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Research Article

Araştırma

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Apoptosis Induction Through Increased TRPV1 Activation by Synergic Effect of Melatonin and Doxorubicin in Human Osteosarcoma and Chondrosarcoma Cell Lines

İnsan Osteosarkoma ve Kondrosarkoma Hücre Hatlarında Melatonin ve Doksorubisinin Sinerjik Etkisi Yoluyla Artan TRPV1 Etkinliği Üzerinden Apopitoz Uyarımı

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ABSTRACT

Aim: We aimed to reveal the role of doxorubicin (Dox), melatonin (Mel) and transient receptor potential Vanilloid 1 (TRPV1) channels in bone and cartilage cancer cells during the treatment process. Human Bone Osteosarcoma (Saos-2/An1) and Human Chondrosarcoma (Hs 819.T) cell lines were used to prepare in-vitro experiment models.

Methods: Both cell lines were cultured at 37°C. We have separated each cell line into five groups as follows: Controls, Dox, Dox+Capsazepine (Cpz), Dox+Melatonin (Mel), and combined Dox+Mel+Cpz given group. Capsaicin and capsazepine were added to cell culture mediums to activate or inactivate the TRPV1 channels, respectively. Cytosolic calcium, apoptosis, intracellular reactive oxygen, mitochondrial depolarization, caspase-3 and caspase-9 levels were measured.

Results: Increased apoptotic activity was detected in doxorubicin given cell lines (Group II) when compared with the controls ($p<0.001$). There was also a significantly higher apoptotic level in Dox+Mel group (Group IV), when compared with only Dox given group ($p<0.001$). TRPV1 inhibition applied groups (Group III and V) have had lower apoptotic levels than other drug administered groups ($p<0.001$).

Conclusion: This study has indicated that apoptotic effects of Dox and Mel on both osteosarcoma and chondrosarcoma were strictly associated to TRPV1 channels, and that TRPV1 channels played an important role in whole mitochondria dependent pathways of apoptosis, which in turn may lead to increased intracellular Ca+2 levels and mitochondrial depolarization.

Key Words: Osteosarcoma, Chondrosarcoma, TRPV1, Doxorubicin, Melatonin

ÖZET

Amaç: Kemik ve kıkırdak kanseri hücrelerinde doksorubisin (Dox) ve melatonin (Mel) ile birlikte geçici reseptör potansiyeli olan Vanilloid 1 (TRPV1) kanallarının tedavi sürecindeki rolünü ortaya çıkarmayı amaçladık. İn-vitro deney modellerinin hazırlanmasında İnsan Kemik Osteosarkomu (Saos-2/An1) ve İnsan Kondrosarkomu (Hs 819.T) hücre hatları kullanıldı.

Gereç ve Yöntem: Her iki hücre hattı da 37°C'de kültürlendi. Her hücre hattını aşağıdaki gibi beş gruba ayırdık: Kontroller, Dox, Dox+Kapsazepin (Cpz), Dox+Mel ve kombine Dox+Mel+Cpz verilen grup. Kapsaisin ve kapsazepin, sırasıyla TRPV1 kanallarını etkinleştirmek veya inaktive etmek için hücre kültürü ortamlarına eklendi. Sitozolik kalsiyum, apoptoz, hücre içi reaktif oksijen, mitokondriyal depolarizasyon, kaspaz 3 ve kaspaz 9 seviyeleri ölçüldü.

Bulgular: Doksorubisin (Dox) verilen hücre hatlarında (Grup II) kontrollere göre artmış apoptotik aktivite saptandı ($p<0.001$). Ayrıca Dox+Mel grubunda (Grup IV), sadece Dox verilen grupla ($p<0.001$) karşılaştırıldığında anlamlı derecede daha yüksek bir apoptotik seviye vardı. TRPV1 inhibisyonu uygulanan gruplar (Grup III ve V) diğer ilaç uygulanan gruplara göre daha düşük apoptotik düzeylere sahiptir ($p<0.001$).

Sonuç: Bu çalışma, Dox ve Mel'in hem osteosarkom hem de kondrosarkom üzerindeki apoptotik etkilerinin TRPV1 kanalları ile kesin olarak ilişkili olduğunu ve TRPV1 kanallarının apoptozun tüm mitokondriye bağımlı yolaklarında önemli bir rol oynadığını ve bunun da hücre içi Ca+2 düzeylerinde artışa ve mitokondriyal depolarizasyona yol açabileceğini göstermiştir.

Anahtar Kelimeler: Osteosarkom, Kondrosarkom, TRPV1, Doksorubisin, Melatonin

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Introduction

Osteosarcoma and chondrosarcoma cases are the common malignant tumors of the musculoskeletal system [1-3]. Osteosarcoma originates from the bone cells and is generally detected in long bones. For this reason, bones of upper and lower extremities are frequently involved. Other bone or soft tissue involvements have also been reported [2,3]. Whereas, chondrosarcoma is a less commonly encountered condition rather than osteosarcoma in bones and surrounding soft tissues. Chondrosarcoma is more commonly seen in pelvic, femoral, acetabular or acromioclavicular regions in contrast to osteosarcoma. Besides, spinal involvement has also been reported [3]. Radiotherapy, surgery and chemotherapy are used in medical management of these tumors, although the chemotherapeutic approach is still controversial in chondrosarcoma cases [1].

Osteosarcoma is more sensitive to chemotherapy and radiotherapy, when compared to chondrosarcoma [2]. Data established from studies conducted in recent years suggest that favorable outcomes have been established from chemotherapy applications in various types of cancer, especially osteosarcoma and Ewing cancer [3]. However, chemotherapy resistance seen in chondrosarcoma cells restricts treatment strategies in this area. Doxorubicin is a very effective first-line treatment agent used in high-grade osteosarcoma cases. Although cisplatin is widely administered in combined treatments in osteosarcoma, doxorubicin has recently been preferred as a chemotherapy agent in chondrosarcoma cases which are resistant to cisplatin [4]. Melatonin is an antioxidant hormone which is released mainly from epiphysis gland as well as testes, retina, ovary, skin, and intestines. Melatonin plays a crucial role in regulation of various biological pathways such as reproduction and circadian rhythms in the human body. Protective effect of melatonin has been reported in healthy cells, and its pro-apoptotic effect is a well-known fact in cancer cells [5]. Transient receptor potential (TRP) channels are tetramers assembled from sub units with six membrane-spanning domains, are permeable to monovalent cations and calcium ions, and are comprised of mammalian large six subfamily of ion channel proteins including TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin) and TRPP (polycystin) and TRPV (vanilloid) [6]. There are various factors affecting TRP channels such as body temperature changes, blood pH level, injury healing, cations, cytokines and mechanical stress [7]. Moreover, reactive oxygen species (ROS), reactive nitrogen species and other electrophilic compounds are also important mediators for TRP channels [8]. It has been reported that TRPV1 channels play an active role in pathogenesis and treatment of diseases just like other TRP channels. Stimulation of TRPV1 channels induces apoptosis in cancer cell populations and prevents their development in various tissues such as osteosarcomas, gliomas, colon and pancreas [9]. However, we have not come across any research article about the relationship between chondrosarcoma and TRPV1 channels in the literature. In this study, we aimed to investigate the possible effect of combined doxorubicin and melatonin use in osteosarcoma and chondrosarcoma cell cultures, and the role of TRPV1 channels in mitochondria-dependent intracellular apoptotic pathway mechanism.

Materials And Methods

Cell Preparation and Culture

Human Chondrosarcoma (Hs 819.T) cell line was purchased from ATCC. Human Bone Osteosarcoma and (Saos-2/An1) cell lines were obtained from Ankara ŞAP (Culture Collection of Animal Cells, Foot and Mouth Disease) Institute, Ankara, Turkey. The protocols were designed according to the standard process for cell cultures. Cells were cultured with 1% penicillin / streptomycin and 10% fetal bovine serum containing Dulbecco's modified Eagle's medium (DMEM). The distribution of cells was as follows: 1×10^6 cells in each of 6-8 flasks (sterile filter cap, 5 ml, 25 cm^2). Saos-2 and Hs 819.T cells were incubated at 37°C at 5% CO_2 in a humidified incubator. Cell lines were then incubated with chemical compounds when 75–85% confluence level was reached. Flasks that did not grow during standard time were excluded from the study. Additionally, triple repetitions were made during the analysis, and in-group agreement was observed in established data.

Reagents and Stains

DMEM, Trypsin–EDTA, Fetal Bovine Serum (FBS) and penicillin-streptomycin (Pen-Strep) and Dihydrorhodamine-123 (DHR 123), Dimethyl sulfoxide (DMSo) were obtained from Sigma Aldrich (St. Louis, MO), and dye was purchased from a commercially available company (Fura-2 (AM) calcium fluorescent, Calbiochem, Darmstadt, Germany). Likewise, commercially available kits were utilized for mitochondrial stain (5,50, 6,60-tetrachloro-1,10,3,30-tetraethyl benzimi dazolyl carbocyanine iodide (JC-1), probenecid (Santa Cruz, Dallas, Texas, USA), Pluronic® F-127 (Biovision, San Francisco, USA)). Caspase substrates (Caspase 3 (AC-DE-VD-AMC) and Caspase 9 (AC-LEHD-AMC)) were purchased from Enzo (Lausen, Switzerland). Biocolor APOPercentage assay (Belfast, Northern Ireland) was used for obtaining the releasing buffer.

Study Groups

A total of five groups were adjusted for study design as follows:

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Group I (Control): A control group was designed with empty cell culture for observing normal development in routine conditions.

Group II (Dox): In Dox groups, 2 µM doxorubicin was applied to Saos-2 and Hs 819.T cells for 24 hrs [10].

Group III (Dox+Cpz): In Dox+Cpz groups, 2 µM doxorubicin was applied to Saos-2 and Hs 819.T cells for 24 hrs; and then TRPV1 channel antagonist Capsazepine (Cpz, 0.1 mM, 30 min) was applied.

Group IV (Dox+Mel): In Dox+Mel groups, 2 µM doxorubicin and 0.1 mM Melatonin was applied to Saos-2 and Hs 819.T cells for 24 hrs [10,11].

Group V (Dox+Mel+Cpz): In Dox+Mel+Cpz groups, 2 µM doxorubicin and 0.1 mM Melatonin was applied to Saos-2 and Hs 819.T cells for 24 hrs; and then TRPV1 channel antagonist Capsazepine (Cpz, 0.1 mM, 30 min) was utilized.

During calcium signaling analysis (Fura-2/AM), Saos-2 and Hs 819.T cells were stimulated on 20th cycles with 0.1 mM Cap (TRPV1 channel agonist) in the existence of 1.2 mM Calcium and calcium free buffer in extracellular environment. Cells were treated with Cap (0.1 mM, 10 min) for activation of TRPV1 channel prior to intracellular ROS, mitochondrial depolarization, caspase-3 and caspase-9 measurements and analyses.

Measurement of Intracellular Free Calcium Concentration

Free intracellular calcium ion level was determined using Fura-2 fluorescent stain. Saos-2 and Hs 819.T cells were treated with doxorubicin and melatonin as indicated in the group descriptions (no chemicals were applied to the control group) and were treated separately for two times in calcium and non-calcium solutions. Cellular response to agonists (Cap, 0.1 mM) which added with the automated injector during the analysis was detected by evaluating fluorescence emission intensity at 510 nm in individual wells using a plate reader equipped with an automated injection system (Synergy TM H1, Biotek, USA) at alternating excitation wavelengths of 340 and 380 nm every 3 s for 50 acquisition cycles (cycle:3 s; gain:120). $[Ca^{+2}]i$ in cells was expressed as the average emission at 510 nm in individual wells in response to excitation at 340 nm (Ca^{+2} bound) / 380 nm (Ca^{+2} Free Fura 2 AM) normalized to initial fluorescence emission obtained during the first twenty cycles. The $[Ca^{+2}]i$ evaluation and other process such as staining was applied according to the methods described by Martinez et al. [12,13].

Determination of Apoptosis Level

Apoptosis (programmed cell death) is a highly regulated mechanism involved in cell death, and it is essential for development and cellular homeostasis. It is clear that excessive increase in Ca^{2+} concentration by disrupting the intracellular Ca^{2+} balance may affect apoptosis. Apoptosis analysis was performed using the APOPercentageTM commercial assay kit. Apoptosis levels were carried out regarding the producer instruction [13,14]. APOPercentage dye binding to phosphatidylserine lipids has reflected red color in apoptotic cells. Detection of red stained apoptotic cells was achieved using spectrophotometry (multiplate reader) at 550 nm (SynergyTM H1, Biotek, USA).

Measurement of Reactive Oxygen Species (ROS) and Mitochondrial Depolarization Levels

Oxidative stress caused by reactive oxygen species (ROS) can induce activation of some TRP ion channels, induce rapid depolarization of inner mitochondrial membrane potential, and subsequent impairment of oxidative phosphorylation. Damaged mitochondria produce more ROS and the intracellular mitochondria-dependent apoptosis pathway becomes more effective. Saos-2 and Hs 819.T cell lines (10^6 cells/ml per group) were incubated with DHR123 fluorescent oxidant dye [13]. An automatic microplate reader (Synergy™ H1, Biotek, USA) was used for determination of the Rh123 fluorescence intensities. Emission wavelengths of the analysis was 543 nm and excitation wavelengths of the analysis was 488 nm. The data as fold change over the level before treatment was presented. Quantification of the mitochondrial membrane depolarization was performed by measuring the fluorescence intensity of the cationic dye of JC-1 a. Wavelength of 485 nm (green) for excitation, wavelength of 535 nm for emission, and the red signal emitted at 540 and 590 nm wavelengths were measured using SynergyTM H1, (Biotek, USA) [13,14]. Data were shown as fold change over the level before treatment as emission ratios (590/535).

Measurement of Caspase-3 and Caspase-9 Enzyme Activities

Caspase-3 and caspase-9 enzyme activity levels have increased following the increment in mitochondrial depolarization level. Caspase-9 mediates the intracellular apoptotic process, while caspase 3 is the ultimate apoptotic inducer. Caspase-3 and caspase-9 activities were evaluated by using reported literature [13]. Caspase-3 (AC DEVD-AMC) and caspase-9 (AC-LEHD-AMC) substrates cleavages were performed with automatic Synergy™ H1 microplate reader (Biotek, USA) with 360 nm and 460 nm wavelengths. The values were shown as fold change over the level before treatment (experimental / control).

Statistical Analysis

Whole statistical analyses were performed using GraphPad Prism v-7.04 for Windows (San Diego California, USA). Significant variances were evaluated using one-way ANOVA and parametric values were shown as means ± standard deviation (SD). P values less than 0.05 were taken as statistically significant.

Results

There was a significant difference between Dox and Dox+Mel groups in Human Bone Osteosarcoma (Saos-2/An1) and Human Chondrosarcoma cell cultures (Hs 819.T). Dox and Dox+Mel treatments increased the intracellular calcium levels via stimulation of TRPV1 channels in osteosarcoma and chondrosarcoma cells ($p<0.001$). In Dox+Mel treated osteosarcoma and chondrosarcoma cell lines, intracellular calcium levels of TRPV1 significantly increased when compared with Dox applied groups ($p<0.001$). Ca^{2+} levels prominently decreased after administration of TRPV1 channel blocker capsazepine (Cpz) in Dox+Cpz and Dox+Mel+Cpz groups, when compared with doxorubicin (Saos-2: $p<0.001$, Hs 819.T: $p<0.05$) and Dox+Mel groups (Saos-2 and Hs 819.T: $p<0.001$) (Figure 1). In evaluation of the programmed cell death and intracellular ROS levels, we have observed a remarkable increment in apoptosis ($p<0.001$). Also, Dox+Mel treatment significantly increased the intracellular ROS and apoptosis levels by activation of TRPV1 channels compared to the doxorubicin groups ($p<0.001$). However, programmed cell death (apoptosis) and intracellular ROS levels prominently decreased in Saos-2 and Hs 819.T with use of the TRPV1 channel blocker Cpz in Dox+Cpz and Dox+Mel+Cpz groups, when compared with only Dox ($p<0.001$) and Dox+Mel groups ($p<0.001$) for both analyses (Figures 2 and 3). In Dox and Dox+Mel administered groups, mitochondrial membrane potential, caspase-3 and caspase-9 levels have significantly increased via activation of TRPV1 channels, when compared to the control group ($p<0.001$). We have also found that Dox+Mel treatment increased the mitochondrial depolarization, caspase-3 and caspase-9 levels when compared to only Dox given group ($p<0.001$). However in Saos-2 and Hs 819.T cells, pro-

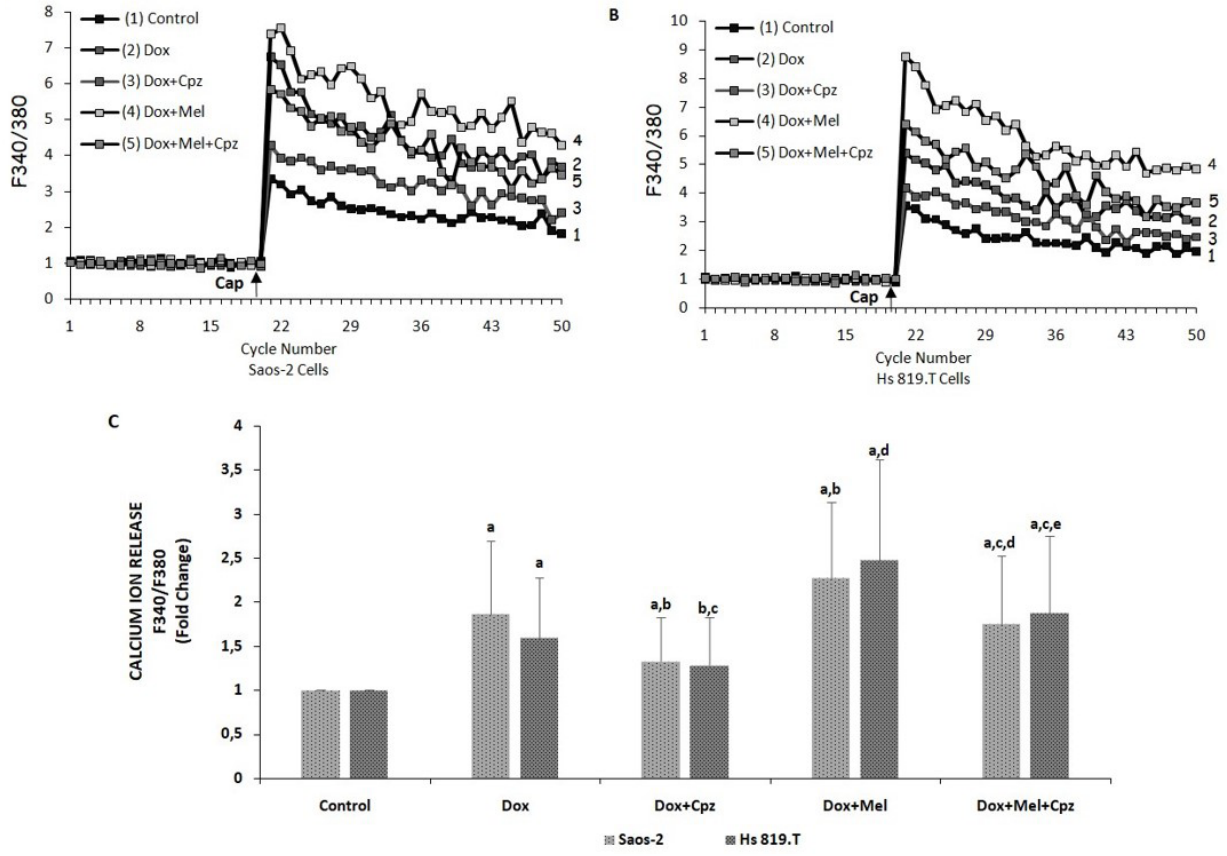

Figure 1. In vitro effect of doxorubicin (2 µM, 24 hrs) and Mel (0,1 mM, 24 hrs) on the free intracellular calcium increase ([Ca2+]i) through TRPV1 channels in Saos-2 (A) and Hs 819.T (B) cells and cellular calcium ion release (C). (n=3 and mean ± SD). The cells are stimulated by capsaicin (Cap and 0.1 mM on 20th cycle) but they were inhibited by capsazepine (Cpz and 0.1 mM for 30 min). Saos-2: ap<0.001 vs control, bp<0.001 and cp<0.05 vs Dox, dp<0.001 vs Dox+Mel. Hs 819.T: ap<0.001 and bp<0.05 vs control, cp<0.05 and dp<0.001 vs Dox, ep<0.001 vs Dox+Mel.

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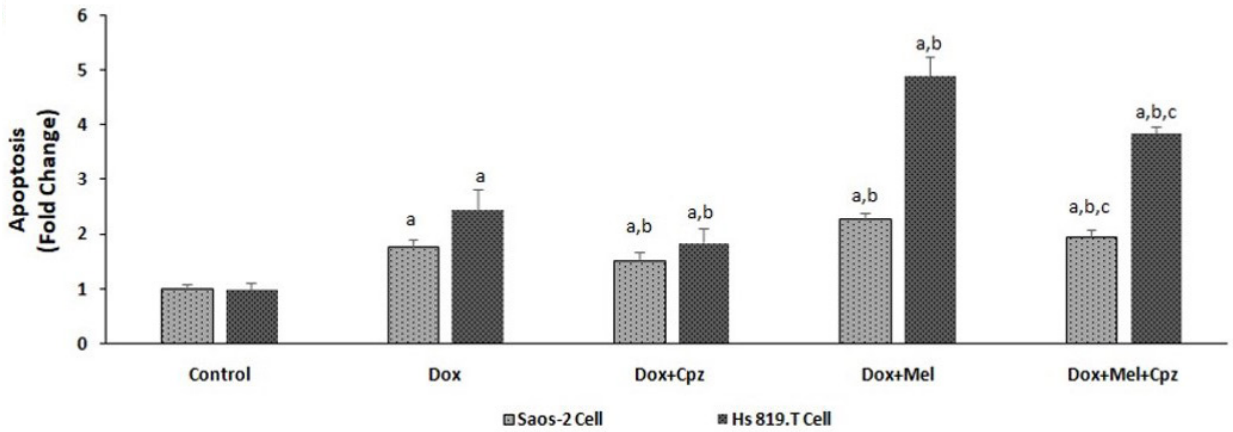

Figure 2. The effect of doxorubicin (2 µM, 24 hrs) and Mel (0,1 mM, 24 hrs) on apoptosis levels in the Saos-2 and Hs 819.T cells (mean ± SD and n=10). ap<0.001 vs control, bp<0.001 vs Dox, Cp<0.001 vs Dox+Mel.

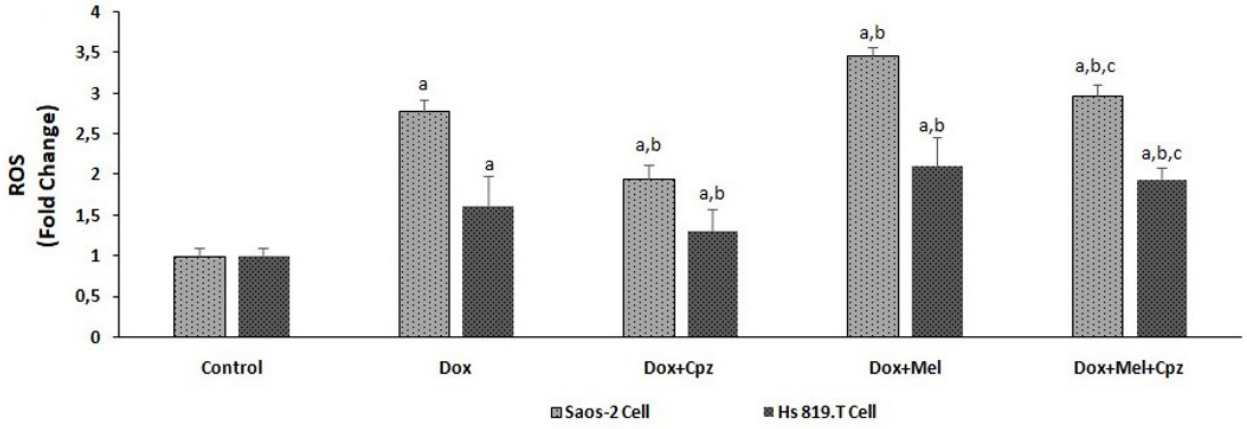

Figure 3. The effect of doxorubicin (2 µM, 24 hrs) and Mel (0,1 mM, 24 hrs) on Reactive Oxygene Species levels in Saos-2 and Hs 819.T cells (mean ± SD and n=10). ap<0.001 vs control, bp<0.001 vs Dox, Cp<0.001 vs Dox+Mel.

grammed depolarization, caspase-3 and caspase-9 levels prominently decreased with use of TRPV1 channel blocker capsazepine (Cpz) in Dox+Cpz and Dox+Mel+Cpz groups, when compared with only Dox ($p<0.05$ and $p<0.001$) and Dox+Mel given groups ($p<0.05$ and $p<0.001$, respectively) (Figures 4, 5 and 6).

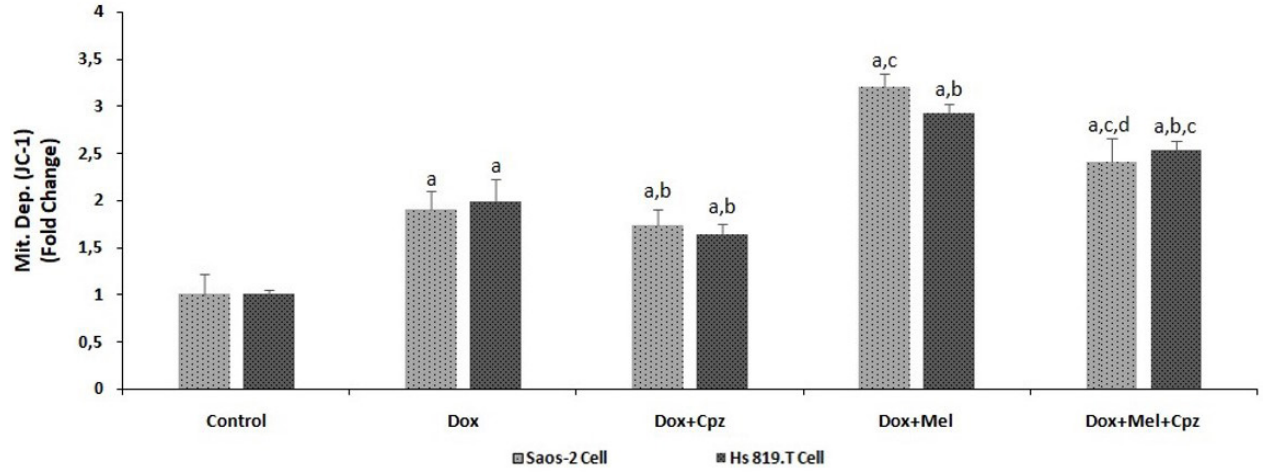

Figure 4. The effect of doxorubicin (2 µM, 24 hrs) and Mel (0,1 mM, 24 hrs) on Mitochondrial Depolarization levels in Saos-2 and Hs 819.T cells. (mean ± SD and n=10). Saos-2: ap<0.001 vs control, bp<0.05 and cp<0.001 vs Dox, dp<0.001 vs Dox+Mel. Hs 819.T: ap<0.001 vs control, bp<0.001 vs Dox, Cp<0.001 vs Dox+Mel.

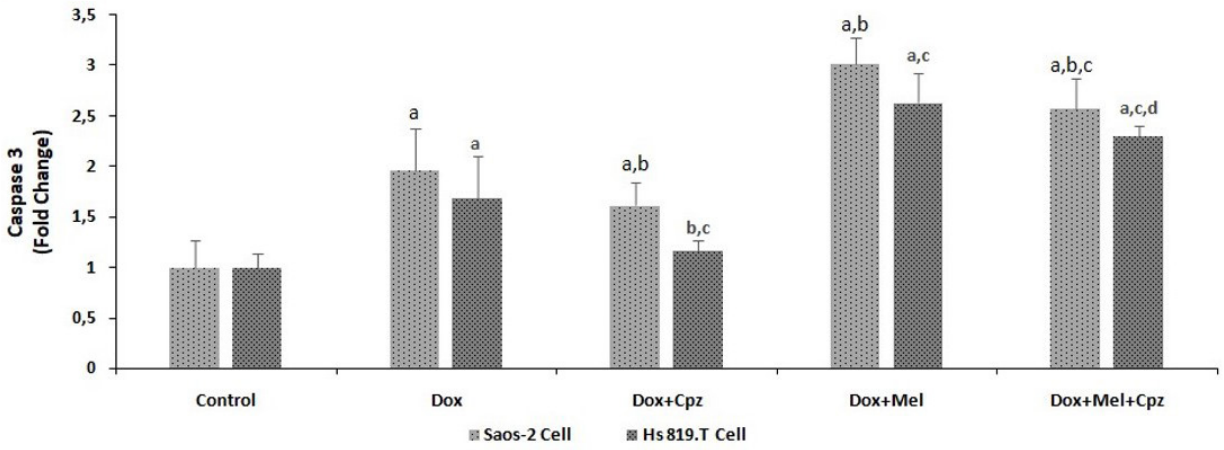

Figure 5. The effect of doxorubicin (2 μM, 24 hrs) and Mel (0,1 mM, 24 hrs) on Caspase 3 levels in Saos-2 and Hs 819.T cells. (mean ± SD and n=10). Saos-2: ap<0.001 vs control, bp<0.001 vs Dox, Cp<0.001 vs Dox+Mel. Hs 819.T: ap<0.001 and bp<0.05 vs control, cp<0.001 vs Dox, dp<0.001 vs Dox+Mel.

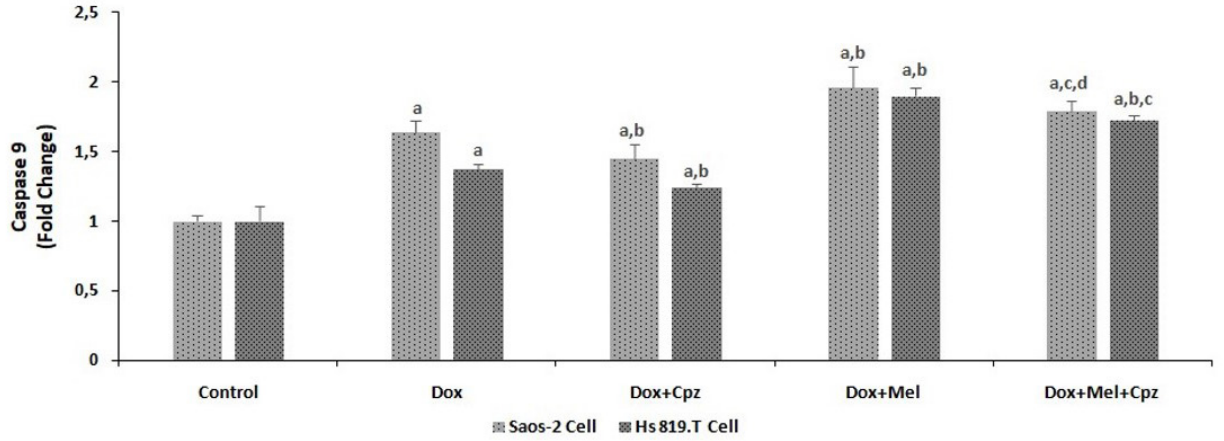

Figure 6. The effect of doxorubicin (2 μM, 24 hrs) and Mel (0,1 mM, 24 hrs) on Caspase 3 levels in Saos-2 and Hs 819.T cells. (mean ± SD and n=10). Saos-2: ap<0.001 vs control, bp<0.001 and cp<0.05 vs Dox, dp<0.001 vs Dox+Mel. Hs 819.T: ap<0.001 vs control, bp<0.001 vs Dox, Cp<0.001 vs Dox+Mel.

Discussion

Osteosarcoma and chondrosarcoma are rarely seen cancer types worldwide, and are among the leading causes of mortality. There are numerous chemotherapy protocols widely used during the preoperative and postoperative periods in osteosarcoma treatment [14]. Chemotherapy agents such as cisplatin and doxorubicin are frequently preferred in both types of cancer. Although no resistance to cisplatin is observed in osteosarcoma, it has been shown in the literature that chondrosarcoma is resistant to cisplatin [15]. TRP channels have six different subtypes in mammals; TRPA, TRPC, TRPM, TRPML, TRPP and TRPV. The selectivity of TRP channels has a wide spectrum, ranging from nonselective cation channels to highly selective Ca^{2+} channels [16]. Increased intracellular oxidative stress level due to the blocking or hyperactivity of intracellular mechanisms of chemotherapeutics used in cancer treatment may activate the channels which are sensitive to oxidative stress. These channels can play an important role in different processes such as intracellular Ca^{2+} metabolism, phagocytosis, cell motility, homeostasis and inflammation [17]. While there are many articles in the literature about the expression and roles of TRP channels in osteosarcoma cells, there is no article on the presence or function of these channels in chondrosarcoma so far. TRPV1 channels have been reported to have effect on invasion, proliferation, differentiation, and vascularization of cancer cells [18]. It has been indicated that TRP channels altered intracellular calcium concentrations and they had influence on regulation of Ca^{2+} release in numerous cell organelles. Intracellular Ca^{2+} concentration is variable and has been reported to increase in cases such as increased proliferation, apoptosis and abnormal differentiation, which are

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important markers of cancer invasion. doxorubicin is an agent which is used therapeutically in many types of cancer including osteosarcoma and chondrosarcoma. It kills cancer cells by blocking the DNA duplication and increasing ROS levels. Increased intracellular ROS levels trigger oxidative stress, which in turn result in irreversible changes in components such as intracellular lipids, proteins and nucleic acids [19,20]. Increase in intracellular Ca^{2+} levels leads to an increase in the amount of ROS, mitochondrial membrane depolarization, and increase in activation of caspase-3 and caspase-9 [18,20]. In our study, only Dox and Dox+Mel were administered to osteosarcoma (Saos-2) and chondrosarcoma (Hs 819.T) cell lines. Afterwards, the effect of these treatments on TRPV1 channels on intracellular mitochondria dependent cell death were investigated. A specific stimulator (Capsaicin) and inhibitor (Capsazepine) for TRPV1 channels were administered, and the intracellular Ca^{2+} level, intracellular ROS, mitochondrial depolarization, caspase-3 and caspase-9 values, as well as the degree of apoptosis were measured by examining the intermediate stages of apoptosis of osteosarcoma and chondrosarcoma cells. Established results were compared to the control group.

We have found that Dox and Dox+Mel treatments have resulted in TRPV1 channel stimulation, and that there was a significant increase in intracellular calcium ion levels, mitochondrial depolarization levels and intracellular ROS levels, which are mediated by TRPV1 channels in Dox and Dox+Mel groups. Oxidative stress-induced apoptosis levels were significantly increased compared to the control group, and melatonin triggered the mitochondria dependent cell apoptosis in both cancer cell lines. However, intracellular calcium, ROS, mitochondrial depolarization and caspase-3 / -9 levels were significantly decreased in Dox+Cpz and Dox+Mel+Cpz, when compared to Dox and Dox+Mel groups. Moreover, these effects of Dox and Dox+Mel on both cancer types achieved through TRPV1 channels and mitochondria dependent cell death processes were higher in Dox+Mel combination groups, when compared to Dox groups in both cancer cells. According to our literature review, there are very limited studies in the literature on the effect of chemotherapeutic agents on TRPV1 channels in osteosarcoma, but no study has conducted to examine the effects of chemotherapeutic agents on these channels in chondrosarcomas to date [18]. Melatonin is an antioxidant hormone released from the pineal gland and some other organs. In addition to its cardioprotective effect, melatonin is also used clinically as a pro-apoptotic agent in cancer treatment. It is well-known in the literature that melatonin is effectively used together with antineoplastic agents in treatment of bone, breast and colon cancer types. Melatonin is used together with chemotherapeutic agents with known efficacy such as cisplatin and doxorubicin [21]. In our study, both synergistic and comparative effects of melatonin and doxorubicin in osteosarcoma and chondrosarcoma were investigated. It has been observed that 0.1 mM of melatonin and 2 µM of doxorubicin combination increased apoptosis in cancer cells, when compared with the controls. Niu et al. have reported that melatonin increased the pro-apoptotic activity of doxorubicin in osteosarcoma cells by obtaining results similar to what we have established in our study [21]. Although there are limited studies conducted on the use of doxorubicin in chondrosarcoma, no reference has been found regarding the combined use of melatonin and doxorubicin. Kumari et al. have reported that the pro-apoptotic activities of doxorubicin increased in cancer cells depending on the dose at variable levels [21,22]. Intracellular cytosolic calcium ion level is fixed at 80-100 nanomolar concentrations through various ion channels. Excessive increase in intracellular calcium leads to increase in intracellular reactive oxygen species production. Besides, it also triggers mitochondrial depolarization and increases the level of caspase-3 and caspase-9. Increased caspase levels lead to increment in intracellular mitochondria-dependent apoptotic pathways and apoptosis [23]. Hsu et al have reported that five microgram/ml doxorubicin led to IC_{50} effect on human osteosarcoma cells, and it triggered apoptosis by increasing intracellular reactive oxygen species production, mitochondrial depolarization, caspase-9 and caspase-3 levels at given dose levels lower than five micrograms [24]. These results were similar to the 2 µM for 24 hours dose we used in our study for the same purpose. Administration of chemotherapeutic agents gives rise to increment in intracellular reactive oxygen species production and causes activation of TRPV1 channels which are sensitive to oxidative stress. Melatonin treatment given in combination with various chemotherapeutic agents may cause more activation in TRPV1 channels. However, we have not encountered any related study in the literature conducted to investigate the TRPV1 channel-mediated efficacy of doxorubicin alone or in combination with melatonin in osteosarcoma and chondrosarcoma cell lines. Koşar et al. have reported in a study in 2016 that combined doxorubicin and melatonin treatment administered to MCF7 breast cancer cells induced apoptosis by increasing intracellular calcium concentration, reactive oxygen species production, mitochondrial depolarization, caspase-3 and caspase-9 levels [25]. In our study, we have observed that intracellular calcium concentration, reactive oxygen species production, mitochondrial depolarization, caspase-3 and caspase-9 levels were similarly increased and apoptosis was induced after the combined use of doxorubicin and melatonin in osteosarcoma and chondrosarcoma cell lines.

Conclusion

As a result, we have concluded that apoptosis levels of osteosarcoma and chondrosarcoma cells increased more in both cell lines with the use of melatonin in addition to doxorubicin, and that the apoptotic effects of doxorubicin and melatonin were mediated by the indirect activation of TRPV1 channels. Future and more comprehensive studies are warranted to be conducted in the future to fulfill the aim to uncover the in vivo and in vitro effect of these and other agents which have efficacy on TRPV channels and calcium metabolism.

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ORCID and Author contribution: ***A.K. (0000-0002-2487-2431):*** Literature Search, Writing, Final Approval. ***İ.S.Ö. (0000-0002-0392-4386):*** Concept and/or Design, Interpretation, Writing, Critical Review, Final Approval. ***A.G. (0000-0002-9012-8053):*** Analysis, Literature Search, Writing, Final Approval.

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