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Nano Ozonized Oil Trigger ROS Production and γH2AX Cell Positivity of B-16 Melanoma and OV-90 Ovarian Cells

Nano Ozon Yağının B-16 Melanoma ve OV-90 Over Hücrelerinde ROS Üretimi ve γH2AX Hücre Pozitifliğini Tetiklemesi

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Abstract

Tumor hypoxia is a restrictive factor for cancer treatment. Ozone therapy, used to decrease or prevent the hypoxia in tumor. In this study, it is aimed to use ozonized oil nanoemulsions (OZNEs) to overcome the limitations of ozone therapy in cancer research. The influence of OZNE against cancer cell lines is evaluated by Reactive Oxygen Species (ROS) and γH2AX assays. For both B-16 melanoma and OV-90 cell lines, ROS production levels increased due to the increase in OZNE doses (0.85% to 14.88% and 1.94% to 58.58% for B-16 and OV-90 cell lines, respectively). 5-fold higher γH2AX cell positivity's determined in OV-90 cells compared to B-16 melanoma cells. OZNE treatment could provide a new effective method to damage cancer cells in the future.

Keywords: Ozonized oil, Nano, γH2AX, ROS, B-16 melanoma, OV-90 ovarian

Öz

Tumör hipoksisi, kanser tedavisinde tedaviyi kısıtlayan bir faktördür. Ozon terapi, tumor hipoksisini azaltmak ya da önlemek amaçlı kullanılır. Bu çalışmada, kanser tedavisinde ozon terapinin kısıtlamalarının üstesinden gelmek için, ozon yağı nanoemülsiyonu (OZNE) kullanımı amaçlanmaktadır. OZNE'nin kanser hücre hatlarına olan etkisi, Reaktif Oksijen Türevleri (ROS) ve γH2AX testleri ile değerlendirilmiştir. B-16 melanoma ve OV-90 over hücre hatlarının her ikisinde de, ROS üretimi OZNE doz artışı ile artmıştır (B-16 melanoma için %0.85'den %14.88'e, OV-90 over için %1.94'den %58.58'e), OV-90 over hücreleri γH2AX hücre pozitifliği B-16 melanom ile kıyaslandığında 5 kat fazla artış göstermiştir. OZNE tedavisi gelecekte kanser hücrelerine hasar verecek yeni etkili bir yöntem sağlayabilir.

Anahtar Kelimeler: Ozon yağı, Nano, γH2AX, ROS, B-16 melanoma, OV-90 over

1. Introduction

Cancer is most common cause of death, despite a new techniques have been developed to for cancer treatment. Tumor hypoxia is most important factor affecting the success of cancer treatment (Van Meir, 1996) and increase the risk of angiogenesis, metastasis and resistance to treatment (Brizel et al., 1996; Plasswilm et al., 2000; Young et al., 1988). Gray *et al.* have shown that resistance to radiotherapy increased 2.5-3 times and apoptosis decreased in the presence of tumor hypoxia (Gray et al., 1953).

Ozone, which is a reactive molecule, degrades in aqueous mediums and is converted to oxygen (Strickland & Perkins, 1995). In clinical applications ozone is used in high concentrations for disinfection and at low concentrations to accelerate wound healing and epithelization. It is also used as an alternative therapy in the treatment of advanced ischemic diseases, diabetic foot, viral infections, fungal infections, burns, dentistry, cancer, etc (Bocci et al., 2000; Clavo et al., 2004; Stoker, 1902). In recent years, there have been

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pilot studies examining ozone therapy and tumor hypoxia. These studies show that ozone increases oxygenation in hypoxic tumors (Clavo et al., 2004). To date, few studies have demonstrated the effect of ozone on cancer cells. Among all, ozone has been shown to decrease cellular viability by apoptosis (Klestadt et al., 2005). Sweet *et al.* reported the effect of different concentrations of ozone on the growth of lung, breast, and uterus cancer cells. The presence of 0.3 ppm, 0.5 ppm, and 0.8 ppm ozone inhibited cancer cell growth 40%, 60%, and more than 90%. In the presence of 0.5 ppm ozone, growth rates of cancer cells are lower than noncancerous cells (Sweet et al., 1980). In another study, Simonetti *et al.* investigated the influence of ozone with 5-Fluorouracil and cisplatin in human colon cancer cell (HT29) and reported that ozone with 5-FU and cisplatin decrease cell viability and increase cytotoxicity by 15-20% (Simonetti et al., 2017). Moreover, to date, most of these studies have focused on DNA synthesis and metastatic potential, which inspire alternative cancer therapy applications nowadays (Young et al., 1988).

Ozonized oils are obtained from reaction of ozone with vegetables oils and preferred more than ozone as they have high solubility, absorption and easy application (Serio et al., 2017; Travagli et al., 2010). Ozone affects the intracellular signaling systems (Travagli et al., 2010) and ozonized oils are frequently used in medical applications since ozonized oils overcome the difficulties of ozone during its applications (i.e. high reactivity of ozone gas, low solubility, and low absorption)(Serio et al., 2017; Travagli et al., 2010).

In this study, we aimed to investigate the influence of ozonized oil nanoemulsions (OZNEs) on cancer cells because the effects of hypoxia and the therapeutic activity of oxygenation in cancer cells are critical. The novelty of this study relies on the ozone nanoemulsion dose-dependency effect in different cancer cell lines, allowing us to evaluate ROS production and γH2AX cell positivity. In this regard, B-16 melanoma and OV-90 ovarian cell lines were incubated with OZNE at different concentrations. Subsequently, *in vitro* cellular activities of both cell lines have been determined by ROS, and γH2AX assays (Figure 1).

2. Materials and Methods

Ozonized oil was obtained from Aktifoks Ozonid (İzmir, Turkey). Peroxide value, iodine value, and acidity of ozonized oil were determined as described previously (analyzed by TÜBİTAK-MAM, Turkey) (Aydın & Kazancı, 2018). Nanoemulsions were prepared using the emulsion inversion point (EIP) method as described in our previous study (Aydın & Kazancı, 2018). In this study, the OZNE has been prepared under pre-defined optimized conditions (750 rpm mixing rate, surfactant/oil (2:1)). Zeta potential and size distribution of nanoemulsions were analyzed with dynamic light scattering (DLS) with Zetasizer Nano-S (Malvern, England). Scanning electron microscopy (SEM) was conducted on nanoemulsions for imaging the nanoemulsion morphology using a field emission scanning electron microscope (FEI Quanta, FEG450) with an operating voltage of 7 kV. Malignant melanoma mouse B-16 cell line and human ovarian OV-90 cell line have been purchased from ATCC (Germany).

2.1. In Vitro Cell Culture Studies

B-16 mouse melanoma (B16, ATCC CRL-6475) cells and OV-90 ovarian cancer cells (OV-90, ATCC CRL-11732) were cultured in RPMI 1640 medium (Sigma, Germany) supplemented with 10% fetal calf serum (FCS, (Sigma, Germany), %10 L-glutamine (Sigma, Germany) and 1% penicillin/streptomycin (Sigma, Germany). After the cells reached 80–85% confluence, before cell seeding, the cells were digested with 0.25% trypsin–EDTA (Sigma,

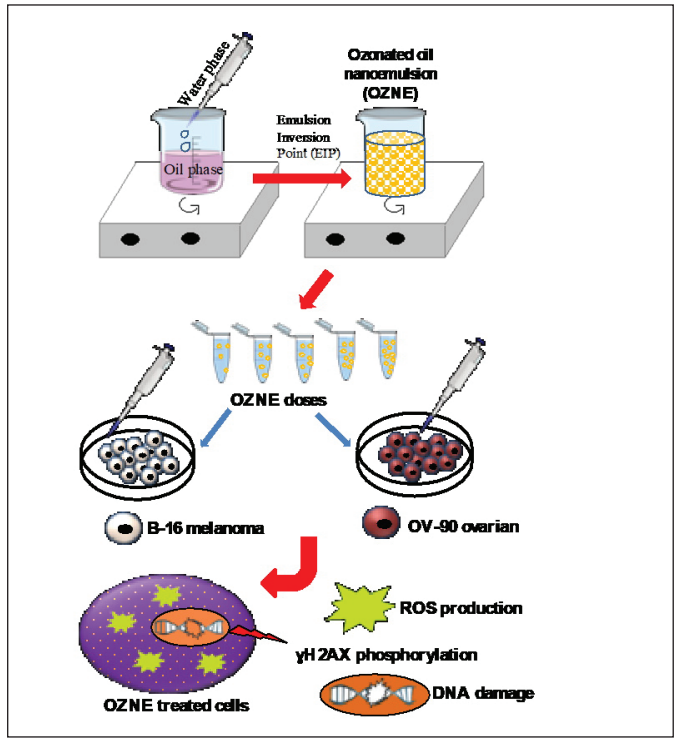

Figure 1. Schematic illustration of OZNE treated B-16 melanoma and OV-90 ovarian cell lines assessed DNA damage, ROS, and γH2AX assays.

Germany), centrifuged, and resuspended in medium. Then, the cells were conducted in sterile 12-well tissue culture polystyrene (TCPS) dishes in stationary conditions at 37°C in a humidified CO_2 (5%) atmosphere (Heraeus Instruments, Germany). 2 mL of cell suspension at a density of $5x10^5$ cells ml^{-1} (RPMI 1640 supplemented with 10% FBS (and 1% penicillin–streptomycin) was incubated in 12-well plates for 3 days. Then, B-16 melanoma cells and OV-90 ovarian cells were incubated with (OZNE) doses (1 µl OZNE dose=0.015 µl ozonized oil) sterilized by filtering through 0.22 µm filter (Milipore, Sigma) were for 60 min at 37°C according to our previous study (Yalçın et al., 2021). Experimental groups were defined as control (without OZNE), OZNE5 (5 µl/well), OZNE10, (10 µl/well), OZNE20 (20 µl/well), OZNE50 (50 µl/well), and OZNE100 (100 µl/well) and optical microscopy images of the cells exposed to different concentrations of OZNEs were shown in our previous study (Yalçın et al., 2021).

2.2. Reactive Oxygen Species (ROS) by Flow Cytometry

ROS determination by flow cytometry was assessed with the principle of determining the conversion of 2'7'-dichlorofuorescin d, acetate (DCFDA) to 2'7'-dichlorofluorescein (DCF). B-16 and OV-90 cells treated with all OZNE groups were incubated with DCFDA (10 µM, Sigma) at 37°C for 30 minutes, and then conducted to flow cytometry device (Beckman coulter, USA). The ROS assay kit (Sigma, Germany) was used according to the instructions of manufacturer. The percentage of fluorescence caused by DCF was determined.

2.3. Evaluation of γH2AX Positivity by Flow Cytometry

B-16 and OV-90 cells treated with all OZNE groups were incubated on the 12 well plates using flow cytometry. Phosphorylated H2AX histone proteins have interacted with primary antibodies. After 30 minutes of incubation at room temperature, staining has been done with labeled with FITC. The H2AX DNA damage assay kit (Beckman Coulter, FL) was used according to the instructions of manufacturer.

2.4. Statistical Analysis

Data are expressed as means ± standard deviation of three similar experiments carried out in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons using Graph-Pad Instant (GraphPad Software) statistics program. $p>0.05$, $p<0.05$, $p<0.01$, and $p<0.001$ represent statistically no significant values, statistically significant, very significant, and extremely significant values, respectively.

3. Results and Discussion

Figure 2 demonstrates DLS results and SEM image of ozonized oil nanoemulsion. SEM image approved homogenously distributed and spherical nanoemulsions (Figure 2A), confirmed by polydispersity index (PDI) value

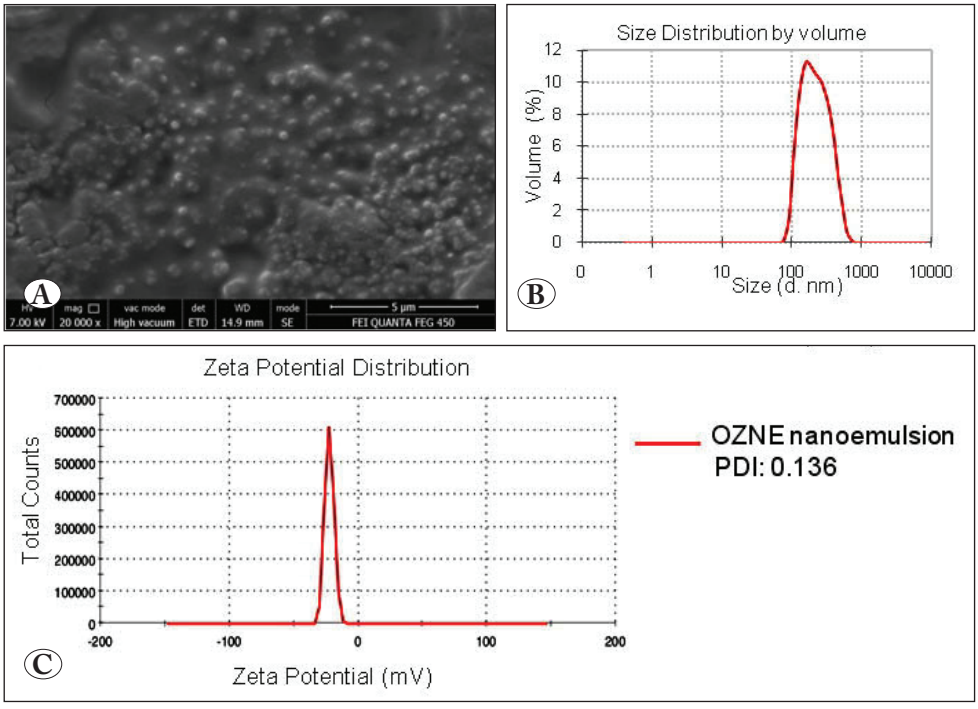

Figure 2. A) SEM image of OZNE, **B)** mean size distribution of OZNE and **C)** zeta potential distribution of OZNE.

of nanodroplets dispersed in emulsion (PDI: 0.136) (Figure 2B). Prepared nanoemulsion average size and zeta potential values were 212.9±0.7 nm -22.5±05 mV, respectively (Figure 2B and 2C).

3.1. Evaluation of Reactive Oxygen Derivatives (ROS) by Flow Cytometry

The increase of ROS production in the cell plays an significant role in the initiation of apoptosis by causing damage to the cell membrane, deterioration in intracellular protein structure and functions, structural damage in DNA and loss of function in the cell (Bayr, 2005). ROS affects DNA and rapid cellular division more. After the discovery of these qualifications of ROS, ROS release concentration has become more and more important. ROS is a side product of normal cell metabolism and can be either beneficial or harmful depending on the concentration and location (Bhardwaj et al., 2016). In this study, the percentage of fluorescence caused by DCF was measured by flow cytometry in order to determine ROS derivatives on both B-16 melanoma and OV-90 ovarian cells treated with OZNE nanoemulsions. OV-90 ovarian cells were used in order to investigate the effect of OZNE treatment regarding different cancerous cell types by exploring ROS derivatives and γH2AX positivity.

Figure 3 demonstrates ROS histograms of B-16 melanoma cells. As seen from Figure 3, ROS levels in B-16 melanoma cells were gradually increased from 0.85% in control group to 14.88% in the OZNE100 group due to the increase in nanoemulsion concentration. Additionally, it is noteworthy that a significant increase was determined in ROS levels of OZNE50 and OZNE100 groups (~9-fold and ~17-fold, respectively) compared to the control group. Thus, ROS levels in B-16 melanoma cells were evaluated as OZNE concentration-dependent. Previously, Alarifi *et al.* studied the cellular ROS production and apoptosis efficiency of Pd nanoparticles in human malignant melanoma cells. When the fluorescence percentages of cells with different concentrations of Pd nanoparticles were examined after 24 and 48 hours, an increase in ROS was observed depending on the increase in nanoemulsion concentration (Alarifi et al., 2017).

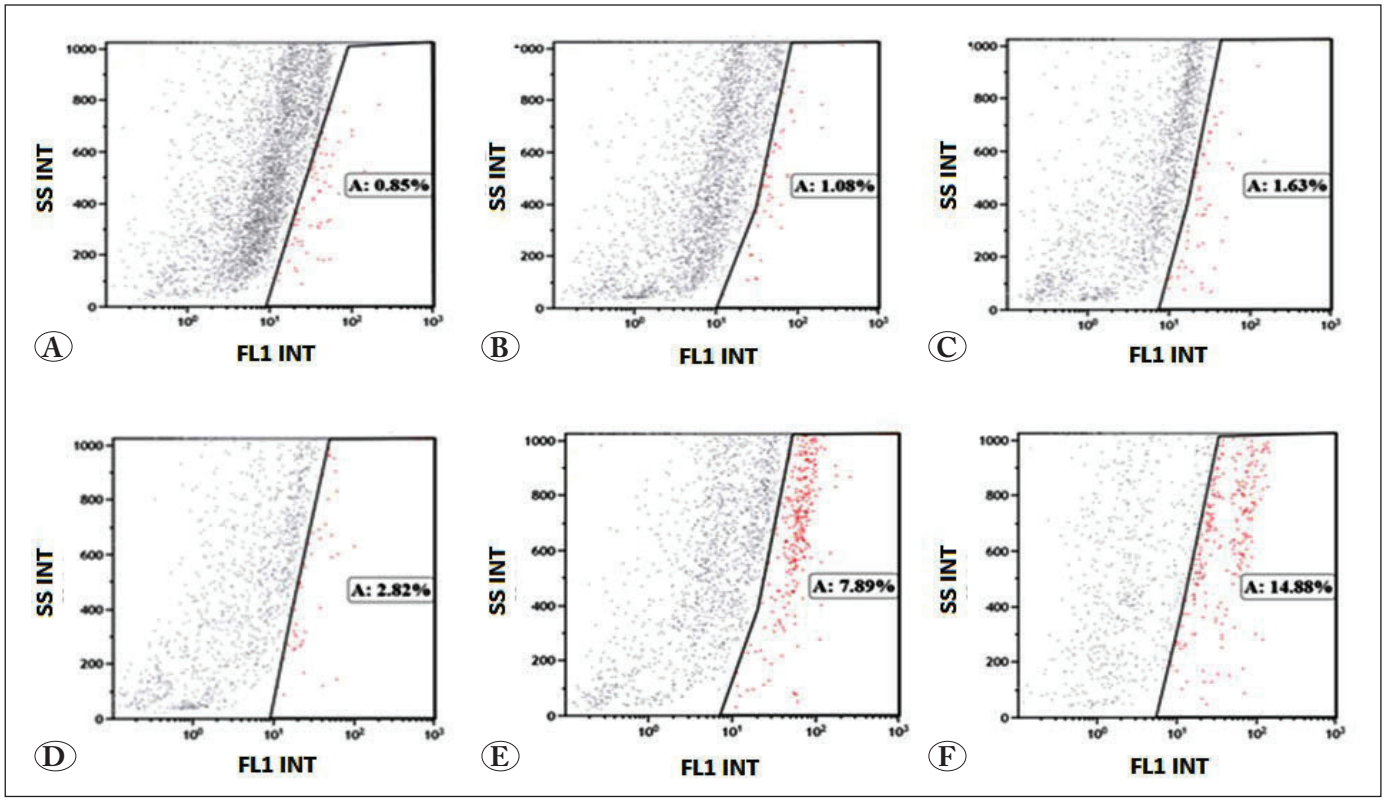

Figure 3. ROS Histograms of B-16 melanoma cells treated with different concentrations of OZNE; **A)** Control **B)** OZNE5, **C)** OZNE10, **D)** OZNE20, **E)** OZNE50, **F)** OZNE100 (X-axis (FL1 INT) represents the intensity of fluorescence in the red spectrum and Y-axis (SS INT) represents the intensity of side scatter).

Essential oils and their components have high anticancer potential due to the fact that they induce ROS. However, there are not many studies on the nanoemulsion forms of these oils. Khan *et al.* investigated the anticancer potential of the carvacrol nanoemulsion in human adeno carcinoma A549 cells and the carvacrol nanoemulsion treatment showed an increase in ROS levels, and this induced apoptosis (Khan et al., 2018). Our results showed in a similar manner with previous reports in the literature. Thus, it can be suggested that the increase of ROS levels in OZNE nanoemulsion treated cells induced apoptosis, which also confirms apoptosis studies.

Figure 4 demonstrates flow cytometry histograms showing ROS levels of OV-90 cells treated without and with OZNE nanoemulsions. Results showed an increase in ROS levels from 1.94% (control) to 58.58% (OZNE100 group) due to the increase of OZNE concentration within OV-90 cells (Figure 4). OV-90 cells displayed ~22- and ~30-fold higher levels in OZNE50 and OZNE100 groups, respectively, compared to control groups.

3.2. Evaluation of γH2AX Positivity by Flow Cytometry Method

Double-stranded DNA break is the most dangerous DNA damage. Even a single double-stranded break that is not repaired results in cell death (Jackson, 2002; Sonoda et al., 2006). Many anticancer drug studies are also being studied as an inducer of DNA double-stranded break. γH2AX is a new cancer biomarker to evaluate DNA damages (Bassing et al., 2003) in cancer treatment and follow-up (Kuo & Yang, 2008). γH2AX positivity is a method that allows the physical locations of DNA damage to be viewed by fluorescent methods (Nikitaki et al., 2020). H2AX is a member of the H2A family, it is phosphorylated at regions where double-stranded DNA breaks occurs and is named γH2AX. The presence of γH2AX in the environment means DNA damage and double-stranded break (Kuo & Yang, 2008; Rogakou et al., 1998; Stiff et al., 2004). In the current study, we hypothesized that while undergoing OZNE treatment, double-strand DNA breaks were induced in B-16 melanoma and OV-90 cell lines. Here we benefited by the γH2AX assay to investigate the phenomenon. The investigation also compares the cumulative cell damage

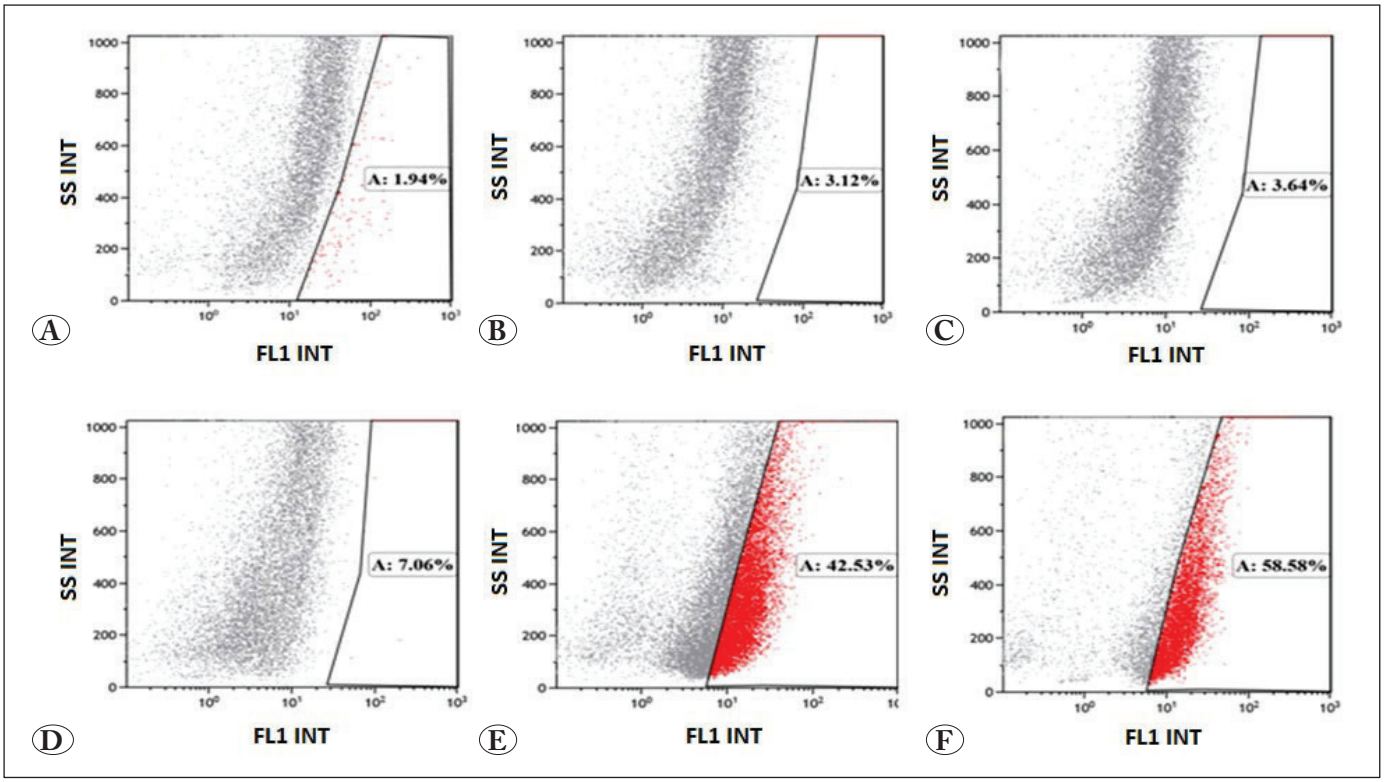

Figure 4. ROS Histograms of OV-90 ovarian cells treated with different concentrations of OZNE; **A)** Control **B)** OZNE5, **C)**OZNE10, **D)** OZNE20, **E)** OZNE50, **F)** OZNE100 (X-axis (FL1 INT) represents the intensity of fluorescence in the red spectrum and Y-axis (SS INT) represents the intensity of side scatter).

which is hypothesized to occur depending on OZNE concentration.

Figure 5 demonstrates H2AX positive B-16 cell population levels without and with OZNE nanoemulsions. According to the histograms, γH2AX positive cells were 0.52% in control group, the positive cells increased to a value of 12.55% and 17.06% in OZNE50 and OZNE100 groups, respectively (Figure 5). This is ~24- and ~33-fold increase suggesting that the γH2AX is expressed at a higher level with the recruitment of repair enzymes, and their active participation in the DNA damage repair pathway. When the cells exposure OZNE nanoemulsion, γH2AX positive cells gradually increased (Figure 5). This suggests that DNA damage was caused by exposure to high OZNE concentrations, which is associated with programmed cell death.

γH2AX positive OV-90 cell levels without and with OZNE nanoemulsions were represented in Figure 6. According to the histograms, a significant increase in γH2AX levels was determined in OZNE50 (64.74%) and OZNE100 (71.89%) groups compared to control (3.52%) (Figure 6). This leads to approximately 18- and 20-fold high levels, respectively. The results clearly demonstrate that the increase in OZNE concentration gradually increases γH2AX cell positivity in both B-16 melanoma and OV-90 cell lines.

3.3. Evaluation of Cell Lines Under ROS Production and γH2AX Positivity

Different cell types have comparable DNA-repair capacity. It is well-known that malign melanoma has high a resistivity (Miller & Mihm Jr, 2006; Trott, 1991) throughout the treatment and overcomes sublethal DNA injuries compared to the other tumor cells (Jones et al., 2001). Previously it was shown that B-16 melanoma and OV-90 ovarian cells exhibited different γH2AX positivity and ROS production due to their DNA repair capacity (Böcker & Iliakis, 2006).

In this study, the effects of OZNE nanoemulsion concentration in different cells were highlighted in terms of determining the increase of ROS production in cells. It is likely that OZNE treatment contributed to ROS production in both B-16 melanoma and OV-90 cells (Figure 7A). Moreover, it should be mentioned that high concentrations of OZNE nanoemulsions (OZNE50 and OZNE100) strongly supported ROS production influencing cell apoptosis.

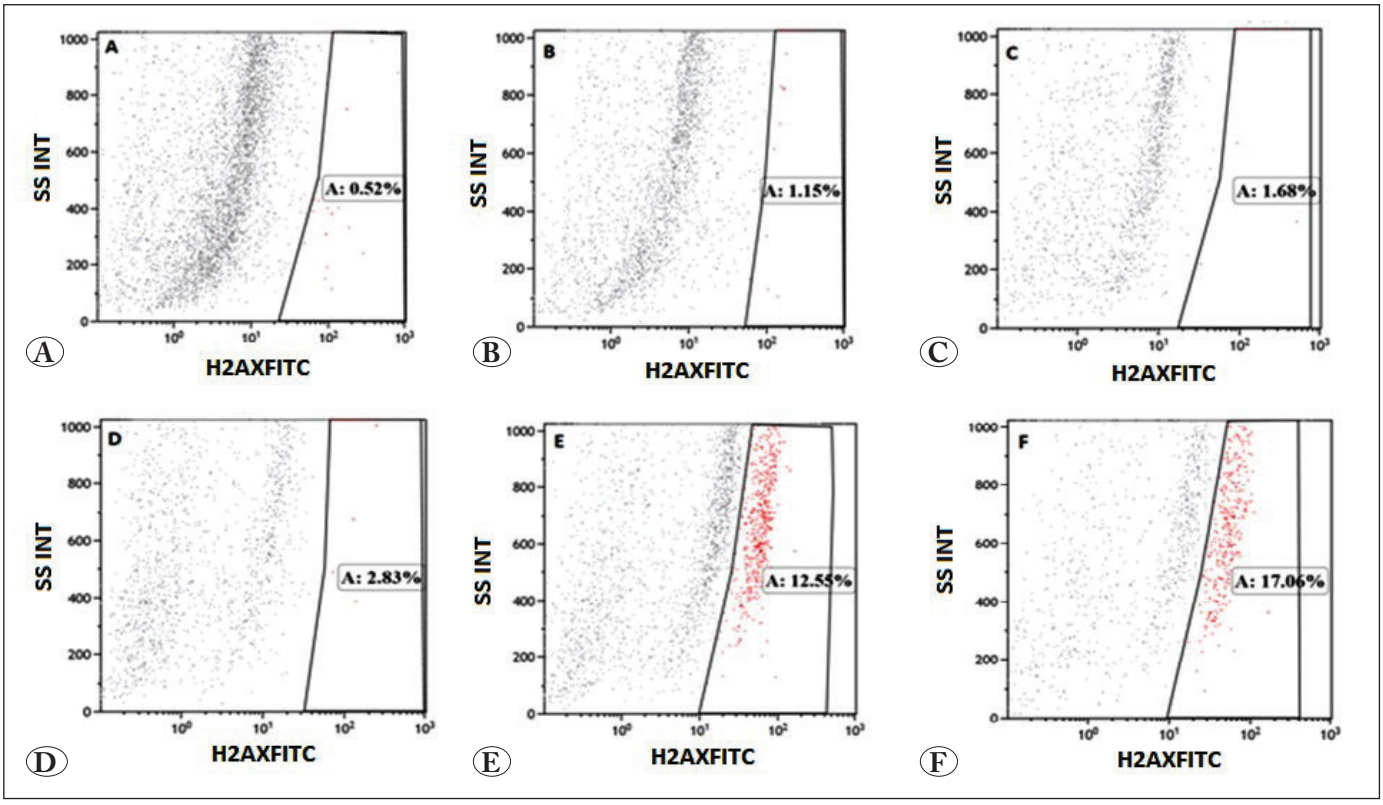

Figure 5. γH2AX Histograms of B-16 melanoma cells treated with different concentrations of OZNE; **A)** Control **B)** OZNE5, **C)** OZNE10, **D)** OZNE20, **E)** OZNE50, **F)** OZNE100.

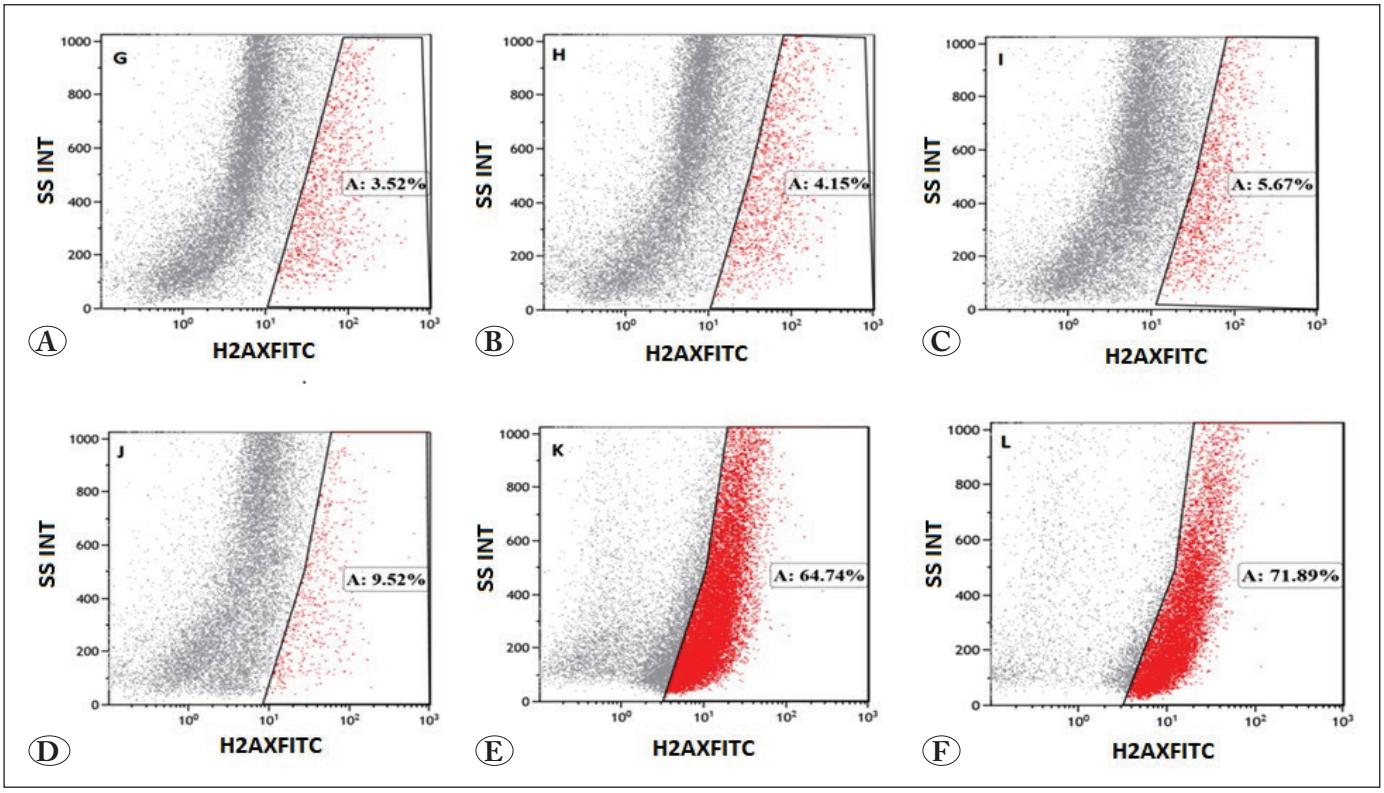

Figure 6. γH2AX Histograms of OV-90 ovarian cells treated with different concentrations of OZNE; **A)** Control **B)** OZNE5, **C)** OZNE10, **D)** OZNE20, **E)** OZNE50, **F)** OZNE100.

Approximately 6- and 4-fold higher ROS production was determined in OV-90 cells compared to B-16 melanoma cells treated with OZNE50 and OZNE100 groups, respectively (Figure 7A). Figure 7B shows approximately 5- and 4-fold higher γH2AX levels were determined in OV-90 cells compared to B-16 melanoma cells treated with OZNE50 and OZNE100 groups, respectively.

Oxygen and its mimics act through a mechanism of damage fixation utilizing electron affinity. Some chemicals developed from free radicals have shown promising prospects or have already been used clinically. Although great progress has been made in this direction, there are still some obstacles hindering clinical translation, such as adverse effects of hyperbaric oxygen, lack of structure types, lack of optional active groups with 'oxygen effect' and lack of tumor specificity (Churchill-Davidson, 1964; Overgaard, 2007). In this study, an emerging nanoplatform, which was developed to regulate the level of ROS such as H_2O_2 and ·OH, served carriers of ozone. ROS can mediate signal transduction, oxidize proteins, damage DNA structure, and induce apoptosis (Reczek & Chandel, 2015; Sfikas et al., 2012; Simon et al., 2000; Stadtman & Berlett, 1997). Since the regulation of ROS levels in tumors enables the accelerated damage of cancer cells (Tang et al., 2020), our results give a new method to damage cancer cells in the future.

However, it is noteworthy to mention that further study would be required to fully understand the impact of ROS levels and the mechanism of cellular damage in vivo, regarding the OZNE studied here. The latter aspect, the inevitable of clinical potential remains a major challenge.

In conclusion, we explored the effects of OZNE on B-16 melanoma and OV-90 ovarian cell lines in terms of ROS production and γH2AX cell positivity. Our results showed that ROS production and γH2AX cell positivity depend on OZNE concentration. Furthermore, both B-16 melanoma and OV-90 cell lines treated with OZNE50 and OZNE100 groups greatly increased ROS production and γH2AX cell positivity. Future studies of OZNE nanoemulsion may further support its use in cancer treatment due to increase of cellular damage.

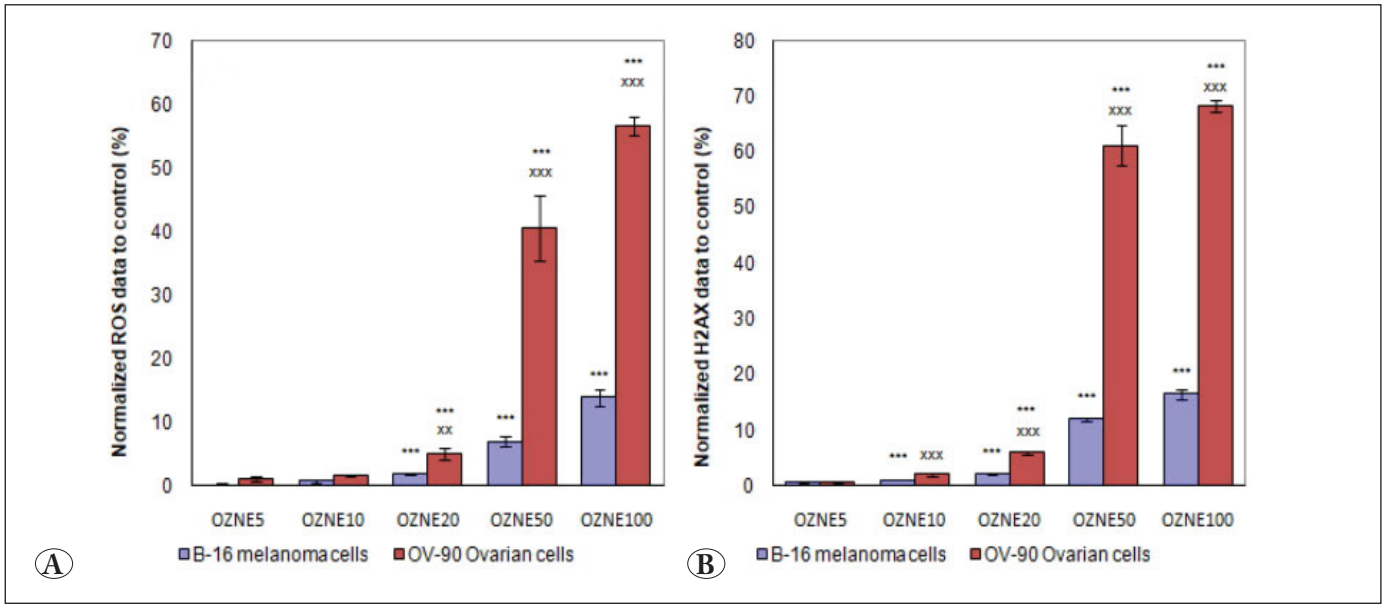

Figure 7. Normalized ROS production **(A)** and γH2AX **(B)** levels of B-16 melanoma and OV-90 ovarian cells treated with OZNE. Statistical differences between groups (n = 6, without OZNE is control group, ***p<0.001, *p<0.05; B-16 melanoma is control group, xxxp< 0.001).

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