Doxorubicin-Induced Apoptosis in Murine Cancer Model: A Gene Expression Profiling Study

Sara Ahmed El-Said[1*] and Iman E. El-Araby[2]
[1]Zoology Department, Faculty of Science, Zagazig University, Egypt and
[2]Animal Wealth Development department, Veterinary medicine, Zagazig University, Egypt
Corresponding authors: Sara Ahmed El-Said, Zoology Department, Faculty of Science, Zagazig University, Egypt
E-mail: Sara.a021@science.zu.edu.eg

Received: 1 November 2024, **Accepted:** 25 November 2024, **Published:** 30 November 2024

Abstract

Background: Objectives: Doxorubicin (Dox) used in cancer treatment in female mice as an experimental animal.

Methods: Mice were treated intraperitoneally with 0.026 mg/ml of doxorubicin chemotherapy once every 7 days ucedfor 30 days. Simultaneously, mice received distilled water by oral gavage or were intraperitonially injected with a single dose of Ehrlich Ascites Carcinoma and kept as negative and positive controls, respectively. Real-time PCR technique were used to evaluate the apoptosis in the liver of treated mice.

Conclusion: The present study dealt with the effect of Dox chemotherapy , findings of this study the therapeutic role of doxorubicin during chemotherapeutic treatment of cancer

Keywords: *Cancer, doxorubicin chemotherapy, PCR; apoptosis*

Background

Doxorubicin (Dox) is one of the chemotherapeutic drugs approved by the Food and Drug Administration as a kind of chemotherapy treatment as a kind of chemotherapy treatment in which the naphthacenequinone nucleus is linked to an amino sugar, daunosamine, by a glycosidic bond[1]; however, its clinical application is limited by dose-related organ damage [2]. It is used in clinical trials and commonly prescribed to treat numerous human malignancies, including breast cancer in postmenopausal women [3, 4,5]. Dox has a highly effective anti-tumor effect for various tumors; however, severe cytotoxic effects, drug resistance, and oxidative damage to the cellular lipid membrane and cellular components were accompanied by its treatment [5, 6], resulting in delaying the relief and outcome of the carcinoma [5]. Therefore, the inhibiting accumulation of Dox in target organs is a promising strategy to attenuate the toxicity of Dox [7].

Materials

Chemicals

Toxorubicin hydrochloride (5,12-Naphthacenedione, 10-[(3-amino-2,3,6-trideoxy-L-lyxohexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8 ,11-trihydroxy-8-(hydroxylacetyl)-1-methoxy-, hydrochloride (8S-cis) was obtained from Khandelwal Laboratories Pvt. Ltd Factory Thane, India (lyophilized Doxorubicin 50 mg for intravenous injection).

Treatment of Animals and Tissue Sampling

The handling of animals and experiments were conducted according to the guidelines set by the Research Ethics Committee at Zagazig University, Egypt (ZU-IACUC/1/F/111/2023). Female adult mice (*Mus musculus*) weighted 20–25 g (10 weeks old) was obtained from the Animal House of the Research Center, Cairo, Egypt, and housed in well-ventilated plastic cages, fed on a commercial diet, and given water ad

libitum. Animals were randomly divided into 3 groups (10 mice each) kept at a temperature of 25 ± 2 °C, 70% humidity, and 12:12 h light to dark, and allowed to acclimatize for one week before starting the study. Animals of the first group received distilled water and were kept as negative controls, while animals of the second group were intraperitoneally injected with a single dose of Ehrlich Ascites Carcinoma, EAC (10% in saline solution) and kept as positive controls [9]. EAC is one of the most prevalent malignancies and can induce undifferentiated carcinoma [9]. The third group was injected intraperitoneally with 0.026 mg/ml of Dox chemotherapy once every 7 days for 30 days, as described by Paget and Barnes [10],.

Trypan Blue Viability Test

Trypan blue is a stain usually used for detecting living cells, where cells without interactions with the dye are viable [11]. In brief, 0.5 ml of the cell suspension was stained with 0.4% Trypan blue stain, mixed thoroughly, allowed to stand at room temperature for 5 minutes, and then subjected to a light microscope to be counted using the hemocytometer. The non-viable cells stained were counted under the microscope during a period of not more than 30 minutes because it is possible to observe an increase in dead cells due to trypan's toxicity.

RNA extraction and qRT- PCR

Total RNA was extracted from livers using an HiSenScript^{TM} RH-ccDNA Synthesis Kit (iNtRON Biotechnology Co., South Korea) following the manufacturer's instructions. In brief, 20 µl reaction volume (10 µl master mix and 10 µl RNA sample containing 1µg RNA), 1µg of extracted RNA was reversely transcripted into cDNA using the High-Capacity cDNA Reverse Transcription Kit cDNA Kit according to the manufacturer's instructions (Applied Biosystems TM USA). The purity and concentration of RNA were performed at 260 nm and 260 nm/280 nm ratios using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, United States). Furthermore, complementary DNA (cDNA) was synthesized from 1 to 2 µg of extracted RNA using the HiSenScriptTM RH [-] RT PreMix Kit (iNtRON Biotechnology), and the reaction was carried out at 37 °C for 60 min. The synthesized cDNA was used as a template for PCR amplification. RT-PCR was performed using Topreal Cyber-Green qPCR Mix Plus (Enzynomics, Korea) in a Rotor-Gene Q PCR System (Qiagen, Germany). Following the manufacturer's instructions, the PCR assay was carried out under the following conditions: Initial denaturation at 95 °C for 12 min, followed by 40 cycles of denaturing at 95 °C for 20 sec, annealing at 60 °C for 30 sec, extending at 72 °C for 30 sec, and then cooling at 40 °C for 30 sec [12]. Further, Rotor-Gene Q series software (Qiagen) was used to analyze the CT values and melting curves of each sample. The PCR products were evaluated using 1% agarose gel electrophoresis. Gel images were analyzed using real-time PCR (SYBR Green qRT-PCR), and the relative level of gene expression in the liver was calculated by using the $2^{-\Delta\Delta CT}$ method to determine relative fold changes in target genes (BAX, BCL2, and caspase 3), and the gene expression level was normalized using GAPDH, which is the internal control. The amount of target gene expression levels was quantified [13] using the formula:
ΔΔCT= (CT target –CT GAPDH) treated- (CT target –T GAPDH) untreated.

The RT-qPCR primers were synthesized by Sangon Biotech (Beijing, China) and listed in Table 1.

Table1: Forward and reverse sequences for the internal control Gapdh and the apoptotic factors BAX, Bcl2, and Caspase 3 genes.

Gene	Forward primer	Reverse primer
BAX	5′ CTGGATCCAAGACCAGGGTG3′	5′ GTGAGGACTCCAGCCACAAA3′
Bcl2	5′ GAACTGGGGGAGGATTGTGG3′	5′ GCATGCTGGGGCCATATAGT3′
CASPASE3	5′ GGGGAGCTTGGAACGCTAAG3′	5′ GAGTCCACTGACTTGCTCCC3′
Gapdh	5′AAATGAGAGAGGCCCAGCTAC3`	5′ GAGGGCTGCAGTCCGTATTTA3′

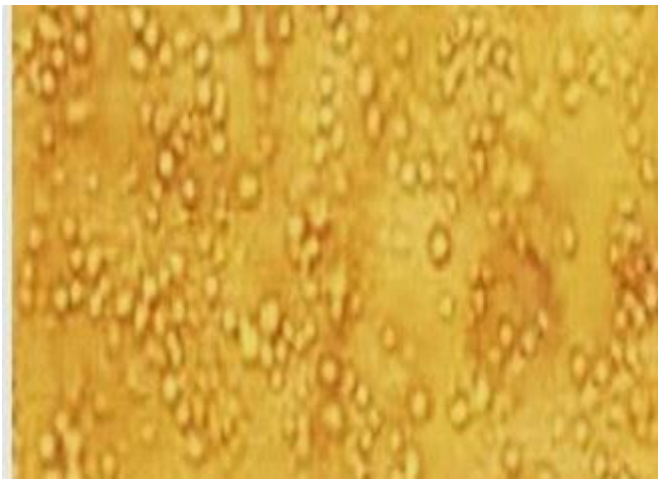
Statistical analysis

Using the statistical package of SPSS version 28.0 (SPSS Inc., Chicago, Illinois, USA), data were statistically analyzed using one-way analysis of variance (ANOVA) at a probability of 0.05. Analyses were carried out at least in 10 replicates. All values were calculated as the mean ± standard deviation (SD).

Results

Cell Viability

EAC showed a high count of 2.5 million obtained from the peritoneal cavity aspiration, crowded cells with rounded morphology in the isotonic suspension, and high viable cells detected by trypan blue stain (Figure 1).



(Figure 1). Trypan Blue Viability

Detection of the concentration and purity of extracted RNA

The isolated RNA was validated prior to cDNA synthesis, and the absorbency 260\280 ratio was calculated to determine the purity and quality of the RNA extract. The yield of RNA extract ranged in liver samples from 4.016 to 7.672 µg/ml, while the isolated RNA had an absorbency 260/280 value ranging between 1.8 and 2.0 indicating high purity.

Apoptotic genes of Caspase3, BAX, Bcl2, and BAX/Bcl-2 expressions detected by qRT-PCR

The apoptotic gene expressions from qRT-PCR for caspase3, BAX, Bcl2, and the BAX/Bcl-2 ratio are shown in Table **2.** The results showed significant up-regulation ($P<0.05$) in caspase3, BAX, and the BAX/Bcl-2 ratio expressions compared to the positive control, while Bcl2 values showed significant up-regulated ($P<0.05$) compared to the positive control.

Table (2) Comparison between studied groups as regard caspase3, BAX, Bcl2, and BAX/Bcl-2

Groups	Negative	Positive	DOX
caspase3	1	1±0.08 [b]	6.83±0.35 [a]
BAX	1	1.02±0.05 [b]	5.16±0.06 [a]
Bcl2	1	1.04±0.08 [a]	0.31±0.02 [b]
BAX/Bcl-2	1	0.99±0.09 [b]	16.73±0.8 [a]

Values in rows followed by the different letter are significantly

Discussion

The present results revealed ,The genetically expressed results Doxorubicin (Dox) is considered an elegant anti-cancer agent contains specific constituents act against the apoptosis pathway that arrest the cancer cell cycle up the normal mitotic division by changing in cancer cell's membrane blebbing. The apoptotic gene expressions found in the present results showed a significant difference among the groups. The caspase3 and Bax apoptotic genes were found to be more significantly induced in mice treated with Dox [12, 13]. On the contrary, the expression of the anti-apoptosis gene Bcl-2 was consequently reduced due to the significant influence of the combined effect of Doxorubicin (Dox) on the downregulation of Bcl-2, hence ,the Bax gene. Flow cytometric analyses of human breast adenocarcinoma cells with Doxorubicin (Dox) for 24 hrs demonstrated cell cycle arrest at 'S' phase; increased p53, Bax protein expression; caspase3 activation and decreased the mitochondrial membrane potential [14].

The activation of caspase expression throughout the change in the integrity of the mitochondrial membrane leads to enhancing the apoptosis mechanism in hepatic carcinoma [14]. Therefore, following treatment of mice, Doxorubicin can bring about cell cycle arrest and inhibit the proliferation of cancer cells [15]. Also, the anti-apoptotic Bcl-2 was found to decrease, while the ratio of Bax/Bcl-2 transcription increased, suggesting that carcinoma cell death following treatment with Doxorubicin was activated by p53-mediated signaling leading to apoptosis [15].

Conclusion

This work is the first research to deal with the Doxorubicin effect, which induce apoptosis and/or modulate apoptotic pathways and have cancer inhibitory properties [15]. In conclusion, the following study recommends using Doxorubicin, to reduce cancer cells.

Statements & Declarations

Conflict of interest Not applicable

Funding Not applicable

Authors contribution All of the authors contributed significantly to the document and agree to its publication. The contributions were performed in the following order: Conceived and designed the experiments: S.A.E., F.A., and I.E.E. Performed the research: S.A.E., F.A., and I.E.E. Analyzed the data:

S.A.E., F.A., .E.E., and S.A. Contributed drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis: S.A.E. The author(s) read and approved the final manuscript.

Acknowledgment Not applicable

Data Availability The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Ethical approval All animal housing, handling, breeding, and bioassays were conducted with the guidelines of The Research Ethics Committee at Zagazig University, Egypt (ZU-IACUC/1/F/111/2023). that could have appeared to influence the work reported in this paper.

Consent to publish Informed consent was obtained from all individual participants included in the study.

Clinical trials registration This study does not contain any studies with clinical trials performed by any of the authors.

References

1. Chen, M., Yi, Y., Chen, B., Zhang, H., Dong, M., Yuan, L., ... and Ma, Z., 2024. Metformin inhibits OCTN1-and OCTN2-mediated hepatic accumulation of doxorubicin and alleviates its hepatotoxicity in mice. Toxicol. 503, 153757.
2. Gilliam, L. A., Lark, D. S., Reese, L. R., Torres, M. J., Ryan, T. E., Lin, C. T., ... & Neufer, P. D., 2016. Targeted overexpression of mitochondrial catalase protects against cancer chemotherapy-induced skeletal muscle dysfunction. *American Journal of Physiology-Endocrinology and Metabolism*, *311*(2), E293-E301.
3. Chabner, B.A., Ryan, D.P., Paz-Ares, L., Garcia-Carbonero, R., Calabresi, P., Hardman, J.G., Limbird, L.E., Gilman, A.G., 2001. Goodman and Gilman's The Pharmacologic Basis of Therapeutics. New York: McGraw-Hill, Medical Publishing Division, p. 1389–1459.
4. Dunleavy, K., Fanale, M.A., Abramson, J.S., Noy, A., Caimi, P.F., Pittaluga, S., Parekh, S., Lacasce, A., Hayslip, J.W., Jagadeesh, D., Nagpal, S., Lechowicz, M.J., Gaur, R., Lucas, A., Melani, C., Roschewski, M., Steinberg, S.M., Jaffe, E.S., Kahl, B., Friedberg, J.W., Little, R.F., Bartlett, N.L., Wilson, W.H., 2018. Dose-adjusted EPOCH-R (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and rituximab) in untreated aggressive diffuse large B-cell lymphoma with MYC rearrangement: a prospective, multicentre, single-arm phase 2 study. Lancet Haematol. 5, e609–e617.
5. Miyahara, K., Nouso, K., and Yamamoto, K., 2014. Chemotherapy for advanced hepatocellular carcinoma in the sorafenib age. World Journal of Gastroenterology.20(15), 4151.
6. Butowska, K., Woziwodzka, A., Borowik, A., and Piosik, J., 2021. Polymeric nanocarriers: a transformation in doxorubicin therapies. Materials, 14(9), 2135.
7. Yi, Y.D., Zhang, H.B., Chen, M.Y., Chen, B.X., Chen, Y.C., Li, P., Zhou, H., Ma, Z.Y., and Jiang, H.D., 2023. Inhibition of multiple uptake transporters in cardiomyocytes/ mitochondria alleviates doxorubicin-induced cardiotoxicity. Chem. -Biol. Inter. 382.
8. Khan, F., Ahmed, F., Pushparaj, P. N., Abuzenadah, A., Kumosani, T., Barbour, E., AlQahtani, M., and Gauthaman, K., 2016. Ajwa date (*Phoenix dactylifera* L.) extract inhibits human breast adenocarcinoma (mcf7) cells *in vitro* by inducing apoptosis and cell cycle arrest. PIoS one. 11(7), e0158963. https://doi.org/10.1371/journal.pone.0158963
9. Saleh, N., Allam, T., Abdelfattah, A., and El-Borai, N., 2022. Review on Ehrlich Ascites Carcinoma in mice and cancer treatment with special reference to the potential protective and therapeutic effects of hesperidin versus cisplatin. J. Curr Vet Res. 4(1), 47-57.
10. Paget, G. E., & Barnes, J. M., 1964. Toxicity tests. Evaluation of drug activities: pharmacometrics, 1, 135-65.
11. Freshney, R. (1987). Culture of Animal Cells: A Manual of Basic Technique, Alan R. Liss, Inc., New York ,117.
12. Ramadan, M. A., Shawkey, A. E., Rabeh, M. A., an Abdellatif, A. O., 2019. Expression of P53, BAX, and BCL-2 in human malignant melanoma and squamous cell carcinoma cells after tea tree oil treatment *in vitro*. Cytotechnol. 71, 461-473.
13. Livak, K. J., & Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, *25*(4), 402-408.
14. Chou, C.C., Yang, JS, Lu, H.F., Ip, S.W., Lo, C., Wu, C.C., et al. 2010. Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. Arch Pharm Res. 33(8),1181–91. doi: 10.1007/s12272-010-0808-y PMID: 20803121.
15. Nihal, M., Wu, J., Wood, G.S., 2014. Methotrexate inhibits the viability of human melanoma cell lines and enhances Fas/Fas-ligand expression, apoptosis and response to interferon-alpha: Rationale for its use in combination therapy. Arch. Biochem. Biophys. 563:101–107. doi: 10.1016/j.abb.2014.04.019.PMID: 24862567.