentiation, Scanning Electron Microscopy (SEM) and Alizarin Red S staining results was used. The mineralization of the MSC was reinforced by the presence of ZOL- GO complex.

Keywords: Zoledronic Acid, Graphene Oxide, Mesenchymal Stem Cells, Breast Cancer, Mineralization.

ÖZET

BİSFOSFONAT / GRAFEN OKSİT KOMPLEKSLERİN MEZENKİMAL KÖK HÜCRE VE MEME KANSERİ HÜCRELERİNİN ÇOĞALMA VE FARKLILAŞMASINA ETKİSİ

Bisfosfonatlar, menepoz sonrası kemik erimesi üzerinde güçlü iyileştirici özellik göstermiştir. Ayrıca meme kanseri hücreleri üzerinde apoptotik etkisi mevcuttur. Zoledronik asit (ZOL) üçüncü jenerasyon (nitrojen içeren) bir bisfosfonattır. Bu çalışmada ZOL'ün, Grafen oksitin (GO) ve ikisinin konjugasyonunun (ZOL-GO) meme kanseri hücreleri (MCF-7) ve mezenkimal kök hücrelerin (MSC) üzerindeki etkileri gözlemlenmiştir. ZOL (Sigma, 10 mg toz) ve GO (Sigma, 2mg/mL) konjugasyonu manyetik karıştırıcı ve sonikatör ile karıştırılarak hazırlanmıştır. Malzemelerin hazırlanması sırasında; 5 mg ultra saf su ile 10 mg ZOL tozu vortekslenerek ZOL stok solüsyonu (2mg/mL) elde edilmiştir. GO stok solüsyonunu hazırlamak için ise 4500 μL ultra saf su ile 500 μL GO (2mg/mL) solüsyonu sonike edilmiştir. Bu çalışmada son kullanılan konsantrasyonlar ZOL için 200 μM, 50 μM, 12.5 μM, GO için 11.7 ng/mL, 2.91 ng/mL, 0.73 ng/mL ve ZOL-GO içinse 200 μM ZOL - 11.7 ng/mL GO, 50 μM ZOL - 2.91 ng/mL GO, 12.5 μM ZOL - 0.73 ng/mL GO şeklindedir. Fourier Dönüşümlü Kızılötesi (FTIR) Spektroskopisi kullanılarak malzemelerin karakterizasyonu yapılmıştır. Malzemelerin hücre çoğalması üzerindeki etkilerini gözlemlemek için Alamar Blue canlılık testi ve Akridin Oranj boyama testi yapılmıştır. Deneyin üçüncü gününde ZOL-GO'nun MSC çoğalmasına negatif etkisi bulunmamıştır ancak MCF-7 üzerinde apoptotik etkisi olduğu gözlemlenmiştir. Malzemelerin hücre farklılaşmasına etkisi Tarayıcı Elektron Mikroskopisi (SEM) ve Alizarin Kırmızı Boyası kullanılarak gözlemlenmiştir. ZOL-GO konjugasyonu ile tedavi edilen hücrelerde mineralleşmenin arttığı gözlemlenmiştir.

Anahtar Sözcükler: Zoledronik Asit, Grafen Oksit, Mezenkimal Kök Hücre, Meme Kanseri, Mineralleşme .

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g	Gram
mg	Milligram
ng	Nanogram
mM	Millimolar
μM	Micromolar
mL	Milliliter
μL	Microliter
cm	Centimeter
nm	Nanometer
°C	Degrees Centigrade

LIST OF ABBREVIATIONS

ZOL	Zoledronic Acid
GO	Graphene Oxide
ZOL-GO	Zoledronic Acid and Graphene Oxide
MSC	mesenchymal stem cells
MCF-7	Michigan Cancer Foundation-7
TCP	Tissue Culture Plate
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate Buffer Saline
DPBS	Dulbecco's Phosphate Buffer Saline
FBS	Fetal Bovine Serum
SEM	Scanning Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
ATCC	American Type Culture Collection
CO_2	Carbon dioxide
$C_{140}H_{42}O_{20}$	Chemical formula of Graphene Oxide
HCl	Hydrochloric Acid
NH_4OH	Ammonium Hydroxide
P	Phosphate
C	Carbon
O	Oxygen
H	Hydrogen
AO	Acridine Orange
ANOVA	Analysis of Variance
HSD	Honestly Significant Difference

1. INTRODUCTION

1.1 Motivation

Bisphosphonates are small sized drugs which can be easily discharged from the body. Therefore, the conjugation of these drugs with hydrophilic nanoparticles or with polymeric nanoparticles provides more controllable drug release [1],[2]. In this study, one of the highly potent third generation bisphosphonates, Zoledronic Acid (ZOL) was used as a drug. ZOL has a high potential for treatment of cancer and high level of osteoporosis [3],[4].

Graphene Oxide (GO) has hydrophilic property which is an important feature for targeting drug treatments [5]. However, treatment with graphene nanostructures shows cytotoxic effect on MSC [6]. GO shows no cytotoxic effect up to 50 μg/mL level [7].

In general, motivation for this study was reinforcing the osteogenesis effect of ZOL with less concentrations by conjugation of ZOL with GO. Thus, by lowering the uptake concentration of ZOL, side effects might be inhibited. Depending on the results of the current study, in vivo studies will be carried out to study the effect of ZOL-GO for cancer and osteoporosis treatment.

1.2 Objectives

In this study, ZOL conjugated to GO in order to improve osteoblast cells proliferation and to observe the effects of this complex on viability of the breast cancer (MCF-7) cells. Objectives of this study presented as followed :

- To successfully conjugate ZOL and GO

- To determine the optimum concentration of ZOL-GO complex in order to increase the viability of Mesenchymal Stem Cells (MSC) while decreasing the viability of MCF-7 cells
- To find the ideal drug combination in order to cure breast cancer and also high level of postmenopausal osteoporosis
- To determine the effects of ZOL-GO complex on mineralization and differentiation of MSC

1.3 Outline

This thesis consists of 6 chapters. The first chapter includes introduction and its subsections are motivation, objectives and outline. In chapter 2, background information of the main topic of this study was given which covers graphene oxide, bisphosphonates, breast cancer and postmenopausal osteoporosis treatments and side effects of zoledronic acid. In chapter 3, the preparation of samples and experimental procedures were clarified. In chapter 4, results of the experiments were given. In chapter 5, the results of the experiments were discussed. Finally, in chapter 6, this study was concluded and possible future studies were remarked.

2. BACKGROUND

2.1 Bone metastasis

Bone metastasis means spread of cancer cells into the bones [8],[9]. Bone metastasis commonly occurred in patients with breast and prostate cancer [8]. Approximately 15% to 20% of breast cancers patients interfaced with osteoblastic bone metastasis [9]. Bone metastasis resulted with bone fractures, osteonecrosis of bones,bone resorption and severe pain in bones [9].

Figure 2.1 shows tumor types and patients rate of incidence with bone metastasis and survival time [10].

Tumor type	Incidence of bone metastasis (%)	Median survival from diagnosis of bone metastasis (months)
Multiple myeloma	90 – 100	NR
Prostate cancer	65 – 75	12 – 53
Breast cancer	65 – 75	19 – 25
Bladder cancer	40	6 – 9
Lung cancer	30 – 40	6 – 7
Kidney cancer	20 – 25	12
Thyroid cancer	60	48

NR: Not reported.

Figure 2.1 Survival time, incidence rate of patients with bone metastasis according to tumor types [10].

2.2 Bisphosphonates

Bisphosphonates contains two phosphonate groups ($(HO)_2P(O)$) [11]. First generation bisphosphonates (etidronate and clodronate) contain short alkyl side chains in their chemical structure [12],[13]. Different then first generation bisphosphonates,

second generation bisphosphonates (alendronate and pamidronate) contain amino terminal groups [12],[13]. Zoledronic acid, ibandronate and risedronate are third generation bisphosphonates that contain nitrogen and cyclic side chain [3],[4].

Bisphosphonates inhibit osteoclast originated bone loss [14]. In addition, bisphosphonates prevent bone turnover and strengthen bones by facilitating osteogenic differentiation of MSC [15]. Bisphosphonates are counted as main drug for osteoporosis treatment [16]. ZOL is the most efficient bisphosphonate in terms of providing rigidity and increasing the density of bones due to its consistence with Hydroxyapatite [16]. ZOL could be taken by intravenous infusion so that it can travel through the whole body via blood vessels [11].

In Figure 2.2, chemical formulas and structures of bisphosphonates are shown.

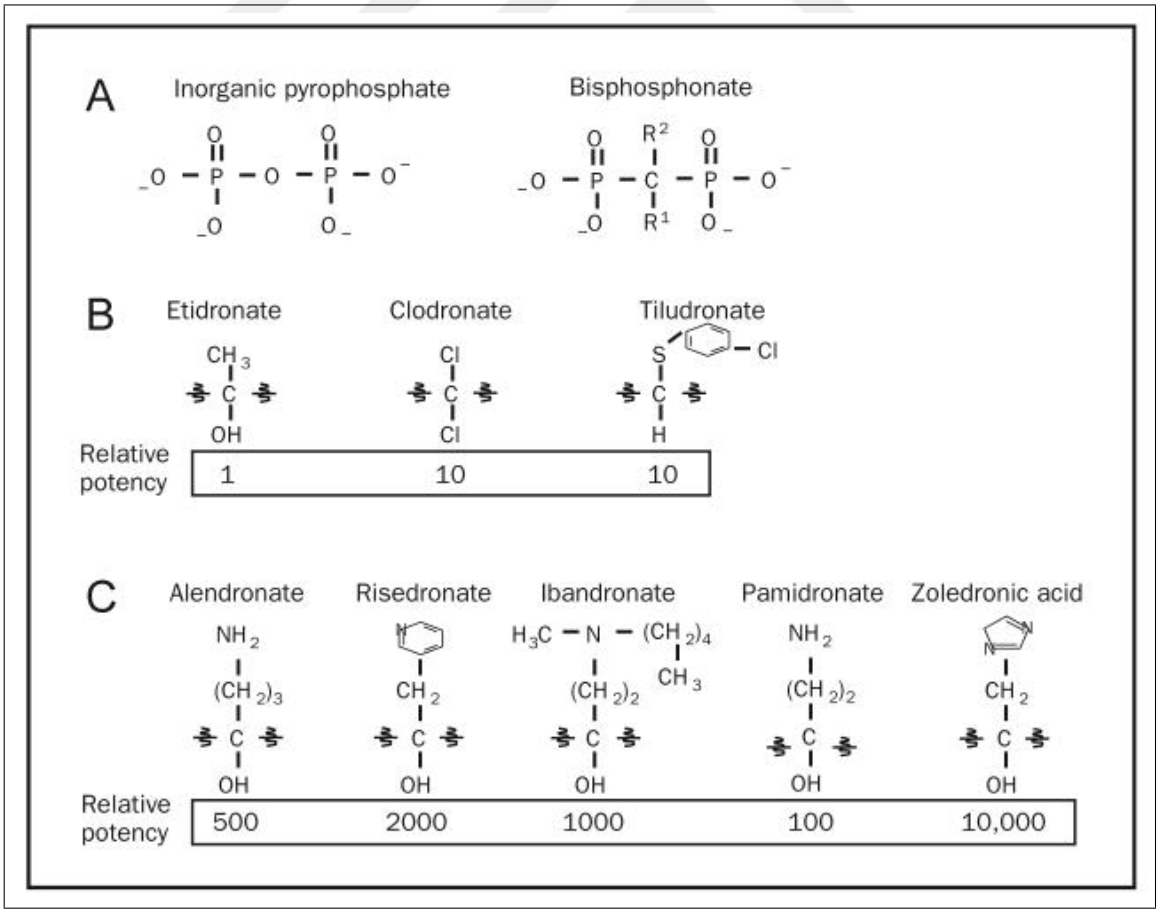

Figure 2.2 Chemical structures of bisphosphonates [4].

2.3 Zoledronic Acid

ZOL is a drug that is used both as an inhibitor of bone loss for osteoporosis and an anti-cancer treatment [3]. The main aim for bisphosphonates to be used in osteoporosis treatment was preventing patients from bone loss and increase the bone density by reinforcing the density of mineralization in bone matrix [17],[18]. For the cancer treatment, the most potent bisphosphonate that reduces the viability of MCF-7 cells was ZOL [19],[18]. ZOL inhibits mobility of MCF-7 cells which leads to apoptosis or necrosis of the cells[20].

Figure 2.3 shows chemical structure of ZOL.

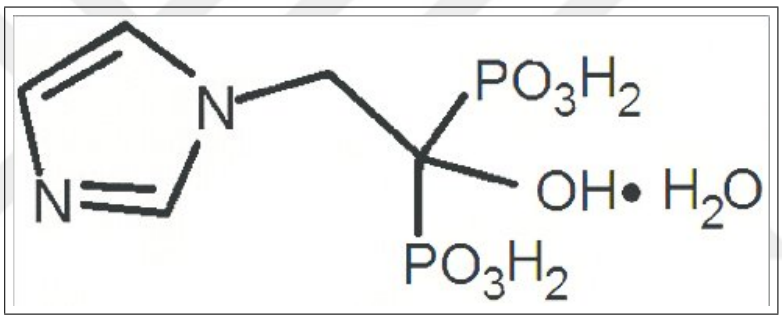

Figure 2.3 Chemical structure of ZOL [21].

2.3.1 Side Effects of Using Zoledronic Acid

Common side effects of treatment of ZOL were headache, pain in muscles,influenza, and fever [22]. There are some rare but serious side effects of taking high concentrations of ZOL treatment. One of the rare side effects of ZOL is osteonecrosis of jaw bone [23]. Osteo means bone and necrosis means death. Bone loss in jaw possibly related to the time and dosage dependent behaviour of ZOL. Although bone matrix mineralization is beneficial for osteoporosis treatment, in vivo studies showed that there were severe side effects of treatment with ZOL such as osteonecrosis of jaw, atrial fibrillation, kidney failure and hypocalcemia [24],[25]. An another serious side effect occurs due to annual intravenous treatment of ZOL for three or more years which might cause cancellous

bone matrix density to be increased [17].

2.4 Graphene Oxide

Graphene Oxide (GO) is a single atomic layer of graphite [26]. The molecular formula of Graphene oxide is $C_{140}H_{42}O_{20}$. GO contains mainly oxygen related carboxyl, epoxyl and hydroxyl groups [5].

Figure 2.4 shows bonding structures and graphene to graphene oxide oxidation and graphene oxide to reduced graphene oxide reduction.

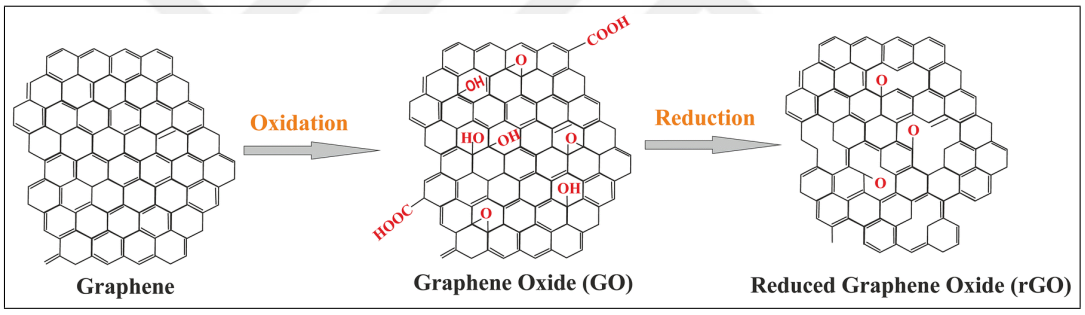

Figure 2.4 Oxidation of graphene to graphene oxide and reduction of graphene oxide to reduced graphene oxide [27].

There are a lot methods to synthesize GO from graphite or graphene. The most common methods are known as Hummers method, chemical vapour deposition (CVD), and epitaxial growth [28],[29]. GO is mostly used in the applications of biosensors. In addition, GO has impressive success when it comes to targeted drug delivery [30],[31],[32]. A number of physical properties are effected from various parameters such as flake size There are some parameters (flake size, oxidation level, temperature, concentration, etc.) for having a better result while using GO with the purpose of treatment [33]. One of those parameters is the flake size of GO. Flake size of GO effects the result of viability of cells, because the dimensions of the cells differ for each cell line. in vitro studies [34],[35]. GO has a high potential to be used as drug carrier

similar with carbon related other carriers such as carbon nanotubes and graphite oxide [30],[36].

Novelty of this study is analysis of the effect of loading ZOL on GO on MSC and MCF-7 cell lines. In this study, ZOL conjugated to GO in order to enlarge the size of the drug so that drug will not be easily eliminated from the body. So that, drug delivery could be slower and the serious side effects of ZOL would be prevented by reducing drug release time [1]. Finally, the efficiency of ZOL would be enhanced by the presence of GO. To characterize the samples, cell proliferation was examined with alamar blue viability test and Acridine Orange (AO) dying. Cell differentiation was observed with Scanning Electron Microscopy (SEM) and Alizarin Red S staining images.

3. MATERIALS and METHODS

3.1 Preparation of Samples (ZOL, GO and ZOL-GO)

The preparation of samples took place in two stages. Firstly, stock solutions were prepared with ultra pure water and secondly samples were freshly prepared from stock solutions with Dulbecco's Modified Eagle Medium (DMEM). For ZOL samples, 5 mg of ultra pure water was added to 10 mg of ZOL powder and mixed via vortex and first stock solution was obtained. Then, the first stock solution was diluted with ultra pure water in order to gain lower concentrations (1mg/mL and 0.05 mg/mL). 200 μM ZOL sample was obtained by adding 17.37 mL DMEM to 1 mL from 1mg/mL stock solution of ZOL. 50 μM ZOL sample was obtained by adding 10.92 mL DMEM to 4.08 mL from 0.05 mg/mL ZOL stock solution. 12.5 μM ZOL sample was obtained by adding 14 mL DMEM to 1 mL from 0.05 mg/mL ZOL stock solution. All of the ZOL samples was magnetic stirrered overnight at dark room. Figure 3.1 shows preparation of ZOL samples.

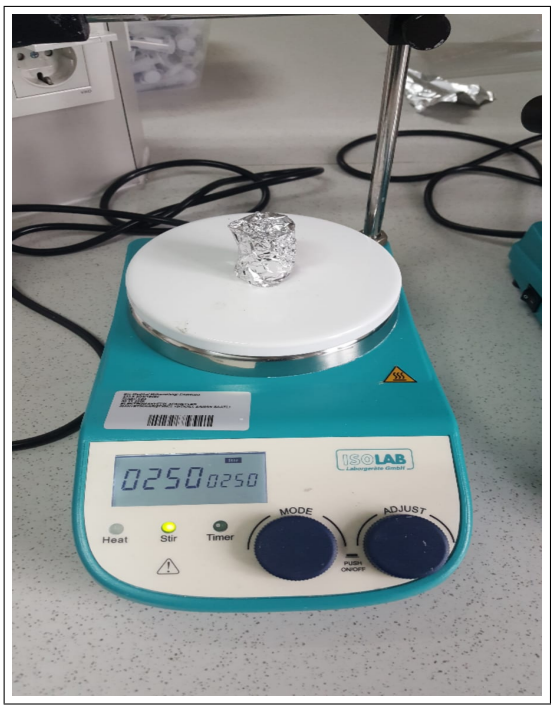

Figure 3.1 Preparation of ZOL samples.

GO stock solutions (0.2 mg/mL, 0.02 mg/mL, 0.002 mg/mL and 0.0002 mg/mL) were obtained by dilution of 2mg/mL GO (Sigma) with ultra pure water. 11.7 ng/mL GO sample was obtained by addition of 1755μL from 0.002 mg/mL stock solution of GO and 28.245 μL DMEM. 2.91 ng/mL GO sample was prepared by addition of 3.73 mL from 11.7 ng/mL GO sample and 11.26 mL DMEM. 0.73 ng/mL GO sample was prepared by adding 3.763 mL from 11.7 ng/mL sample to 11.237 mL DMEM. Then, all of the GO samples sonicated for 30 minutes. Figure 3.2 shows preparation of GO samples.

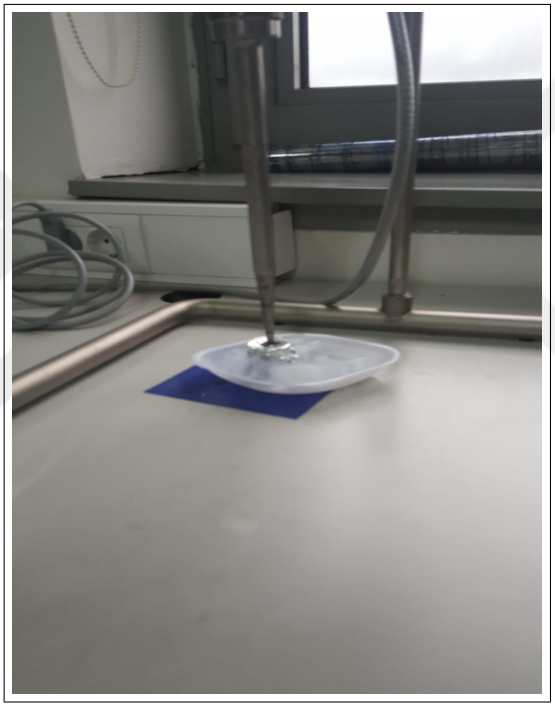

Figure 3.2 Preparation of GO samples.

While preparing 200 μM ZOL - 11.7 ng/mL GO sample, 816 μL from 1mg/mL stock solution of ZOL was added to 8.8 μL from 0.02 mg/mL of GO stock solution and then 14.175 mL DMEM was added. For 50 μM ZOL - 2.91 ng/mL GO sample, 204 μL from 1 mg/mL of ZOL stock solution was added to 2.19 μL from 0.02 mg/mL of GO stock solution and then 14.79 mL DMEM was added. Lastly, for 12.5 μM ZOL - 0.73 ng/mL GO sample, 51 μL from 1 mg/mL of ZOL stock solution was added to 0.55 μL from 0.02 mg/mL of GO stock solution and then 14.94 mL DMEM was added. All of the ZOL-GO samples were sonicated for 15 minutes and then magnetic

stirred overnight in dark room. All of the samples were prepared at room temperature (but after preparation, the samples and stock solutions were kept at +4°C) and freshly prepared before each test.

3.2 Characterization of Samples (GO and ZOL-GO)

3.2.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy (Thermo Scientific Nicolet 380 FT-IR spectrometer) was acquired to understand the chemical structure and properties of GO and ZOL-GO complex. FTIR spectroscopy was performed in Chemistry Department at Boğaziçi University and the spectroscopy results were taken in the range of 400 cm^{-1} to 4000 cm^{-1}. 100 μL of 1 mg/ml ZOL sample which were diluted with ultra pure water was used for FTIR analysis. Then, 100 microliter of 2 mg/ml GO sample was used for FTIR analysis. Finally for ZOL-GO sample, the highest concentration (200μM ZOL - 11.7ng/ml GO) was used. The samples were drop by drop left for drying overnight. This procedure repeated for 3 days.

3.3 Cell Culture Studies

This study was conducted by two cell lines which were human breast cancer cell line MCF-7 (ATCC HTB-22) and bone marrow-derived mesenchymal stem cells (MSC) (ATCC PCS-500-012). For the culturing of the MCF-7 cells, T25 flasks were used and complete growth medium was prepared with DMEM high glucose (Sigma), 10% Fetal Bovine Serum (Sigma) and 1% penicillin/streptomycin (Sigma) and then incubated at 37 °C with 5% CO_2 until 80% confluency of the cells reached. For culturing of the MSC, T25 flasks were used and complete growth medium was prepared with DMEM low glucose (Sigma, D6046), 10% Fetal Bovine Serum (FBS) (Sigma) and 1% penicillin/streptomycin (Sigma) and also differently from the MCF-7 complete growth

medium 4mM l-glutamine was added to MSC complete growth medium. Cells were counted by a hemocytometer before seeded to well plates. According to the test well plates and cell densities varies.

3.4 Cell Proliferation Assays

3.4.1 Alamar Blue Viability Test

Alamar blue viability test was applied to see the effects of samples on viability of the cells. MCF-7 cells were plated on 96-well plate (Sigma) at the cell density of 5000 cells per well. There were 9 sample groups and one control group. Each of the groups had 8 wells repetition. MSC were plated on 96-well plate (Sigma) at the cell density of 2000 cells per well. As for the MCF-7 cells and MSC, the sample groups were the same and alamar blue test applied in the same procedure. 10% Alamar Blue reagent was prepared freshly with medium and 100μL of the solution was added to wells. Then, cells were incubated for 3 hours by preventing from light. Then, the absorbance data was obtained by Microplate reader (BIO-RAD Mark, Microplate Reader) to analize. % viability of the cells were calculated as found in the literature [1].

3.4.2 Acridine Orange (AO) Dying

MSC were seeded on 48-well plate at a density of 7500 cells per well. Then, cells were left for adhering to well plate overnight. Afterwards, the sample groups was added and cells were treated for 3 days before dying with AO. Dyed cells were observed by using fluorescence microscope (Axio Vert A.1-Zeiss).

3.5 Cell Differentiation Assays

3.5.1 Scanning Electron Microscopy (SEM)

Morphology of the cells were monitored by using SEM images. A cover glass was placed inside of a well of 24-well plate (Sigma) and cells were seeded on the cover glass. MCF-7 cells were seeded on cover glass at the density of 50000 cells per well. MSC were seeded on cover glass at the density of 10000 cells per well. Before fixation, cells were washed twice with PBS (Phosphate buffer saline). 1.5 mL glutaraldehyde (Sigma) was added to 48.5 mL of distilled water and therefore 50 mL of 3% glutaraldehyde solution was obtained for fixation of cells. 500 μL of glutaraldehyde solution was added to each well and left for 30 minutes in a dark room at room temperature. Afterwards, cells were washed twice with PBS. Lastly, 500 μL of PBS was added and the cells were preserved at +4°C. Finally, cells were dehydrated by 30%, 50%, 80%, and 95% ethanol for 15 minutes each and afterwards hexamethyldisilazane (HDMS) was dripped on the samples and left for drying overnight. HDMS should be evaporated very slowly so that plates were foiled and small hollows were opened on aluminum. Both of the cell lines were treated for 3 days with the lowest concentrations of 3 sample groups and also one control group seeded.

3.5.2 Alizarin Red S Staining

MSC were seeded on 24-well plate with a density of 10000 cells per well. In order cells to adhere to well plate, cells were incubated overnight. MSC treated for 3 days before osteogenic medium was added. Osteogenic medium was prepared with final concentrations of L-Ascorbic acid-2-phosphate (50 μg/mL), β-Glycerophosphate (5 mM), and Dexamethasone (10 nM) in complete growth medium. Stock solution of L-Ascorbic acid-2-phosphate was prepared by dissolving 2.5 g L-Ascorbic acid-2-phosphate sesquimagnesium salt in 50 mL of 1x Dulbecco's phosphate-buffered saline (DPBS) and 50 mg/mL stock solution was obtained. Then, solution was sterile filtered and stored at -20°C. Stock solution of β-Glycerophosphate disodium salt hydrate was

prepared by dissolving 1.08 g in 10 mL of 1x DPBS and 500 mM stock solution was obtained. Then, the solution was sterile filtered and stored at $-20°C$. Finally, the stock solution of Dexamethasone (10 mM) was prepared by dissolving 0.03925 g in 10 mL 100% ethanol. Then, the second stock solution (with 10 μM) was obtained by diluting 10 μL of the first (10 mM) stock solution with 9.99 mL of 100% ethanol. Both of the stock solutions were sterile filtered and stored at $-80°C$. Lastly, those stock solutions were mixed with concentrations of 50 mL complete growth medium, 50 μL L-Ascorbic acid-2-phosphate (stock of 50 mg/mL), 500 μL β-Glycerophosphate (stock of 500mM), and 50 μL Dexamethasone (stock of 10μM) and osteogenic medium was obtained. After 3 days of treatment, the medium prepared with samples was replaced with osteogenic medium. Osteogenic medium was prepared freshly for each time by using stock solutions. The osteogenic medium was changed every 2 days. The experiment was conducted on 14^{th} day and 21^{st} day in order to understand the effect of mineralization with time. On 14^{th} day and 21^{st} day, freshly made Alizarin Red S solution was prepared by dissolving 2 g of Alizarin Red S in 100 mL distilled water and mixed by vortex (pH of the solution was arranged to 4.2 with HCl and NH_4OH). MSC were washed with DPBS without disturbing the cell monolayer. For the fixation of cells 4% paraformaldehyde (Sigma) solution was prepared and MSC were fixed for 30 minutes at $+4°C$. After the fixation, 2% Alizarin Red S staining solution were added to cover cell monolayer and cells were incubated for 45 minutes at room temperature in the dark room. After that, cells were washed with distilled water 4 times and 500μL of DPBS was added to each well. By using phase-contrast microscope (Axio Vert A.1-Zeiss), the cells were observed on days 14 and 21. The undifferentiated MSC were slightly reddish however differentiated MSC (to osteoblasts) were bright orange-red with extracellular calcium deposits.

4. RESULTS

4.1 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The FTIR spectra of 2mg/mL GO and 200μM ZOL - 11.7ng/mL GO complex produced were shown in Figure 4.1.

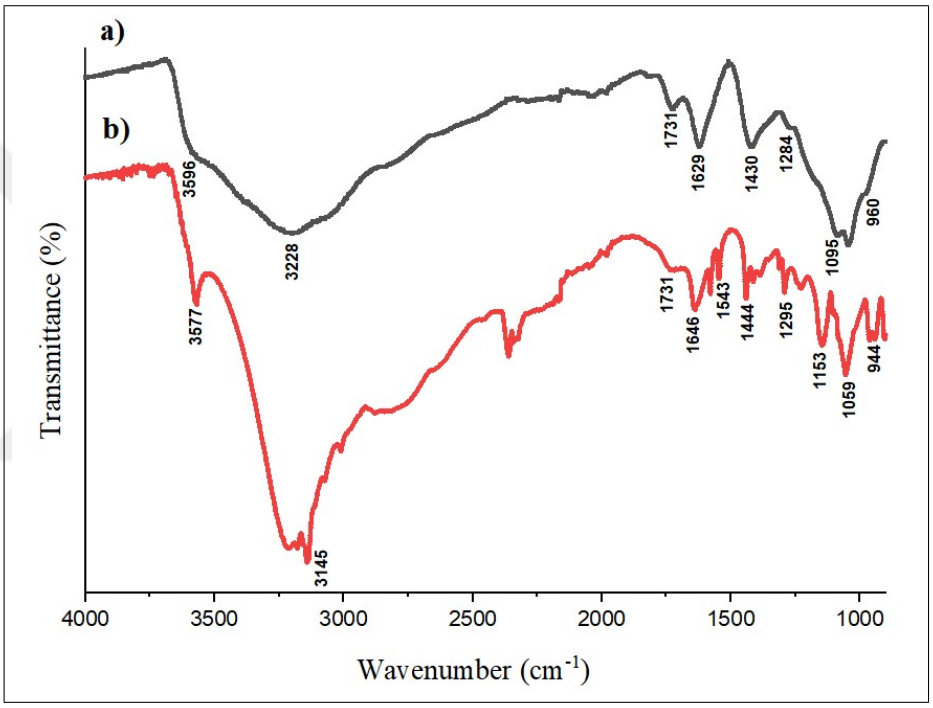

Figure 4.1 FTIR spectra of (a) 2 mg/mL GO and (b) 200μM ZOL - 11.7ng/ml GO.

The analysis of the FTIR spectra of GO was shown in Table 4.1.

Table 4.1
FTIR analysis of GO.

C-O	C=O	O-H	Epoxyl Group
1095 cm^{-1}	1731 cm^{-1}	1284 cm^{-1}, 1629 cm^{-1} 3228 cm^{-1}, 3596 cm^{-1}	960 cm^{-1}

In the FTIR results of GO, the absorption bands at 1284, 1629, 3228, 3596 cm^{-1} were representing stretching and bending vibration mode of O-H [37]. Deformation vibration band of OH group resulted with the band at 1430 cm^{-1} [38]. The band at 1731 cm^{-1} showed (C=O) stretching vibration mode of carboxyl group [39].

The analysis of the FTIR spectra of ZOL-GO complex was shown in Table 4.2.

Table 4.2
FTIR analysis of ZOL-GO.

HC=CH	O-H	C-C	C-H	P=O	P-O	C=O
1646 cm^{-1} 1543 cm^{-1}	3145 cm^{-1} 3577 cm^{-1}	944 cm^{-1}	1444 cm^{-1}	1295 cm^{-1}	1153 cm^{-1}	1731 cm^{-1}

ZOL contains imidazole ring [40]. In the ZOL-GO FTIR absorption bands, C-H stretching vibration was found with the band at 1444 cm^{-1} because of ZOL (imidazole ring). Also, there was the band due to presence of GO (C-O stretching vibration in the band at 1731 cm^{-1}) [39].

4.2 Alamar Blue Viability Assay Analysis

Figure 4.2 shows the viability of MCF-7 cells after treatment with ZOL, GO and ZOL-GO complexes. TCP (Tissue Culture Plate) was used as control groups.

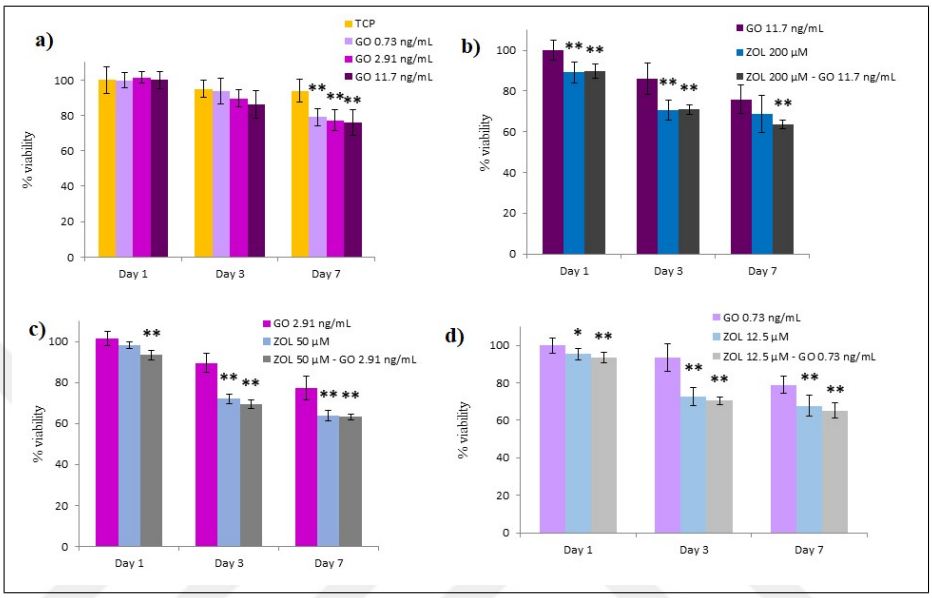

Figure 4.2 % viability of MCF-7 cells after treatment with (a) different GO concentrations, (b) 11.7 ng/ml GO, 200 μM ZOL, 200 μM ZOL - 11.7 ng/ml GO and (c) 2.91 ng/ml GO, 50 μM ZOL, 50 μM ZOL - 2.91 ng/ml GO (d) 0.73 ng/ml GO, 12.5 μM ZOL, 12.5 μM ZOL - 0.73 ng/ml GO (Metabolic activity was normalized to day 1 of TCP.) ($*$ signifies significant difference between TCP and other study groups on each day of the study; $*$ $P < 0.01$, $**$ $P < 0.05$ vs. control.

There were no significant increase or decrease in viability of MCF-7 cells between control group and all concentrations of GO treated groups on day 1 and day 3. However, 200 μM ZOL and 200 μM ZOL - 11.7 ng/mL GO treated groups compared to 11.7 ng/mL GO treated group showed serious decrease in viability of MCF-7 cells on day 1 and 3. On day 7, there was a significant reduction in viability of MCF-7 cells only in 200 μM ZOL - 11.7 ng/mL GO treated group compared to 11.7 ng/mL GO treated group. There were significant decrease in viability of MCF-7 cells 2.91 ng/mL GO treated group compared to 50 μM ZOL - 2.91 ng/mL GO treated group on each days. On day 3 and day 7, there were again a significant decrease in viability 50 μM ZOL treated group compared to 2.91 ng/mL GO treated group. In comparing 0.73 ng/mL treated group to 12.5 μM ZOL and 12.5 μM ZOL - 0.73 ng/ml GO treated groups,

there were again a significant decrease in viability each day. Overall, the most effective decrease was occurred on day 3. Overall, ZOL has dose dependent apoptotic effect on cell proliferation of MCF-7 cells [20].

Figure 4.3 shows the viability of MSC after treatment with ZOL, GO and ZOL-GO complexes.

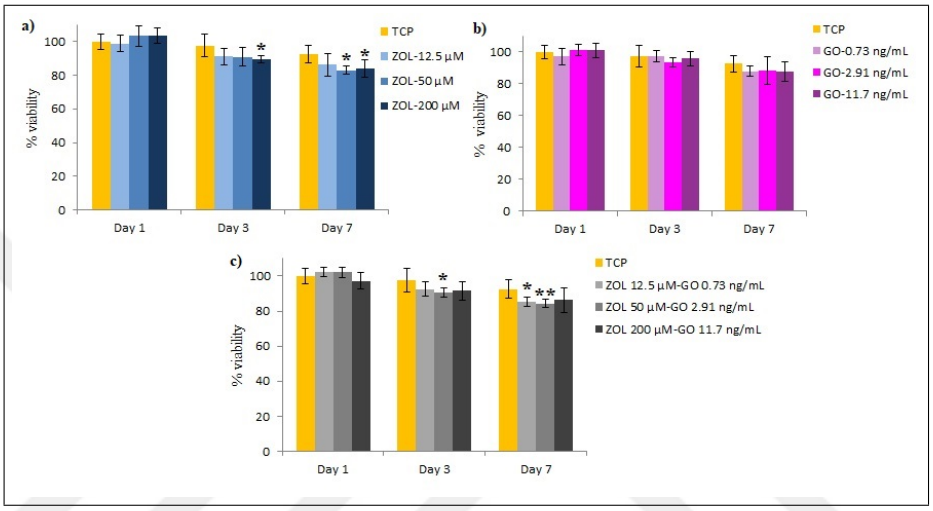

Figure 4.3 % viability of MSC cells after treatment with different concentrations of (a) ZOL (b) GO and (c) ZOL-GO complexes (Metabolic activity was normalized to day 1 of TCP.) ($*$ signifies significant difference between TCP and other study groups on each day of the study; $*$ $P < 0.01$, $**$ $P < 0.05$ vs. control.).

Treatment groups compared to control group (TCP) showed no significant increase or decrease on day 1. On day 3, there were a slight decrease in viability for the group treated with the highest concentration of ZOL (200 μM) and 50 μM ZOL - 2.91 ng/ml GO compared to TCP. On day 7, the groups treated with 50 μM ZOL, 200 μM ZOL, 12.5 μM ZOL - 0.73 ng/ml GO showed a slight decrease compared to control group in viability of MSC. Also, on day 7, there were a significant decrease in viability of MSC treated with 50 μM ZOL - 2.91 ng/ml GO compared to TCP. The dose dependent apoptotic effect of ZOL was possibly the reason for this significant decrease in viability of MSC [20].

Figure 4.4 shows top view of 96-well plate after 3 days of GO treatment and 3

hours of alamar blue reduction.

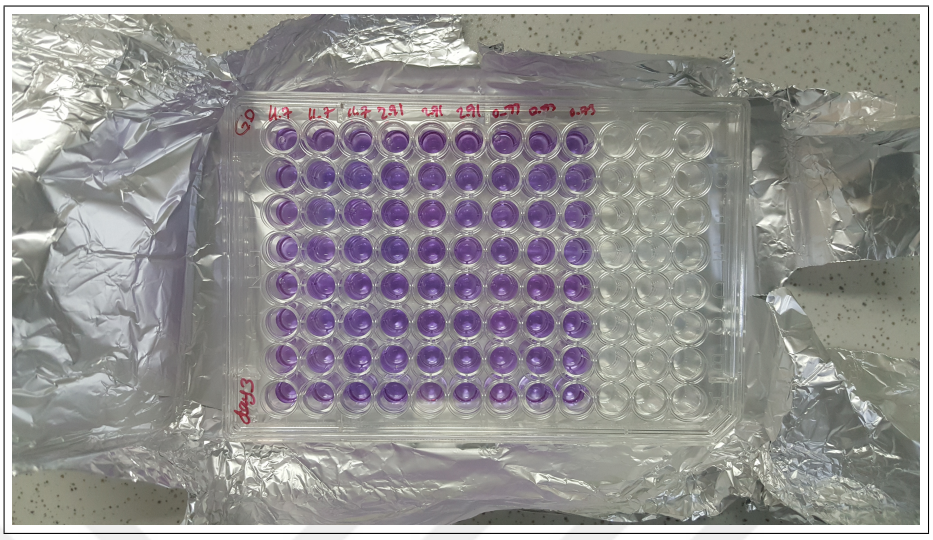

Figure 4.4 The absorbance measurements were taken after treatment of MSC with GO samples (day 3) on 96-well plate

4.3 Statistical Analysis

The statistical analysis of the alamar blue viability test was evaluated by performing one-way analysis of variance (ANOVA) with post-hoc Tukey honestly significant difference (HSD) Test.

4.4 Acridine Orange (AO) Dying Analysis

The images of AO dying of MSC after 3 days of treatment with the lowest concentrations of each sample group was shown in Figure 4.5.

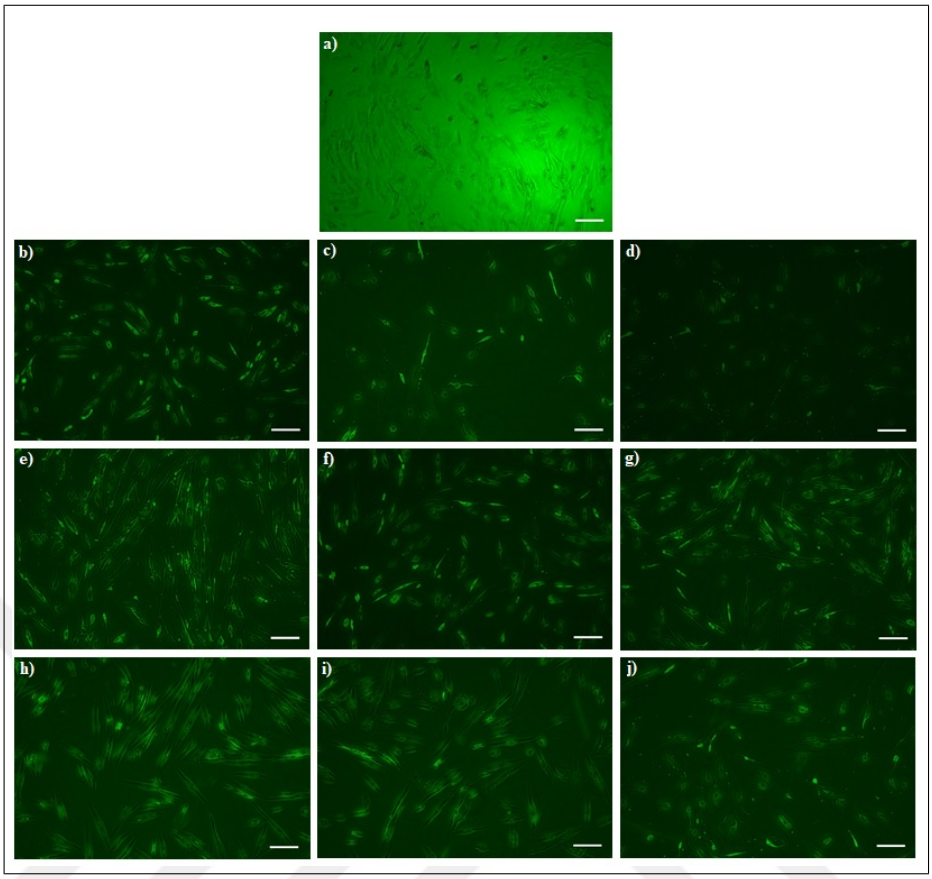

Figure 4.5 AO dying of MSC cells after 3 days of treatment with (a) TCP (b) 0.73 ng/ml GO (c) 2.91 ng/ml GO (d) 11.7 ng/ml GO (e) 12.5 μM ZOL (f) 50 μM ZOL (g) 200 μM ZOL (h) 12.5 μM ZOL - 0.73 ng/ml GO (i) 50 μM ZOL - 2.91 ng/ml GO (j) 200 μM ZOL - 11.7 ng/ml GO (scale bar: 200μm).

There was not a significant increase or decrease in proliferation of MSC after 3 days of treatment with ZOL, GO and ZOL-GO groups. These results confirmed that proliferation and viability of MSC were not drastically affected by existence of treatment groups.

4.5 Scanning Electron Microscopy (SEM) Analysis

The SEM images of MCF-7 cells after 3 days of treatment with the lowest concentrations of each sample group was shown in Figure 4.6.

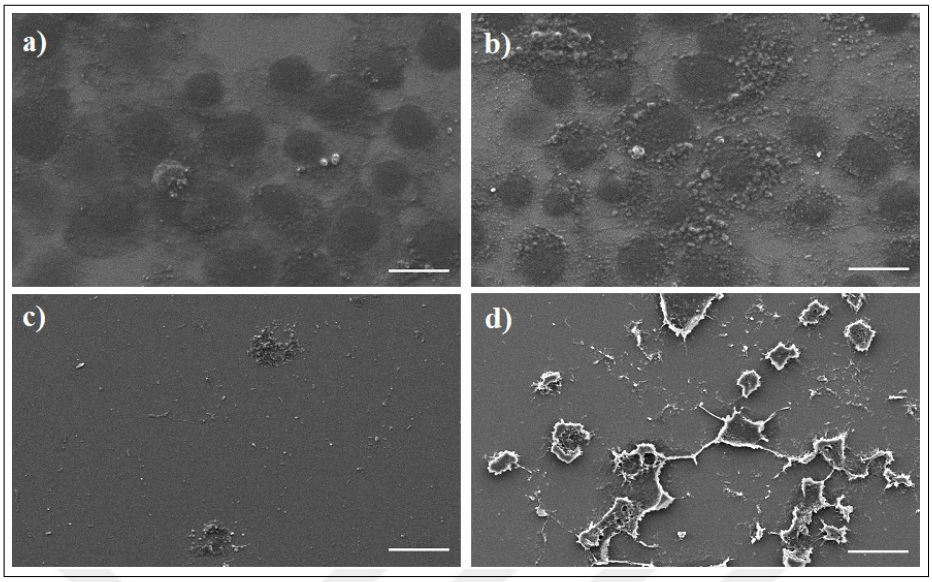

Figure 4.6 SEM images of MCF-7 cells after 3 days of treatment with (a) TCP (b) 0.73 ng/mL GO (c) 12.5 μM ZOL (d) 12.5 μM ZOL - 0.73 ng/mL GO (scale bar: 20 μm).

According to images, the effective treatment groups for apoptosis of the MCF-7 cells after 3 days of treatment were 12.5 μM ZOL and 12.5 μM ZOL - 0.73 ng/mL GO. The existence of ZOL increased the apoptosis of MCF-7 cells. There were no morphological change between control group and the cells treated with 0.73 ng/mL GO. On the other hand, the existence of ZOL probably caused morphological change. The results of MCF-7 cells treated with 12.5 μM ZOL and 12.5 μM ZOL - 0.73 ng/mL GO showed that there were both morphological change and apoptosis.

The SEM images of MSC after 3 days of treatment with the lowest concentrations of each sample group was shown in Figure 4.7.

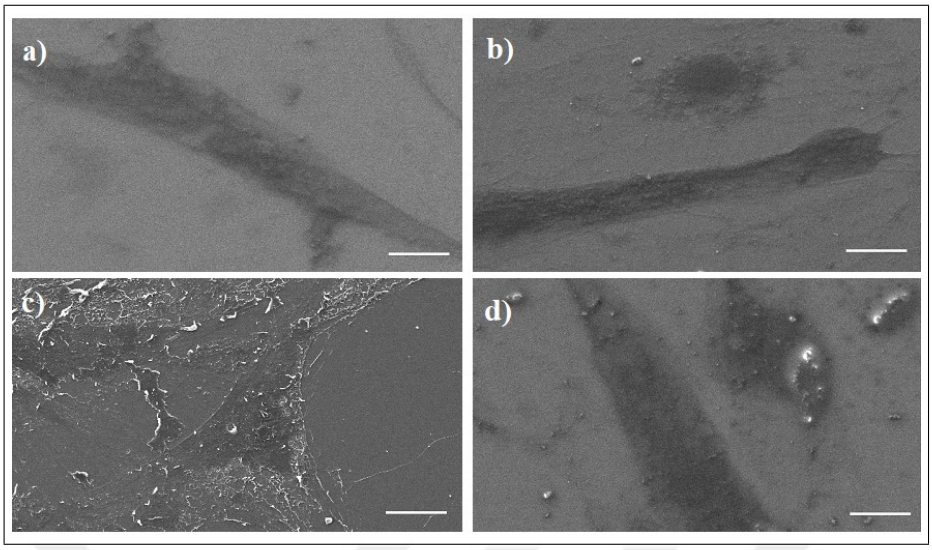

Figure 4.7 SEM images of MSC cells after 3 days of treatment with (a) TCP (b) 0.73 ng/ml GO (c) 12.5 μM ZOL (d) 12.5 μM ZOL - 0.73 ng/ml GO (scale bar: 20 μm).

In Figure 4.7, there were a slight morphological change when MSC treated for 3 days with 12.5 μM ZOL. This might be due to morphological change after ZOL treatment. However, there were no morphological change when MSC was treated for 3 days with 12.5 μM ZOL - 0.73 ng/mL GO. Also, white accumulations around MSC's could be because of the mineralization.

4.6 Alizarin Red S Staining Analysis

Alizarin Red S staining results (after 3 days of treatment) on day 14 for MSC was shown in Figure 4.8.

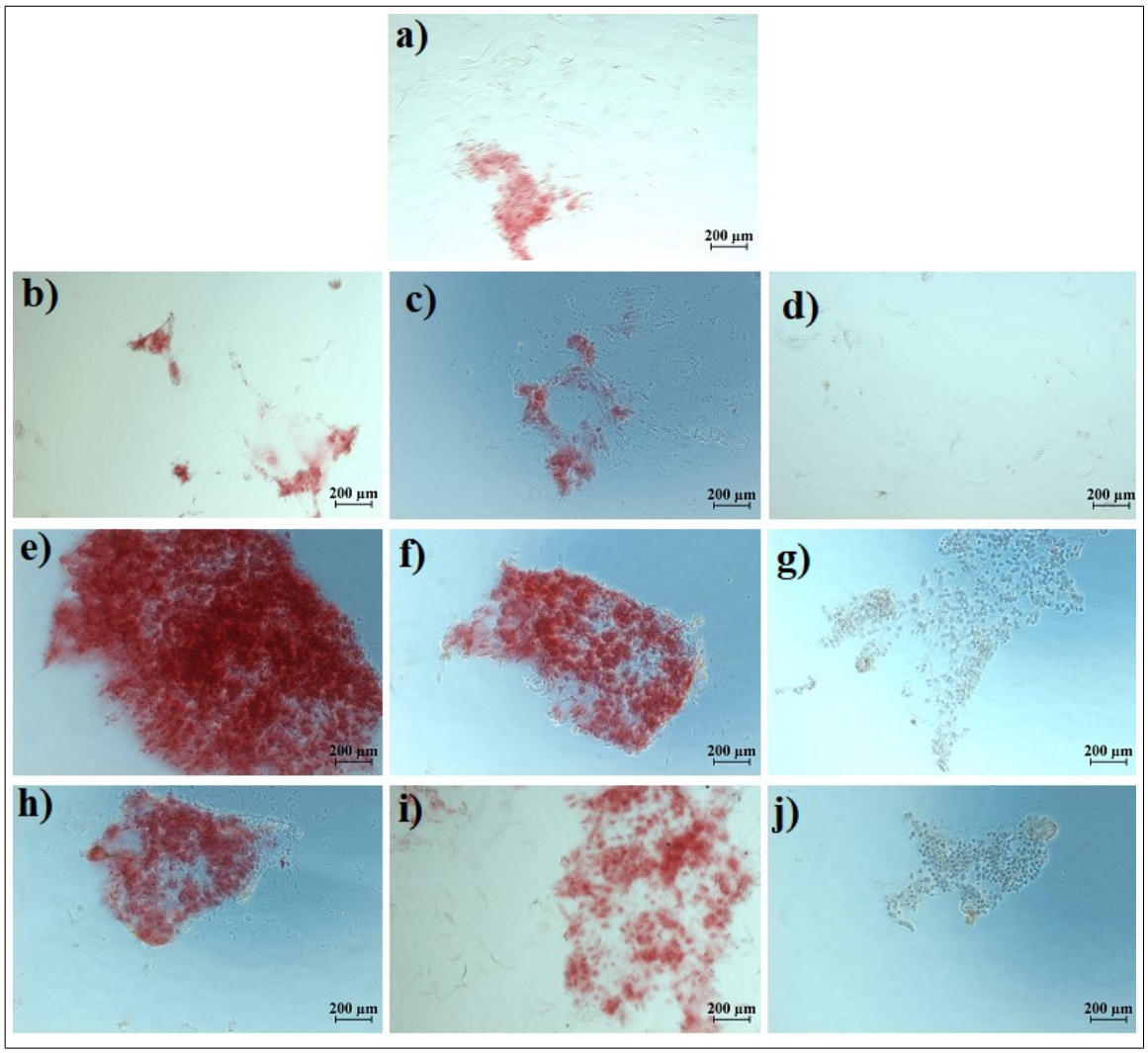

Figure 4.8 Alizarin red results of (a) TCP (b) 0.73 ng/ml GO (c) 2.91 ng/ml GO (d) 11.7 ng/ml GO (e) 12.5 μM ZOL (f) 50 μM ZOL g) 200 μM ZOL (h) 12.5 μM ZOL - 0.73 ng/ml GO (i) 50 μM ZOL - 2.91 ng/ml GO (j) 200 μM ZOL - 11.7 ng/ml GO on day 14.

According to images in Figure 4.8, the lowest concentration of ZOL (12.5 μM) showed the highest mineralization effect on day 14 for MSC. The existence of ZOL reinforced mineralization on day 14. However, the highest concentrations of ZOL (200 μM), GO (11.7 ng/mL), and ZOL-GO complex (200 μM ZOL - 11.7 ng/ml GO) showed no significant mineralization at all.

Alizarin Red S staining results (after 3 days of treatment) on day 21 for MSC was shown in Figure 4.9.

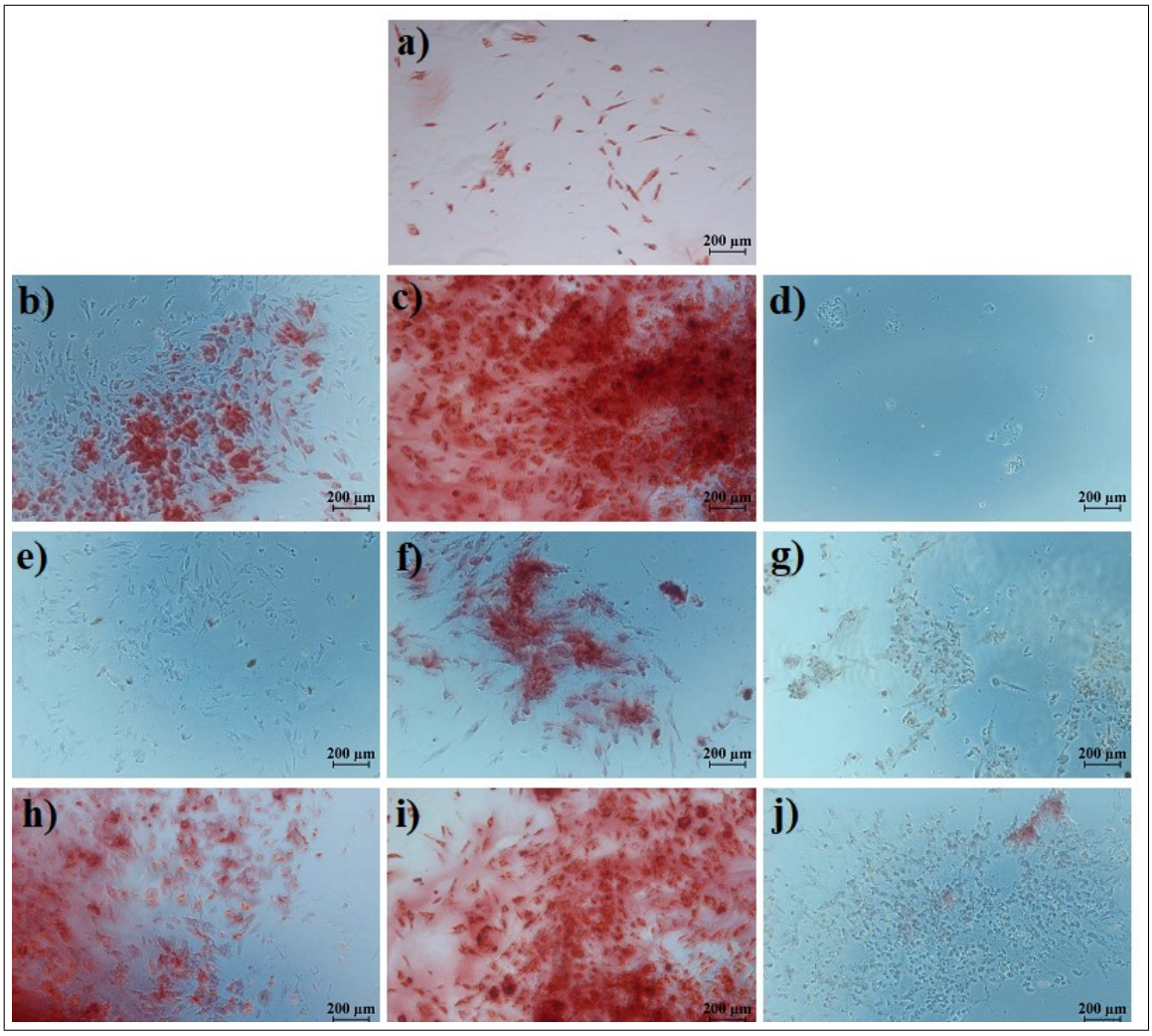

Figure 4.9 Alizarin red results of (a) TCP (b) 0.73 ng/ml GO (c) 2.91 ng/ml GO (d) 11.7 ng/ml GO (e) 12.5 μM ZOL (f) 50 μM ZOL (g) 200 μM ZOL (h) 12.5 μM ZOL - 0.73 ng/ml GO (i) 50 μM ZOL - 2.91 ng/ml GO (j) 200 μM ZOL - 11.7 ng/ml GO on day 21.

On day 21, the highest mineralization occurred in MSC treated 3 days with 2.91 ng/mL GO. That is, GO undoubtedly reinforced mineralization after 21 days. In all of the concentrations of ZOL treated cells, there were diminishing effect of mineralization on day 21 compared to day 14. However, the highest concentrations of all sample groups showed no distinguishable mineralization at all.

5. DISCUSSION

In this study, ZOL and GO conjugated in order to observe effects of this complex on proliferation of MSC and MCF-7 cells and differentiation of MSC. The dose dependent apoptotic effect of ZOL was observed with addition of ZOL to GO. FTIR results were proof of the conjugation was successful. Also, both of the Ultraviolet-visible spectroscopy results from Takavoli et al. and FTIR results in this study confirmed the ZOL-GO conjugation was successful.

The MCF-7 viability test results matched up with the studies in the literature [41],[19],[42]. Cell proliferation assay results clarified that there were a dose and time (day) related apoptotic effect of ZOL on viability of MCF-7 cells [41]. By taking into consideration the effects of time dependant behaviour of ZOL, the optimum treatment duration was found to be 3 days. With regards to MSC and MCF-7 viability, the most effective dosage for 3 days of treatment among the sample groups was 12.5 μM ZOL - 0.73 ng/ml GO. Although 12.5 μM ZOL - 0.73 ng/ml GO and 50 μM ZOL - 2.91 ng/ml GO showed slightly different effects on viability of the MSC and MCF-7 cells, there were a noticeable difference in mineralization results of these two treatment groups.

In the literature, GO reinforces mineralization and plays an effective role on differentiation [43]. In this study, this effect of GO could be seen on day 21 results of alizarin red staining (the presence of GO enhanced mineralization). On the other hand, GO results of alizarin red staining showed no enhancement in the GO sample groups compared to TCP on day 14. It could be due to time dependent effect of GO on differentiation. Future works might explain this effect of GO. ZOL also showed time dependent effect on differentiation of MSC [41]. On day 14, ZOL accelerated the mineralization compared to control group and other groups. However, on day 21, ZOL could not keep its reinforcement effect on differentiation. There were studies in literature which also observed inhibitory effect of third generation bisphosphonates (such as ZOL) on differentiation of MSC when the concentrations of drug ranged between 1

to 5 μM [44],[45]. 200 μM ZOL group showed no mineralization at all on both day 14 and day 21. Reaching or near of the cytotoxicity level for ZOL concentration might be caused this result [46].

Finally, by gathering up the results of viability and mineralization results, the optimum concentration among the ZOL-GO treatment groups was found to be 12.5 μM ZOL - 0.73 ng/ml GO and 50 μM ZOL - 2.91 ng/ml GO. In case of viability, 12.5 μM ZOL - 0.73 ng/ml GO showed better results compared to 50 μM ZOL - 2.91 ng/ml GO. However, mineralization mostly occurred with treatment of 50 μM ZOL - 2.91 ng/ml GO than 12.5 μM ZOL - 0.73 ng/ml GO. Therefore, for cancer treatment optimum concentration would be 12.5 μM ZOL - 0.73 ng/ml GO. For osteoporosis treatment optimum concentration would be 50 μM ZOL - 2.91 ng/ml GO.

6. CONCLUSION And FUTURE STUDIES

Although many researches focused on the effects of ZOL, in this study the conjugation of ZOL and GO was experimented with in vitro. Afterwards, the results were compared with only ZOL and only GO treated groups in order to determine the better outcome. When testing on the viability of MCF-7 cells, it was observed that ZOL-GO conjugation was more effective for apoptosis of MCF-7 cells compared to only ZOL or only GO treated groups since day 1 until day 7.

Moreover, mineralization is an important criteria for the treatment of post-menopausal osteoporosis[47]. Only GO treatments have no effect on the mineralization on the short term but it starts to have positive effect on mineralization starting from day 21. On the other hand only ZOL treated MSC behave completely opposite to only GO treated group. Meanwhile the conjugation of these two materials, reinforces mineralization throughout the whole experimantation process. In the future studies, different ZOL-GO conjugations with the same concentrations may be prepared depending on the change in size of the GO flakes. This may change the effects on cell proliferation, cell differentiation and cell morphology.

The literature about in vivo studies show that ZOL was used annually as a drug treatment for postmenopausal osteoporosis [48]. There were long term side-effects (atrial fibrillation) of this treatment after 3 years of usage [24]. According to in vitro results of ZOL-GO complexes, in vivo studies could be conducted in the future.

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