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ORIGINAL ARTICLE

Medicine Science International Medical Journal

Medicine Science 2026;15(1):89-93

Beta estradiol induces hormone-responsive behavior by regulating Akt-Sox9 signaling pathway in Hepg2 Cells

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Received 31 July 2025; Accepted 12 September 2025
Available online 26 February 2026 with doi: 10.5455/medscience.2025.07.202

Abstract

The varying rates of Hepatocellular carcinoma (HCC) incidence and mortality rates between genders highlight a critical need to understand how β-Estradiol (βEst) affects HCC cell lines. In order to reveal this, the proliferative and migratory characteristics and the protein expressions of the Akt-Sox9 pathway were evaluated in βEst-treated HepG2 HCC cells in vitro. Cell viability and cell migration characteristics, IL-6 levels measurement by ELISA, and Akt and Sox9 protein expression level measurement by Western blotting were performed in βEst-treated HepG2 cells. Results: In βEst-treated cells, migration rate (%/h) and overall migration rate (μm/h) were lowered to 0.5 and 2.6, respectively, and IL-6 levels were reduced to about 40 percent of that of control cells. In addition, the relative protein expression level of Akt (0.88-fold) and Sox9 (0.64-fold) was found to be downregulated following βEst treatment. According to the results of our in vitro study, HepG2 cell behaves as potentially hormone-responsive cells, and the results also support the clinical study findings about gender disparity in HCC.

Keywords: Hepatocellular carcinoma, gender, β-Estradiol, Akt, Sox9

Introduction

Liver cancer ranks as the sixth most prevalent malignancy, constituting 4.6% of annual total cancer cases among individuals, yet it is the third deadliest cancer, with 7.8% in global cancer deaths, following lung and colorectal cancer. Hepatocellular carcinoma (HCC) comprises 75-85% of cases worldwide, with intrahepatic cholangiocarcinoma being the next most common type. The factors contributing to the development of HCC include Hepatitis B and C viruses, chronic infection, aflatoxin, alcohol, obesity, and type 2 diabetes [1]. Besides these factors, incidence differences have been observed according to the geographic location and gender. There are gender based differences in incidence and fatality rates of HCC, with a 1.2 to 3.6 times higher prevalence in men [2].

Significant gender differences in the clinical course and prognosis have been observed in various cancers, including liver and intrahepatic bile duct, urinary bladder, oral cavity, and pharynx [3]. For HCC, women experience less aggressive disease and a more favorable outcome compared to men, with a two to three times lower mortality rate [4]. Female patients with HCC were diagnosed with less advanced HCC, smaller tumor diameter, and had better overall survival than male patients [5].

Unlike androgens, which promote tumor development, estrogen has been shown to have protective and preventive properties in both epidemiologic and animal studies [6,7]. The gender-based variations imply that estrogen levels may influence the initiation and development of HCC, indicating that signaling pathways regulated by estrogen could be involved in the mechanisms underlying HCC [8]. Regardless of the underlying cause of the HCC, chronic inflammation increases the risk in the initial phase of HCC, and estrogen may exert its antitumor effect by counteracting the inflammatory process [9]. Interleukin-6 (IL-6) level, which is produced during the inflammation by liver-resident macrophages, is markedly elevated and is associated with both the development of HCC and disease prognosis [4]. Through estrogen receptor (ER)α, estrogen can suppress IL-6 by reducing MyD88-mediated NF-κB activation [9]. Upon binding of β-Estradiol (βEst) to ERα leads to the activation of

CITATION
Satilmis B, Beta estradiol induces hormone-responsive behavior by regulating Akt-Sox9 signaling pathway in Hepg2 Cells. Med Science. 2026;15(1):89-93.

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DOI: 10.5455/medscience.2025.07.202

phosphatidylinositol-3-kinase / Akt pathway [10]. Expression of Sex Determining Region Y-box transcription factor 9 (Sox9), which is highly expressed in liver cancer stem cells (CSCs), is regulated by activated Akt with two different mechanisms by increasing the expression and preventing proteasomal degradation [11,12].

Knowledge about the inequalities between genders in the incidence and mortality rates in HCC suggests a need to clarify the impact of the βEst treatment on the molecular level. Thus, the current study investigated how βEst affects HepG2 cells, specifically assessing cell proliferation and migration, and quantifying protein levels of the Akt-Sox9 pathway.

Material and Methods

Cell culture

HepG2 cell (ATCC, HB-8065) was cultured in Minimum Essential Medium Eagle (MEM) (M5650, Sigma) with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin-Neomycin and incubated under standard cell culture conditions. This study was carried out using a human cancer cell line, so there was no need for ethics committee approval.

Cell viability assay

For the determination of viability, HepG2 cells were resuspended in MEM. For each well, 10000 cells were seeded in a 96-well cell culture plate, incubated overnight, and treated with doxorubicin (Dox) (Onko İlaç, Turkey) (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 µg/mL) and beta-estradiol (βEst) (Sigma, E2758) (1.95, 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 µM) in MEM for 24 h. 10 µL of MTT solution (5 mg/mL) was added to well. Following 4 h incubation, the well content was aspirated, 100 µL of DMSO was added, and absorbance was detected at 570 nm by a microplate reader (Biotek, Synergy H1m) [13].

Determination of cell migration characteristics

HepG2 cells in a petri dish were cultured overnight, a scratch was made with a pipette tip, cells were treated with 30 µg/mL Dox or 500 µM βEst, and cell migration was recorded for 24 h by a Paula cell imager (Leica Microsystems). Migration and overall migration rates were obtained from the software [14].

Determination of IL-6 level

The IL-6 level was measured using a Human IL-6 ELISA Kit (BT Lab, E0090Hu) according to the kit protocol. 40 µL of the sample, 10 µL of biotinylated primary antibody, and 50 µL of streptavidin-horseradish peroxidase solution were added. Following incubation, the wells were aspirated and washed. 50 µL of Substrate A and B was pipetted into the well, the reaction was stopped, and absorbance was detected at 450 nm.

Western blotting

After treating cells with Dox or βEst for 24 h, the washed cells were lysed in ice-cold radioimmunoprecipitation assay buffer containing 1% protease and phosphatase inhibitors. A Protein Assay Kit (Thermo Fisher, 23225) was used to determine the amount of protein. Equal volumes of sample and loading buffer (Serva, 42526) were combined and denatured by heating at 95 °C for 5 min. Samples were run on a 4–20% Precast Gel and moved onto polyvinylidene fluoride (PVDF) membrane by using PVDF Transfer Kit (Bio-Rad, 1704272). The membranes were prepared by blocking with EveryBlot and probed with primary antibodies, Akt (Cell Signaling, 4691), Sox9 (Cell Signaling, 82630), and β-Actin (Cell Signaling, 4970). Then, horseradish peroxidase-linked (HRP)-linked secondary antibody (Cell Signaling, 7404) was applied. The ChemiDoc imaging system and Image Lab software were used for the visualization and quantification of the bands, respectively.

Statistical analysis

We expressed our findings as median ± interquartile range. Protein expression level was reported as fold changes. The comparison of the continuous variables was carried out using a pairwise Kruskal-Wallis test and $p<0.05$ indicated statistical significance. All statistical computations were conducted by SPSS software version 27 (IBM).

Result

The Determination of Viability in Dox and βEst-tretaed HepG2 Cells

An MTT assay was carried out to quantify the viability of HepG2 cells. For Dox, a concentration-dependent decline in the viability was observed. The cell viability at 6.25, 12.5, 25, 50, and 100 µg/mL was found to be 77.93%, 65.69%, 57.4%, 36.87%, and 22.41%, respectively (Figure 1). In contrast to Dox, there was no dose-dependent cell viability for βEst. Cell viability for most of the βEst concentrations between 3.9 and 250 µM was around 80%. A sharp decline to 44.57% in cell viability was seen at a concentration of 500 µM (Figure 2). Therefore, 30 µg/mL for Dox and 500 µM for βEst were chosen as application doses for the following experiments.

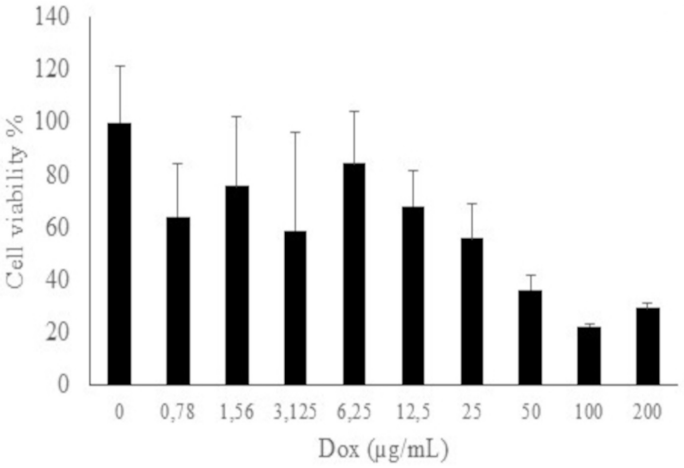

Figure 1. To determine the viability of Dox and βEst-treated HepG2 cells, an MTT assay was performed. Results were expressed as median±interquartile range

DOI: 10.5455/medscience.2025.07.202

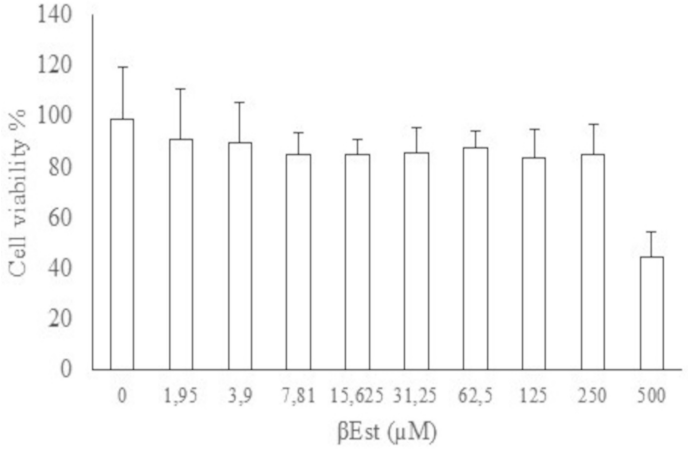

Figure 2. To determine the viability of Dox and βEst-treated HepG2 cells, an MTT assay was performed. Results were expressed as median±interquartile range

Migratory Characteristics of HepG2 cells treated with Dox and βEst

The results of the migratory characteristics of HepG2 cells treated with Dox and βEst for 24h were summarized in Figure 3. The migration rate (%/h) was 2.06, 0.2, and 0.5, and the overall migration rate (μm/h) was 6.5, 0.9, and 2.6 for control, Dox, and βEst, respectively. The migration rate and overall migration rate values in Dox-treated cells were significantly lower than those of control cells ($p<0.05$).

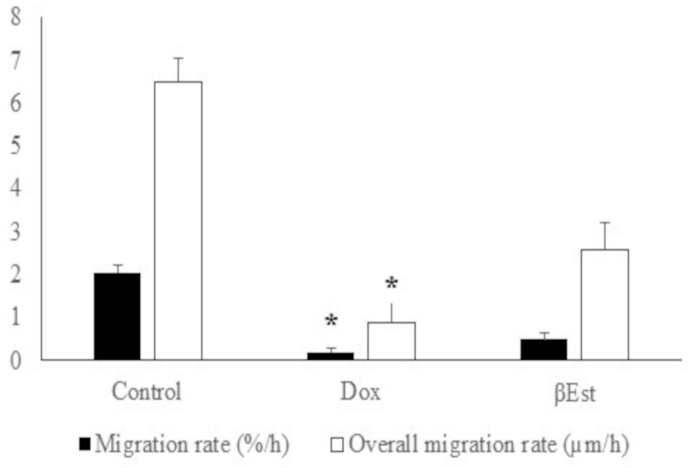

Figure 3. At the end of 24h treatment with Dox (30 μg/mL) or βEst (500 μM), the migratory characteristics of HepG2 cells. After an initial scratch, the movement of HepG2 cells was observed under a cell imager. The migration rate parameters of the cells were calculated by the software of the cell imager. *p<0.05 versus control

The effect of Dox and βEst treatment on IL-6 levels

The results of the effect of 24h Dox or βEst treatment on IL-6 levels in HepG2 cells were shown in Figure 4. IL-6 levels were found to be 433.44, 143.18, and 175.81 ng/L for control, Dox, and βEst, respectively. IL-6 levels in Dox-treated cells were significantly lower when compared to control cells ($p<0.05$).

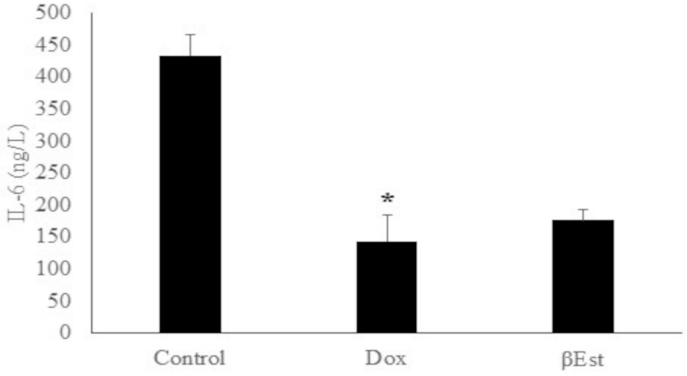

Figure 4. IL-6 levels in HepG2 cells treated with Dox (30 μg/mL) or βEst (500 μM) for 24 h were measured by ELISA. Measurements were repeated three times. *p<0.05 versus control

The effect of Dox and βEst treatment on Akt and Sox9 protein expressions in HepG2 cells

The effects of the 24-hour Dox and βEst treatment on Akt and Sox9 protein levels in HepG2 cells were shown in Figures 5 and 6. Akt protein level was downregulated in both Dox-treated (0.29-fold) and βEst-treated cells (0.88-fold) compared to control cells. Sox9 protein level was downregulated in βEst-treated cells (0.64-fold), while no protein expression was detected in Dox-treated cells.

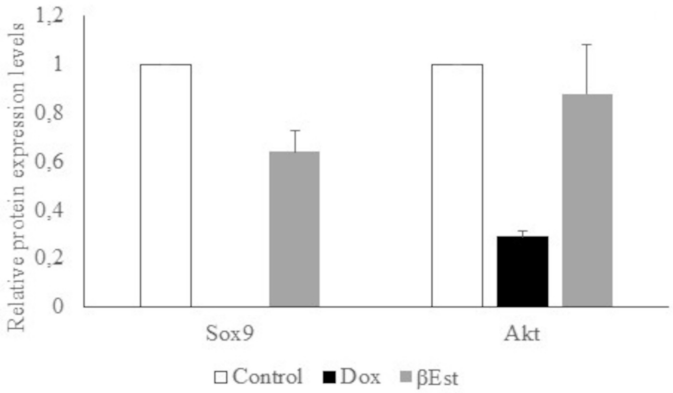

Figure 5. Protein expression of Akt and Sox9 in HepG2 cells following Dox (30 μg/mL) or βEst (500 μM) treatment for 24 h was determined by Western blotting with β-Actin as a reference protein. Experiments were performed in duplicate

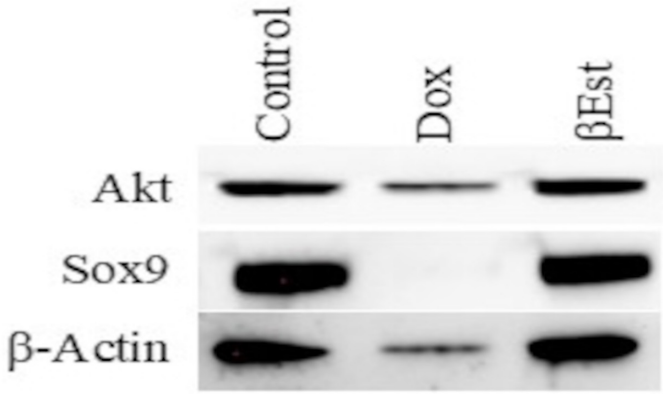

Figure 6. Protein expression of Akt and Sox9 in HepG2 cells following Dox (30 μg/mL) or βEst (500 μM) treatment for 24 h was determined by Western blotting with β-Actin as a reference protein. Experiments were performed in duplicate

DOI: 10.5455/medscience.2025.07.202

Discussion

This study examined the effects of βEst treatment on HepG2 HCC cells. Treatment with βEst resulted in a decrease in the viability and migration rates of HepG2 cells. Together with lower IL-6 levels, Akt and Sox9 protein levels were also found to be decreased in βEst-treated cells. In line with the results, we can suggest that βEst treatment reduced the proliferative and migratory characteristics of HepG2 cells by downregulating the Akt-Sox9 pathway.

As a sexually dimorphic organ, the liver has differences in gene expression, cellular function, and immune response in terms of gender. These differences lead to a disparity between genders and determine the response of the organ. In women, estrogens provide a protective effect against HCC by reducing inflammation and fibrosis [4]. According to the results of a study conducted with 1087 participants who underwent hepatectomy for HCC, males had significantly higher recurrence risk than females, and gender was found as an independent risk factor for early recurrence [15]. In a study carried out with 1716 HCC patients, at the time of diagnosis, female patients had smaller tumor size, lower portal vein tumor thrombosis, fewer distant metastases, and earlier Barcelona Clinic Liver Cancer stages compared to male patients [5].

The cell viability in βEst-treated HepG2 cells was not dose-dependent. It remained almost stable at around 80% up to 250 μM and decreased to half of the previous concentration at 500 μM. Barjesteh et al. showed that treatment of HepG2 cells with βEst resulted in a dose-dependent reduction in cell viability and an increase in the intensity of apoptosis. Following 24-hour treatment of HepG2 cells with 350 μM βEst, the percentage of total apoptosis, as a sum of the early and late apoptosis, was 46.5% [16].

IL-6 is an inflammatory cytokine with diverse functions that play a crucial role in the initiation and regulation of inflammation and proliferation. Thanks to the hepatocyte mitogen effect, IL-6 contributes to the development of neoplasms and liver regeneration. Activation of IL-6/signal transducers and activators of transcription 3 axis causes cells to gain anti-apoptotic, proliferative, migratory, and invasive characteristics [4,17]. There is no statistical difference in IL-6 levels in βEst-treated cells compared to control cells, despite a decrease in half of the control levels. However, the decrease in IL-6 levels explains the reduction in the viability and migration rate characteristics in βEst-treated cells. In addition, following the binding of estrogen to ERα, IL-6 levels decrease by suppressing NF-κB activation, and this mechanism also reveals the reduction in IL-6 levels in βEst-treated cells [9].

HCC is complex and has a heterogeneous nature with distinct biological characteristics and molecular profiles. In the development of HCC, CSCs, which are a small portion of tumor cells, have a key function with the properties of self-renewal and differentiation. Different CSC subpopulations exhibit unique functions and shape the behavior of the disease and response to the treatment options [18]. Akt signaling activation is recognized as a key factor in the initiation of HCC and serves as an indicator of gaining CSC character. Akt increases the Sox9 expression through the regulation of different mechanisms by increasing expression at the transcription level and by increasing protein stabilization via inhibition of glycogen synthase kinase 3β [11]. High mRNA levels of Sox9 are associated with worse disease-free survival (DFS) and are evaluated as an independent prognostic risk factor for DFS in an HCC cohort with different etiologies [19]. Dox treatment downregulated Akt expression strongly, to nearly 30% of the control, and completely suppressed the level of Sox9. The downregulation of Akt and especially Sox, almost half of the control level, implies that treatment of HepG2 cells with βEst suppresses CSC characteristics of HCC cells through the Akt-Sox9 pathway. Furthermore, a reduction in the protein level of Akt also contributes to lowering the cell migration characteristic of HepG2 cells.

The main limitations of the current study are the lack of expression level of ERα in the HepG2 cell line and the use of only one hepatocellular carcinoma cell line. The limitations highlight that in future studies, the expression level of the receptor and the precise mechanism of action should be explained through the use of specific receptor inhibitors and different cell lines.

Conclusion

The results of this study reveal that treatment of HepG2 cells with βEst downregulated of Akt-Sox9 pathway, induced more cell death, and suppressed cell migration characteristics compared to the control. When the results of our in vitro study are combined with the data obtained from clinical studies, it can be concluded that HepG2, isolated from a male youth with liver cancer, behaves as a potentially hormone-responsive HCC cell.

Conflict of Interests

The authors declare that there is no conflict of interest in the study.

Financial Disclosure

The authors declare that they have received no financial support for the study.

Ethical Approval

This study was carried out using a human cancer cell line, so there was no need for ethics committee approval.

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DOI: 10.5455/medscience.2025.07.202

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