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Original Article

Colchicine Enhances the Apoptotic, Anti-tumor Efficacy, Survival of Doxorubicin and Lowers Associated Toxicity in an Ehrlich Ascites Cancer Mouse Model

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ABSTRACT

Doxorubicin (DOX) is an effective chemotherapeutic drug but induces serious adverse effects. The anti-inflammatory drug colchicine (COL) was found to inhibit inflammasome activity involved in DOX side effects. The purpose of this research is to investigate COL impact when being added to DOX if decreases side effects or enhances its anti-tumor efficacy. To this end, we used Ehrlich ascitic carcinoma (EAC)-bearing CD1 female mice and treated them with high and low doses of DOX (DOX^{high} and DOX^{low}) in the presence or absence of COL. Mice were inoculated intraperitoneally with 0.25×10^6 EAC-cells/mouse and then treated with DOX^{high} (2 mg/kg), DOX^{low} (1 mg/kg), COL (5 µmol/kg), DOX^{high}/COL and DOX^{low}/COL. On day 8 of tumor injection, 50% of the mice were sacrificed to evaluate tumor volume, total tumor cell count, EAC cell apoptosis, cell cycle, hematological, and biochemical parameters, including liver and kidney function tests, oxidative stress (OS) markers, C-reactive protein (CRP), and interleukin 1-beta (IL-1β). The remaining 50% of mice were left to determine the survival of the groups. Co-treatment of COL with DOX^{high} or DOX^{low} enhanced the overall antitumor effect of DOX as evidenced by an enhancement in the tumor parameters, an increase in EAC cell apoptosis, and induction of cell cycle arrest. Additionally, their co-treatment ameliorated DOX adverse effects as evidenced by an improvement in the measured markers. **Conclusion**: Combination of COL with DOX^{high} or DOX^{low} enhanced the antitumor effect and decreased the adverse effects. This study opens a new avenue to their use in the clinical setting.

Keywords: *Colchicine; Doxorubicin; Ehrlich Ascites Carcinoma (EAC); Antitumor; Apoptosis; Cell cycle.*

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1. Introduction

Doxorubicin (DOX) (PubChem CID: 31703) https://pubchem.ncbi.nlm.nih.gov/compound/31703 is an anthracycline antibiotic extracted from the bacterium *Streptomyces peucetius* **[1]**. It is usually prescribed for the management of various types of tumors **[2]**. DOX performs its anticancer activity by several pathways including DNA intercalation and topoisomerase II inhibition, histone expelling, production of reactive oxygen

species (ROS), overproduction of ceramide, and modulation of calcium and iron homeostasis **[3]**. Although effective as an anti-cancer therapy, DOX has many side effects as bone marrow suppression, alopecia, bladder problems, and liver and kidney toxicities **[4]**. Being an essential organ for the metabolism and detoxification of several drugs, both the liver and kidney are the primary body organs that are mostly affected by chemotherapy in cancer patients **[5]**. Hepatic cells are among the various tissues of the body in which DOX is accumulated **[6]**. Moreover, DOX increases proteinuria, plasma creatinine, and glomerular capillary permeability in the kidney **[7]**. Furthermore, DOX alters the cell apoptotic signaling via increasing the pro-apoptotic protein Bax expression and decreasing the anti-apoptotic protein Bcl2 expression in cancer as well as in healthy cells **[7]**. Finally, and most importantly, tumor relapse may occur due to resistance to DOX **[8]**. One of the main mediators of DOX resistance and DOX role during cardiotoxicity is the nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome pathways that function both in cancer and cardiac cells **[9]**. The NLRP3 inflammasome is a cytoplasmatic multiprotein complex belonging to the innate immune system, consists of receptors, adaptor protein (apoptosis-associated specks-like protein containing a CARD (ASC)), and caspase-1 that triggers pro-IL-1β and pro-IL-18 conversion into their activated cleaved forms **[9, 10]**. Beyond IL-1β and IL-18 production, NLRP3 stimulation results in the formation of the gasdermin D (GSDMD) pore and finally pyroptosis **[10]**. In general, NLRP3 inflammasome has a pivotal role in a broad spectrum of diseases, including cardiac diseases **[11]** and cancer **[9]**. In cancer, NLRP3 and IL-1β drive cancer progression involving tumorigenesis promotion, angiogenesis, immunosuppression, and metastasis **[12, 13]**. Colchicine (COL) (PubChem CID: 6167) https://pubchem.ncbi.nlm.nih.gov/compound/616 7 originally isolated from *Colchicum autumnale,* is a tricyclic alkaloid drug **[14]** clinically used for inflammatory diseases treatment as the gouty arthritis and familial Mediterranean fever (FMF) **[15]**. COL binds to an unpolymerized tubulin **[16]**, resulting in the inhibition of microtubule polymerization **[17]**. This explains the potent antitumor activities of COL and its derivatives **[18]**. COL antitumor activity could, also, be mediated by its modulatory effect on immune cells, leading to the development of effective cancer immunotherapies **[19]**. Moreover, COL inhibits tumor necrosis factor-alpha activity, leukotriene B4, prostaglandin E2, thromboxane A2, and cyclooxygenase-2 **[15]**. Interestingly, COL also inhibits NLRP3 inflammasome **[15, 20]** by inhibiting caspase-1 and transport of ASC as well as the expression of the pyrin gene responsible for NLRP3 receptor expression, IL-1β, and P2X7, resulting in an increased intracellular potassium **[15]**. Interestingly, COL treatment has been shown to ameliorate cardiotoxicity induced by chemotherapeutic drugs such as 5-fluorouracil in rats **[21]**. Therefore, COL might ameliorate toxicities in other body organs. Given these unique biological effects of COL, it could be used as a potential anti-cancer drug with potential antitoxic effects.

Study aim: to investigate whether or not COL can enhance the antitumor activity of DOX and lower DOX used dose as well as its associated toxicities. To test this hypothesis, we used the Ehrlich Ascites Carcinoma mouse model; a widely used model for establishing tumors in mice **[22]**.

2. Materials and Methods

2.1. Drugs/Chemicals/Kits

DOX was obtained as Adricin from Hikma Pharmaceuticals Company (Cairo, Egypt) and COL from El-Nasr Company (Cairo, Egypt). Phosphate buffered saline (PBS) and trypan blue

(PubChem CID: 135903069) https://pubchem.ncbi.nlm.nih.gov/compound/135903069 were purchased from Sigma-Aldrich Co (St Louis, MO, USA). Other substances or reagents/solvents were of the highest quality. Annexin V-FITC and Propodium iodide (PI) were obtained from Becton Dickinson BD Pharmingen™ (Heidelberg, Germany). CycleTEST™ PLUS DNA Reagent Kit was purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Serum glutamate-pyruvate transaminase (SGPT) and aspartate transaminase (AST) (Cat. No. AT103445), serum urea (Cat. No. UR2110), serum creatinine (Cat. No. CR1250), serum total cholesterol (TC) (Cat. No. CH1220), triacylglycerol (TAG) (Cat. No. TR2030), low-density lipoprotein-cholesterol (LDL-C) (Cat. No. CH1231), high-density lipoprotein-cholesterol (HDL-C) (Cat. No. CH1230), OS markers; superoxide dismutase (SOD) (Cat. No. SD2521), catalase (CAT) (Cat. No. CA2517) and malondialdehyde (MDA) levels (Cat. No. MD2529), and CRP (Cat. No. E0053Ra). ELISA kits were all determined by using Bio-Diagnostics kits purchased from Bio-Diagnostics, Cairo, Egypt. Mouse IL-1β ELISA kit (Cat. No. CSB-E08054m) was purchased from Eagle Biosciences, Inc., Nashua, USA.

2.2. Animals

The research committee of ethics (REC) agreed on the protocol (ENREC-ASU-2020-7). The research was conducted with minimal harm to animals following the ARRIVE guidelines for animal handling and welfare. **CD-1® IGS Mice:** female white Swiss albino mice bred using the Charles River (Crl) International Genetic Standardization (IGS) system. **Crl: CD1(ICR):** Charles River CD1 Institute for Cancer Research (ICR). 2 months, 25±3 g BW female mice purchased from the National Institute of Cancer (NCI, Cairo, Egypt) initially imported from the USA. Mice were housed in the facility of animals at Pharmacy Faculty, University of Ain Shams for a week for adaptation, with accommodation, and relative humidity was kept at 22±1.0 °C and 55±5.0%, correspondingly, as well as light-dark cycles. Tap water and standard pelleted animal food were provided to mice ad libitum.

2.3. Experimental design was done on the top of a Pilot Study

A pilot experiment was conducted to study the additive and the mechanistic impact(s) of giving COL with DOX in an experimental tumor mouse model (EAC), to decide whether it is better to administer DOX and COL simultaneously or not. Blood was collected from mice to measure CRP and CBC.

2.4. Experimental Design

After 1 week of preparation, in the animal house at the Faculty of Pharmacy, Ain Shams University, 140 mice were, randomly divided, using a random number generator https://www.graphpad.com/quickcalcs/randomize1/, into 7 groups (N = 20/group) **[23, 24]**, (to minimize the standard errors between groups) as shown:

Group 1: mice were treated with 200 µL of sterile PBS i.p. injected on days 3, 5, and 7 (control),

From Group 2 to Group 7, mice were inoculated with 0.25×10^6 of EAC-cells/mouse i.p. on Day 0 as follows: [N.B. for comparison, tumor growth in PBS-treated control animals was taken to be 100%].

2.5. EAC-cells inoculation

2 stock mice injected with EACs, provided by the National Institute of Cancer (NCI, Cairo University, Egypt), after 7 days post-EAC's challenge were used as a source for EAC cells. EAC cells were obtained from the mice and the count was adjusted to 0.25×10^6 cells/mouse for intraperitoneal (i.p.) injection;

Group 2: mice didn't receive any treatments (EAC),

Group 3: mice were treated with DOX^{high} (2 mg/kg BW) i.p. **[25]** (EAC/DOX^{high}),

Group 4: EAC-bearing mice received COL (5 µmol/kg BW) orally **[20]** (EAC/COL),

Group 5: EAC-harboring mice were injected intraperitoneally with DOX^{high} and given COL orally (EAC/DOX^{high}/COL),

Group 6: EAC-harboring mice were treated with DOX^{low} (1 mg/kg BW) i.p. (EAC/ DOX^{low}),

Group 7: EAC-harboring mice were injected intraperitoneally with DOX^{low} and given COL orally (EAC/DOX^{low}/COL),

All treatments were given on days 3, 5, and 7 of the EAC tumor inoculation **[25]**.

On the eighth day of EAC tumor inoculation, first, 50% of all groups were anesthetized to obtain samples of blood for hematological and biochemical analysis. Blood was obtained from mice for sera separation as previously described **[26]**, then sera were kept aliquoted at -80 °C. Second, the sacrifice of mice was performed by cervical dislocation to harvest the ascetic tumor fluid to determine the total ascite volume and the total live and dead tumor cell count. Early, late apoptotic, and necrotic percentages (%) of EAC-cells and EAC-cell cycle analysis were determined via flow cytometry (FC). Third, the liver was extracted and homogenized in PBS to measure the oxidative stress markers. The remaining 50% of mice in each group were left alive, from day 8 to day 24 for mice survival rate determination (endpoint). No humane endpoints were established. The study included all the animals with no exclusions.

2.5.1. Determination of the % of body weight changes

The percentage of body weight changes **(**% BW) was calculated as previously described **[27]**.

2.5.2. Determination of the total tumor volume and count

Tumor volume and count were determined as previously described **[27]**.

2.5.3. Estimation of Animal Survival

The effect of different treatment protocols on survival was monitored until day 24 (endpoint) after EAC cell inoculation (day zero), by recording mortality daily of 50% of mice (N= 10/group) for the Kaplan Meir curve.

2.5.4. Apoptosis Analysis of EAC-cells

Annexin V-FITC was used to analyze the apoptotic cells (Becton Dickinson BD Pharmingen™, Heidelberg, Germany) in the ascetic fluid cell harvest.

Shortly, EAC-cells were obtained from EAC-harboring mice in the different groups and washed two times in PBS for 20 min each. Next, EAC-cells from untreated and treated groups were re-suspended in 100 µL of binding buffer with the introduction of 1 µL of FITC-Annexin V, then 40 min. keeping at 4 °C. Cells were then rinsed and re-suspended in 150 µL of binding buffer with the introduction of 1 µL of PI. Then, the FC BD FACS Caliber (BD Biosciences, San Jose, CA, USA) was employed for analysis.

2.5.5. Analysis of EAC-cells cycle

Additionally, CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used to perform the analysis. The EAC-cells from untreated and treated groups were stained with PI (Becton Dickinson BD Pharmingen™, Heidelberg, Germany) as described by the kit and then run on the cytometer. CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) was employed to study the cell cycle distribution **[28].**

2.5.6. Determination of Blood Parameters

Hematological parameters were analyzed as described earlier **[29]**.

2.5.7. Determination of Biochemical Parameters

Serum ALT, AST, lipid profile, urea, creatinine, and CRP in addition to mouse IL-1β levels determination were performed using appropriate kits. The liver was extracted and homogenized in PBS to measure the SOD and CAT enzyme activities and MDA levels.

2.6. Statistical Analysis

Scatter plots with bars were employed to represent the results showing the respective mean ± S.D. One-way ANOVA test and Tukey's *post hoc* test were employed. All analyses, including the Kaplan-Meier survival curve, were performed using GraphPad Prism version 8.00 (GraphPad Software, San Diego California USA). At a *p*-value less than 0.05, results are considered significantly different.

3. Results

3.1. Colchicine lowers the doxorubicin-induced decrease in body weight

Initial body weight (initial BW), final body weight (final BW), and the percent change in body weight are shown in **Table 1**. After 8 days of EAC-cells inoculation in mice, the final BW. was 1.2-fold (P<0.0001) greater in EAC mice when put in comparison with negative control mice. Administration with DOX^{high} or DOX^{high}/COL led to a 1.5-fold (P<0.0001) and 1.4-fold (P<0.0001) decrease in the final BW, correspondingly, relative to the positive control.

Treatment of EAC-mice with COL reduced the final BW by 1.1-fold (P<0.01) when put in comparison with positive control. Treatment of EAC-injected mice with DOX^{low} or DOX^{low}/COL decreased the final BW by 1.1-fold (P<0.001) and 1.2-fold (P<0.0001) correspondingly, when put in comparison with the positive control. Taken together, the combination of COL with DOX lowered the decrease in BW.

Table 1. Impact of drugs on the body weight and the percentage (%) of body weight change

Parameters/Groups	Control	EAC	EAC/COL	EAC/DOX^{high}	EAC/DOX^{high}/COL	EAC/DOX^{low}	EAC/DOX^{low}/COL
Initial BW (g)	27.6 ± 1.3	28.2± 0.57	27.0 ± 1.1	27.2± 1.1	27.5± 0.83	28.6 ± 1.1	27.2± 0.76
Final BW (g)	30.6 ±1.2	35.4 ± 1.5[a]	32.1± 1.3[b, c]	23.9± 1.1[a, b]	25.5± 0.97[a, b, d]	31.22± 1.73[b, c]	29.2±1.4[b, c]
% BW change	11 %	25.4 %	18.7 %	-12.3 %	-7.5 %	9.0 %	7.5 %

The values show the mean ± SD of the initial body weight, final body weight, and the % body weight change of control mice, EAC-harboring mice, and EAC-injected mice given COL (EAC/COL), DOX^{high} (EAC/DOX^{high}), DOX^{high} combined with COL (EAC/DOX^{high}/COL), DOX^{low} (EAC/DOX^{low}), or DOX^{low} combined with COL (EAC/DOX^{low}/COL). N= 10/group and with [a]P<0.05 against control, [b]P<0.05 against EAC, [c]P<0.05 against EAC/$DOX^{high,}$ and [d]P<0.05 against EAC/DOX^{low}, using ANOVA then Tukey test. EAC, Ehrlich ascites carcinoma; DOX, Doxorubicin; COL, Colchicine; BW, body weight.

3.2. Colchicine increases the doxorubicin-induced antitumor effects

Tumor parameters are presented in **Table 2**. EAC-harboring mice treated with DOX^{high} and COL exhibited an 8.1-fold (P<0.0001) and 1.8-fold (P<0.0001) decrease in the total tumor volume (the total ascitic fluid volume) when contrasted with EAC-bearing mice, respectively.

Administration with DOX^{high}/COL showed the highest effect (18.3-fold decrease; $P<0.0001$). DOX^{low} or DOX^{low}/COL treated EAC-injected mice revealed a 2.6-fold ($P<0.0001$) and 4.3-fold ($P < 0.0001$) decrease in the total tumor volume relative to EAC-harboring mice, correspondingly. EAC-bearing mice treated with DOX^{high} showed a 14.8-fold reduction ($P<0.0001$) in the total tumor count (25±1.6 x 10^6/mouse) when compared to EAC-bearing mice (370 ± 6.98 x 10^6/mouse). COL-treated EAC-bearing mice showed a 3.7-fold ($P<0.0001$) decrease in the total tumor count (100±3.4 x 10^6/mouse) when opposed to EAC-injected mice. Administering a combination of DOX^{high} /COL showed the highest reduction in the total tumor count; leading to 37-fold ($P<0.0001$) lower tumor cells when put in comparison with the control group. The treatment of EAC-injected mice with DOX^{low} or DOX^{low}/COL led to a 6.2-fold ($P<0.0001$) and 10.6-fold ($P<0.0001$) decrease in the total tumor count in comparison with the control group. Compared to mice given DOX^{low}, mice that were given a combination of DOX^{low} and COL showed a 1.7-fold ($P<0.0001$) greater decline in the total tumor count (35±3.4 x 10^6 vs 60±3.2 x 10^6/mouse). Compared to the control group, all the treated groups showed a major reduction in the total live EAC-cells ($P<0.0001$). Taken together, COL optimizes the antitumor effects induced by DOX.

Table 2. Effect of drugs on the tumor volume, total tumor count, and total live and dead cell counts

Tumor parameters/Groups	EAC	EAC/COL	EAC/DOX^{high}	EAC/DOX^{high}/COL	EAC/DOX^{low}	EAC/DOX^{low}/COL
Tumor volume (mL)/Mouse	7.3 ± 0.25	4.0 ± 0.30 $^{b, c, d}$	0.9 ± 0.17 ^{b}	0.4 ± 0.07 $^{b, c, d}$	2.8 ± 0.23 $^{b, c}$	1.7 ± 0.15 $^{b, c, d}$
Total tumor count ($\times10^6$/mouse)	370 ± 6.98	100 ± 3.4 $^{b, c, d}$	25 ± 1.5 ^{b}	10 ± 17 $^{b, c, d}$	60 ± 3.2 $^{b, c}$	35 ± 3.4 $^{b, c, d}$
Total live cell count ($\times10^6$/mouse)	350 ± 15.8	92 ± 1.58 $^{b, c, d}$	9.0 ± 1.6 ^{b}	2.0 ± 1.0 $^{b, d}$	25 ± 2.2 $^{b, c}$	5.0 ± 1.6 $^{b, d}$
Total dead cells count ($\times10^6$/mouse)	20 ± 2.2	8.0 ± 1.2 $^{b, c, d}$	16 ± 2.0 ^{b}	8.0 ± 1 $^{b, c, d}$	35 ± 3.5 $^{b, c}$	30 ± 1.6 $^{b, c, d}$

Values represent the mean ± SD of tumor volume/mouse (mL), total tumor count ($\times10^6$/mouse), live cells count ($\times10^6$/mouse), and dead cells count ($\times10^6$/mouse) of EAC-bearing mice, and EAC-injected mice treated with COL (EAC/COL), DOX^{high} (EAC/DOX^{high}), DOX^{high} combined with COL (EAC/DOX^{high}/COL), DOX^{low} (EAC/DOX^{low}), or DOX^{low} combined with COL (EAC/DOX^{low}/COL). N= 10/group and with $^{b}P<0.05$ against EAC, $^{c}P<0.05$ against EAC/$DOX^{high,}$ and $^{d}P<0.05$ against EAC/DOX^{low}, using ANOVA then Tukey test. EAC, Ehrlich ascites carcinoma; DOX, Doxorubicin; COL, Colchicine.

3.3. Doxorubicin and colchicine extend the survival of EAC-injected mice

To assess the impact of different treatments on the survival of EAC-harboring mice, we monitored the survival until day 24 after EAC cell inoculation. On day 12 after EAC inoculation, 100% of the untreated mice died.

COL-treated EAC-harboring mice showed extended survival until day 17, whereas 100% of EAC-injected mice given DOX^{high} were still alive at day 24 post-EAC inoculation. In contrast, only 85.7% of EAC-injected mice given DOX^{high}/COL or DOX^{low}/COL were alive on day 24 after EAC injection, and only 57.1% of mice treated with DOX^{low} (**Fig. 1**).

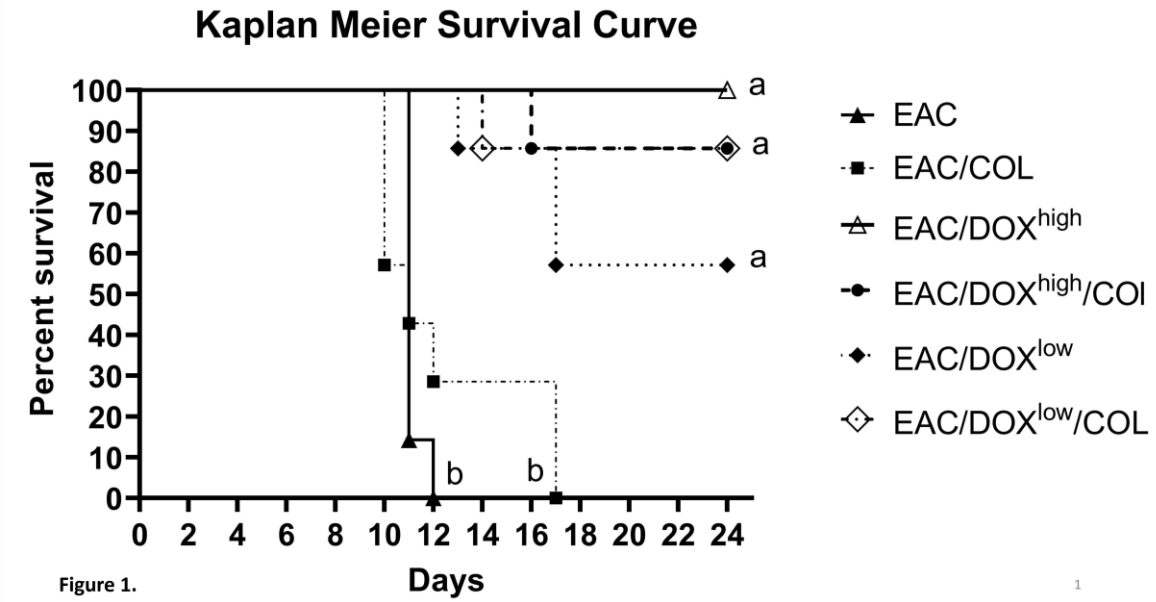

Fig. 1. Kaplan-Meier curve depicting the percentages of survival of untreated and treated EAC-bearing mice. Survival was monitored over 24 days in EAC-bearing mice, and EAC-injected mice treated with COL (EAC/COL), DOX^{high} (EAC/DOX^{high}), DOX^{high} combined with COL (EAC/DOX^{high}/COL), DOX^{low} (EAC/DOX^{low}), or DOX^{low} combined with COL (EAC/DOX^{low}/COL) where N= 10/group. EAC, Ehrlich ascites carcinoma; DOX, Doxorubicin; COL, Colchicine.

3.4. Cotreatment of doxorubicin with colchicine increases the number of early, late apoptotic, necrotic EAC-cells and EAC cell arrest at the G2M phase

The % of necrotic, early apoptotic, and late apoptotic EAC-cells obtained from EAC-injected mice were 0.5±0.1%, 1.9±0.4%, and 0.8±0.2%, respectively. Early and late apoptotic EAC cell percentages harvested from mice treated with DOX^{high} or COL were 7.5-fold (P<0.05) and 4.7-fold (P<0.05) higher, correspondingly, than those of mice injected with EAC cells only. However, treatment with a combination of DOX^{high} and COL did not show significant differences in the percentages of those cells when put in comparison with DOX^{high}-treated mice. Co-administration of DOX^{low} and COL increased the apoptotic cell percentage by 1.6-fold (P<0.05) contrasted with those harvested from DOX^{low}-treated mice (**Fig. 2a**).

EAC-cells Cell cycle Analysis from EAC-bearing mice showed that the % of G1, S, and G2/M phases were 55±1.7 %, 30±1.0 %, and 15± 2.0 %, respectively. % of cells harvested from DOX^{high}-treated EAC-bearing mice was 1.3-fold (P<0.001) lower at the G1 phase, while 1.5-fold (P<0.0001) greater at the S phase, relative to mice injected with EAC cells only. The percentage of EAC cells in the G2/M phase did not differ between EAC cells from DOX^{high}-treated mice and EAC cells from untreated EAC-injected mice. The % of EAC cells from COL-administered mice was 1.3-fold (P<0.001) lower in G1, while 2.0-fold higher (P<0.001) in the G2/M phase, contrasted with control EAC cells. COL did not alter the % of EAC cells in the S

phase. However, treatment with DOX^{high}/COL reduced the % of cells in the G1 phase by 1.7-fold (P<0.0001) and increased cells at the G2/M phase by 2.0-fold (P<0.0001) and at the S phase by 1.2-fold (P<0.01) relative to EAC-harboring mice group. Percentages of cells at G1 and G2M phases harvested from DOX^{low}-treated EAC-bearing mice were not significantly different from those in control EAC cells. By contrast, the % of cells at the S phase was 1.3-fold (P<0.01) higher. Administration with DOX^{low}/COL showed a 1.4-fold (P<0.0001) decrease in the % of EAC cells at the G1 phase and a 1.7-fold (P<0.01) increase in the % of EAC cells at G2/M compared to control EAC cells (**Fig. 2b**). % of cells at the G2M phase was 2.2-fold (P<0.0001) greater in the DOX^{high}/COL group compared to the DOX^{high} group, whereas it was 2.1-fold (P<0.001) greater in the DOX^{low}/COL group relative to DOX^{low} group.

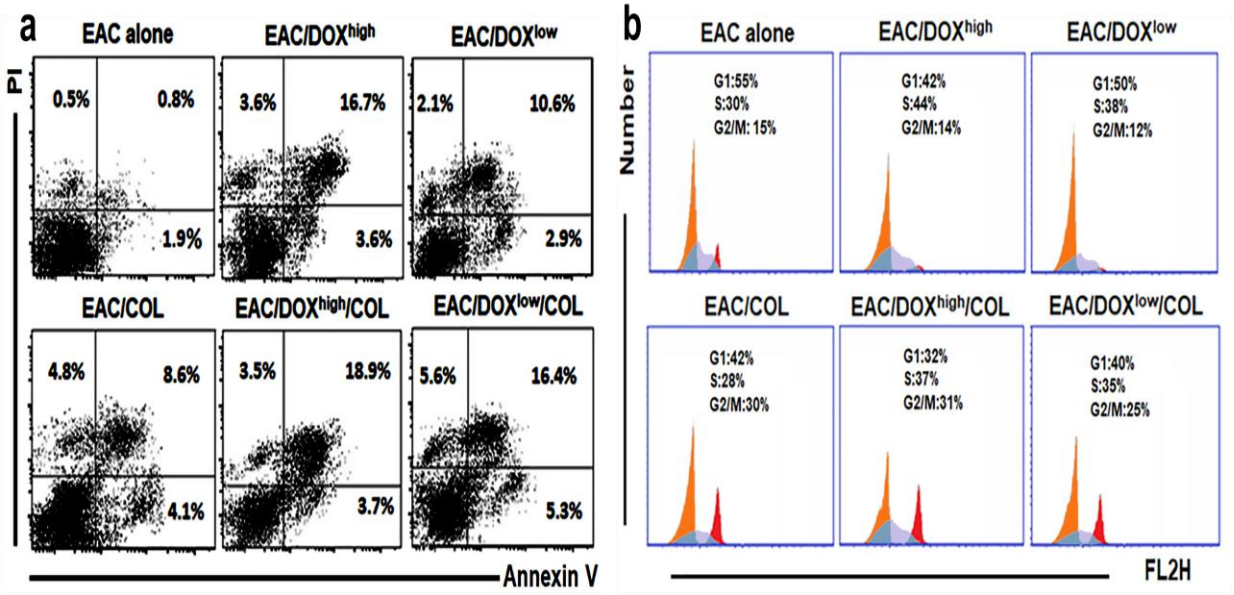

Fig. 2. Impact of the co-treatment on the percentages of apoptotic, and necrotic cells and EAC-cell cycle. a Representative flow cytometry charts of Annexin V/PI-stained EAC-cells derived from untreated EAC-bearing mice, and EAC-injected mice treated with COL (EAC/COL), DOX^{high} (EAC/DOX^{high}), DOX^{high} combined with COL (EAC/DOX^{high}/COL), DOX^{low} (EAC/DOX^{low}), or DOX^{low} combined with COL (EAC/DOX^{low}/COL). The four quadrants show viable cells (lower left), early apoptotic cells (lower right), late apoptotic cells (upper right), and necrotic cells (upper left), respectively. b representative histograms of EAC cells in the G1, S, and G2/M phases, isolated from untreated EAC-bearing mice, and EAC-injected mice treated with COL (EAC/COL), DOX^{high} (EAC/DOX^{high}), DOX^{high} combined with COL (EAC/DOX^{high}/COL), DOX^{low} (EAC/DOX^{low}), or DOX^{low} combined with COL (EAC/DOX^{low}/COL). N= 3. EAC, Ehrlich ascites carcinoma; DOX, Doxorubicin; COL, Colchicine; PI, propidium iodide.

3.5. Colchicine alleviates tumor-associated hematological toxicity

Total RBC count, Hb concentration, and Hct % were 1.5-fold (P<0.05) lower in EAC-possessing mice contrasted with the data of the control mice (**Table 3**). Administration of DOX^{high} to EAC-possessing mice increased these factors by 1.6-fold (P<0.05), 1.4-fold (P<0.05), and 1.4-fold (P<0.05), sequentially, contrasted with untreated EAC-possessing mice. COL treatment of EAC-bearing mice increased those parameters by 1.7-fold (P<0.05), 1.3-fold (P<0.05), and 1.3-fold (P<0.05), respectively. The combination of DOX^{high} with COL raised total RBC count, Hb concentration, and Hct % by

1.5-fold (P<0.05), 1.4-fold (P<0.05), and 1.6-fold (P<0.05) relative to EAC-bearing mice. The number of platelets was slightly decreased in EAC-possessing mice relative to the control group. This count was also slightly decreased after the treatment with DOX^{high} or after combination with COL or COL alone relative to EAC-harboring mice. Total WBCs count increased by 2.8-fold (P<0.0001) in EAC-bearing mice contrasted with control mice. EAC-bearing mice treated with DOX^{high}, COL or DOX^{high}/COL or DOX^{low} or DOX^{low}/COL displayed 1.5-fold (P<0.0001), 1.2-fold (P<0.001), 1.9-fold (P<0.0001), 1.3-fold (P<0.0001), and 1.5-fold (P<0.0001) lower total WBCs count compared to control EAC-bearing mice, respectively. Collectively, COL induced alleviation in the hematological toxicities associated with the tumor as well as tumor treatment when combined with DOX.

Table 3. Effect of COL and DOX single or combined administration on the hematological parameters

Parameters/ Groups	Control	EAC	EAC/COL	EAC/DOX^{high}	EAC/DOX^{high}/COL	EAC/DOX^{low}	EAC/DOX^{low}/COL
RBCs (×10^6/µL)	88± 0.46	5.7± 0.73[a]	9.7 ± 1.0 [b]	9.0 ± 0.57[b]	8.7 ± 1.1 [b]	9.8 ± 1.45[b]	9.5 ± 1.3[b]
Hb (g/dL)	13.8± 0.73	9.3± 0.76[a]	12.4 ± 1.5[b]	12.9 ± 0.7[b]	13.3 ± 1.1 [b]	12.9 ± 0.6[b]	13.0± 1.7[b]
PCV (%)	38.2± 1.65	25.4± 1.1[a]	34.1 ± 4.3[b]	36.4 ± 2.07[b]	39.8 ± 2.6 [b]	37.4 ± 1.6[b]	38.3 ± 2.4[b]
Platelets(×10^3/µL)	771± 46.5	739 ± 49.6	648.6± 88.3	615.8± 81.3[a]	607 ± 64.6 [a]	683.8± 46.8	675.8± 71.9
WBCs (×10^6/µL)	6.88± 0.95	19.0± 1.2[a]	15.6± 0.96[a, b, c]	12.5 ± 1.2[a, b]	10.2 ± 0.86 [a, b, c, d]	14.7± 1.5[a, b]	12.8± 1.0[a, b]

Values represent the mean ± SD of RBC (x 10^6/µL), Hb (g/dl), PCV(%), platelets (x 10^3 /µL), and WBCs (×10^6/µL) of control mice, EAC-bearing mice, and EAC-injected mice treated with COL (EAC/COL), DOX^{high} (EAC/DOX^{high}), DOX^{high} combined with COL (EAC/DOX^{high}/COL), DOX^{low} (EAC/DOX^{low}), or DOX^{low} combined with COL (EAC/DOX^{low}/COL). N= 10/group and with [a]P<0.05 against control, [b]P<0.05 against EAC, [c]P<0.05 against EAC/DOX^{high}, and [d]P<0.05 against EAC/DOX^{low}, employing ANOVA test then applying Tukey test. EAC, Ehrlich ascites carcinoma; DOX, Doxorubicin; COL, Colchicine; RBCs, Red blood cells; Hb, Hemoglobin; PCV, Packed Cell Volume; WBCs, White blood cells.

3.6. Colchicine alleviates tumor-associated kidney and liver toxicities

The liver transaminases (ALT and AST) and the kidney biomarkers (urea and creatinine) were 3.1-fold, 3.0-fold, 1.9-fold, and 2.0-fold elevated in EAC-harboring mice in comparison with the control group, respectively (P<0.0001). EAC-carrying mice administered DOX^{high}, COL, or DOX^{high}/COL revealed a significant decrease in the levels of these biomarkers contrasted with untreated EAC-carrying mice at P<0.05. Although DOX^{low}-treated EAC-carrying mice showed a 1.3-fold (P<0.0001) and 1.4-fold (P<0.05) decrease in ALT and creatinine levels, correspondingly, but not in AST or urea. Combination treatment of DOX^{low} with COL decreased all levels of ALT, AST, urea, and creatinine by 1.4-fold (P<0.0001), 1.1-fold (P<0.05), 1.3-fold (P<0.0001) and 1.6-fold (P<0.001), correspondingly (**Fig. 3**).

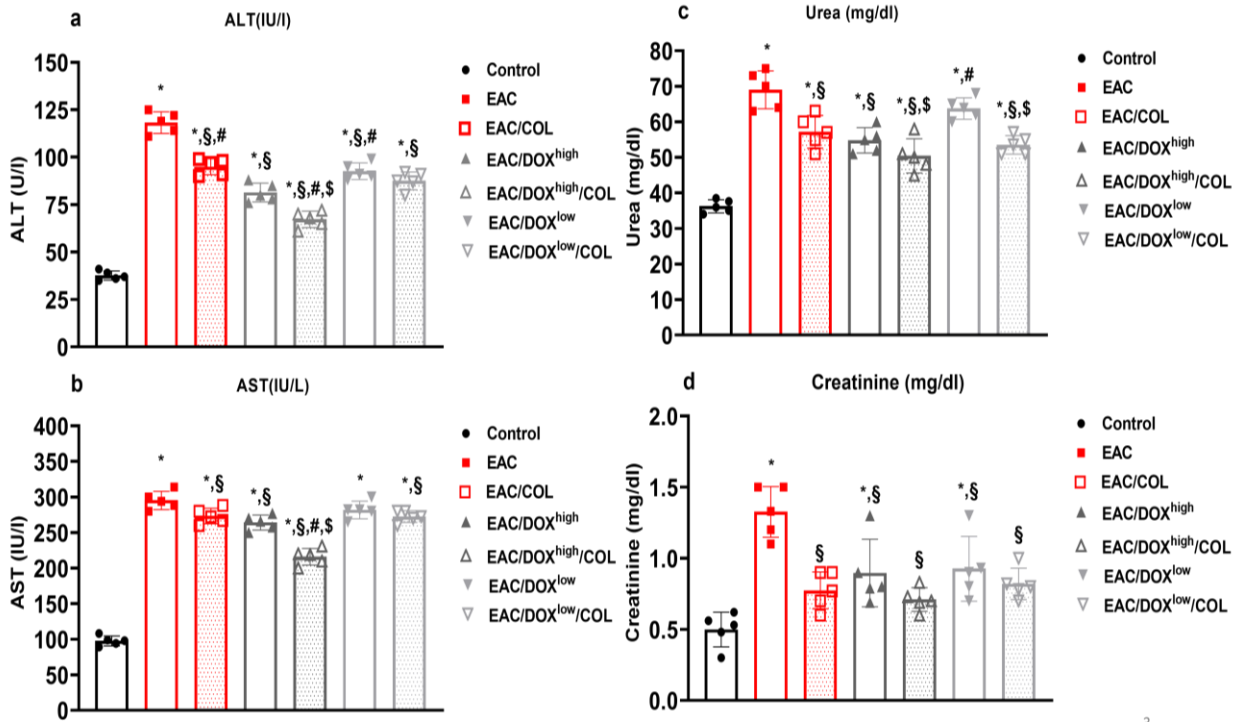

Fig. 3. Impact of the co-treatment on ALT, AST, urea, and creatinine levels in EAC cell-bearing mice. Scatter plots with bars show individual data points and the corresponding mean±SD of an ALT (IU/L), b AST (IU/L), c urea (mg/dL), and d creatinine (mg/dL) in control mice, EAC-bearing mice, and EAC-injected mice treated with COL (EAC/COL), DOX^{high} (EAC/DOX^{high}), DOX^{high} combined with COL (EAC/DOX^{high}/COL), DOX^{low} (EAC/DOX^{low}), or DOX^{low} combined with COL (EAC/DOX^{low}/COL). N= 5/group with **P*<0.05 versus control, § *P*<0.05 versus EAC, #*P*<0.05 versus EAC/$DOX^{high,}$ and $*P*<0.05 versus EAC/$DOX^{low}$, using one-way analysis of variance (ANOVA) followed by Tukey as a post-hoc test. EAC, Ehrlich ascites carcinoma; DOX, Doxorubicin; COL, Colchicine; ALT, Alanine transaminases; AST, Aspartate transaminases.

3.7. Co-treatment of doxorubicin with colchicine ameliorates the lipids profile

The lipids profile of the different mice groups is presented in **Table 4**. Serum TC, TAG, and LDL-C values were 2.0-fold (*P*<0.0001), 1.6-fold (*P*<0.0001), and 2.3-fold (*P*<0.0001) higher, correspondingly, while the HDL-C values were 1.8-fold lower (*P*<0.0001) in EAC-harboring mice relative to the control group. Treatment of EAC-harboring mice with DOX^{high} or DOX^{high}/COL or DOX^{low}/COL resulted in a notable decrease in the TC and TAG values. Treatment with COL or DOX^{low}, however, significantly decreased TC levels by 1.2-fold (*P*<0.0001) and 1.1-fold (*P*<0.001), correspondingly, but not the TAG levels, as opposed to the EAC-carrying mice group. Subjecting EAC-carrying mice to DOX^{high} or COL or DOX^{low} or DOX^{low}/COL did not alter LDL-C levels. Only treatment with DOX^{high}/COL decreased LDL-C levels by 1.3-fold (*P* < 0.001) relative to the EAC-bearing group. None of the treatments induced changes in HDL-C levels. In conclusion, co-treatment of COL with the high dose of DOX improved the lipids profile.

3.8. Co-treatment of colchicine with doxorubicin decreases the tumor-associated oxidative stress

Tumor burden decreased the activities of SOD and CAT by 4.1-fold and 2.5-fold at *P*<0.0001, correspondingly, but increased those of MDA by 2.7-fold (*P*<0.0001). Treatment of EAC-bearing mice significantly increased SOD

and CAT activities and decreased MDA levels in all treatment groups relative to the EAC-harboring mice group. Interestingly, administering DOX^{high}/COL improved SOD and CAT activities by 1.3-fold (P<0.01) and 1.4-fold (P<0.0001), correspondingly, and decreased MDA levels by 1.5-fold (P<0.0001) when contrasted with EAC-carrying mice that received DOX^{high}. In contrast, treatment of EAC-carrying mice with DOX^{low}/COL only led to a 1.1-fold (P < 0.05) decline in MDA levels when contrasted with the DOX^{low}-treated group. Therefore, co-treatment of DOX with COL decreases the oxidative stress (**Fig. 4 a, b, and c**).

Table 4. Effect of COL and DOX single or combined administration on the blood lipids profile

Lipids Profile/ Groups	Control	EAC	EAC/COL	EAC/DOX^{high}	EAC/DOX^{high}/COL	EAC/DOX^{low}	EAC/DOX^{low}/COL
TC (mg/dL)	74.5 ±3.3	146.4 ±7.1[a]	127.2 ± 5.9[a, b]	117.6 ± 5.0[a, b]	97.2 ± 5.6[a, b, c, d]	129.0 ±5.5[a, b, c]	108.8 ±8.1[a, b, d]
TAG (mg/dL)	86.0 ±4.06	137.4 ±5.0[a]	127.0 ±8.7[a, c]	111.4 ±6.8[a, b]	103.4±7.6[a, b, d]	130.3 ±7.5[a, c]	112.0 ±9.9[a, b, d]
LDL-C (mg/dL)	16.2±1.4	36.8 ±3.5[a]	35.4 ±3.4[a]	32.0 ±2.6[a]	28.4 ±2.1[a, b, d]	35.5 ±2.7[a]	36.0 ±3.2[a]
HDL-C (mg/dL)	72.6 ±3.7	40.8 ±1.9[a]	43.0 ±7.4[a]	47.4 ±3.9[a]	45.4 ± 3.4[a]	41.8 ±2.8[a]	44 ±2.8[a]

Values represent the mean ± SD of cholesterol (mg/dL), TAG (mg/dL), LDL-C (mg/dL), and HDL-C (mg/dL) of control mice, EAC-carrying mice, and EAC-injected mice given COL (EAC/COL), DOX^{high} (EAC/DOX^{high}), DOX^{high} combined with COL (EAC/DOX^{high}/COL), DOX^{low} (EAC/DOX^{low}), or DOX^{low} combined with COL (EAC/DOX^{low}/COL). N=10/group and with [a]P<0.05 against control, [b]P<0.05 against EAC, [c]P<0.05 against EAC/$DOX^{high,}$ and [d]P<0.05 versus EAC/DOX^{low}, employing ANOVA test then Tukey's post-hoc test. EAC, Ehrlich ascites carcinoma; DOX, Doxorubicin; COL, Colchicine; TC, Total Cholesterol; TAG, Triacylglycerol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, High-density lipoprotein-cholesterol.

3.9. Colchicine decreases doxorubicin-induced inflammation

CRP and IL-1β are known as typical inflammatory mediators. Our results indicate that serum CRP and IL-1β were 5.7-fold (P<0.0001) and 3.6-fold (P<0.0001) greater in EAC-bearing mice versus control mice, sequentially. DOX^{high}-treated EAC-harboring group further showed a 1.4-fold (P<0.0001) and 1.2-fold (P<0.0001) elevation in CRP and IL-1β levels, sequentially, while COL-treated EAC-harboring group showed a 1.8-fold (P<0.0001) and 1.4-fold (P<0.0001) decline, correspondingly. Although co-treatment with DOX^{high} and COL did not change CRP, they decreased the IL-1β levels by 1.2-fold (P<0.0001) when contrasted with EAC-harboring mice. While subjecting EAC-harboring mice with DOX^{low} did not affect the levels of CRP and IL-1β, treatment with DOX^{low}/COL resulted in 1.1-fold (P<0.001) lower IL-1β levels. Administration of DOX^{high}/COL resulted in a 1.2-fold (P<0.01) and 1.5-fold (P<0.0001) decline of CRP and IL-1β, sequentially, when contrasted with EAC-harboring mice that received DOX^{high} only. Whereas their treatment with DOX^{low}/COL decreased IL-1β levels by 1.2-fold (P<0.0001) when contrasted with EAC-bearing mice that received DOX^{low}. In conclusion, COL decreases DOX-induced inflammation (**Fig. 4 d, and e**).

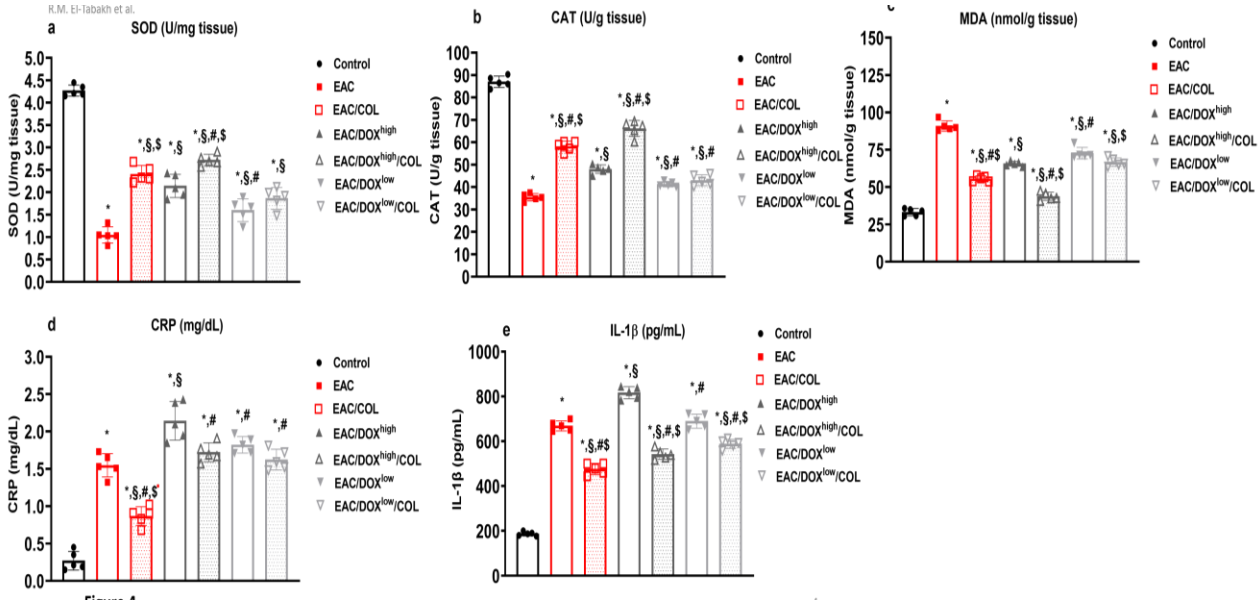

Fig. 4. Impact of the co-treatment on antioxidants/oxidants, C-reactive protein, and interleukin 1-β levels in EAC-bearing mice. Scatter plots with bars show individual data points and the corresponding mean±SD of a SOD activity (U/mg tissue), b CAT activity (U/g tissue), c MDA level (nmol/g tissue), d CRP (mg/dL), and e IL-1β (mg/dL) in control mice, EAC-bearing mice, and EAC-injected mice treated with COL (EAC/COL), DOX^{high} (EAC/DOX^{high}), DOXhigh combined with COL (EAC/DOX^{high}/COL), DOX^{low} (EAC/DOX^{low}), or DOX^{low} combined with COL (EAC/DOX^{low}/COL). N= 5/group with *P<0.05 versus control, §P<0.05 versus EAC, #P<0.05 versus EAC/$DOX^{high,}$ and $P<0.05 versus EAC/$DOX^{low}$, using one-way analysis of variance (ANOVA) followed by Tukey as a post-hoc test. EAC, Ehrlich ascites carcinoma; DOX, Doxorubicin; COL, Colchicine; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

4. Discussion

Resistance to DOX treatment and its adverse effects remain the major problems of its use **[30]**. Therefore, decreasing its resistance and adverse effects, without limitation to its efficacy is recommended. Therefore, the present study aimed to investigate the effect of combining COL and DOX on the antitumor efficacy and side effects. This study revealed that EAC-harboring mice exhibited a notable elevation in the final BW. relative to control mice, which could be due to the rapid proliferation of the tumor cells inside the abdominal cavity of the mice. Treatment with DOX decreased the BW in the EAC-harboring mice group relative to untreated EAC-harboring mice. DOX^{high} even led to a lower final BW than the control mice group, which could be attributed to DOX **[31]** efficacy and toxicity as an anticancer therapy **[2, 31]**. COL decreased the BW in EAC-bearing mice, reflecting its antitumor effect, as previously shown by **[32]**. Consequently, the combination of DOX^{high} or DOX^{low} with COL in EAC-harboring mice led to a decrease in BW., a decrease in tumor volume, and a decrease in tumor count relative to the untreated EAC-harboring mice group. Eradication of the tumor increases survival **[33]**, as the tumor affects the functionality of vital organs **[34]**. In frame with DOX's known efficacy as an effective chemotherapeutic drug **[35]**, DOX prolonged the survival of EAC-harboring mice. Other than the DOX survival effect, also COL enhanced the survival, but only slightly, when compared to the treatment with DOX $^{high\ or\ low}$. Co-treatment of DOX^{low} with COL increased mice survival when compared to the DOX^{low} treatment group. In parallel to the improvement in survival, the % of early and late apoptotic EAC-cells was increased in the EAC-harboring mice group given DOX^{high}, COL, or

DOX^{low} or their combinations. Co-administration of COL with DOX^{high} didn't affect the % of apoptotic EAC-cells, significantly, when compared to DOX^{high} alone, indicating that the maximum anticancer effect was reached by the high dose. In contrast, co-administration of COL with DOX^{low} raised the % of early and late apoptotic cells when compared to DOX^{low} alone, supporting the hypothesis that COL enhances the apoptotic potential of DOX^{low}. These observations are in line with the described dose-dependent potential of DOX, where an increased % of the early and late apoptotic cells occurred using several breast cancer cell lines **[36]**. Cell cycle analysis, further, showed that treatment with either DOX^{high} or DOX^{low} arrested EAC-cells at the S-phase, whereas treatment with COL arrested the EAC-cells at the G2/M phase. This is in line with the previous papers that report DOX causes cell cycle arrest at the S-phase via topoisomerase II poisoning **[37]** and that COL inhibits the survival of MCF-7 cells and induces stoppage of the cell cycle at the G2/M phase **[38, 39]**. The addition of COL to the high or low DOX doses increased the stoppage of the cell cycle in the G2M phase, which could explain the increase in the antitumor effect after treatment with either combination. Concerning total RBC count, hemoglobin, and hematocrit, all these blood indices decreased in the EAC-carrying mice **[40]**, which may be overcome via treatment with DOX and COL. Platelet count did not differ in the EAC-carrying mice group relative to the control group. Inoculation of EAC tumor increased the total WBCs count as previously shown **[41]**, whereas, administration with $DOX^{high\ or\ low}$ doses reduced the total WBC count. Treatment with COL also decreased WBCs count which is in line with a previous study **[42]**. Inoculation of EAC cells in different mice groups caused an increase in AST, ALT activities, urea, and creatinine levels, pointing to hepatic and renal toxicities, following tumor inoculation as in accodance with the previous studies **[43, 44]**. Subjecting EAC-carrying mice to DOX, COL, or their combination diminished liver and kidney toxicities when compared to the EAC-carrying mice group. DOX^{low} had the same effect as DOX^{high}, but to a lesser extent. In line with its known hepatic and renal protective properties **[21, 45]**, COL reduced the increased liver and kidney function tests (AST and ALT, urea and creatinine levels). Serum TC, TAG, and LDL-C levels were elevated in the EAC-bearing mice group relative to the control mice group, whereas HDL-C levels were decreased in EAC-harboring mice vs the control group, supporting the findings of **[46]**. Administration with DOX^{high} resulted in a decrease in serum TC and TAG relative to EAC-harboring mice, whereas treatment with COL or DOX^{low} decreased TC but not TAG levels. This shows that COL or DOX^{low} has a potential antitumor effect, but not as DOX^{high}. However, a previous study showed that COL does not affect TC levels **[47]**. Treatment with either combination of DOX and COL significantly decreased both TC and TAG. The presence of stressful diseases such as diabetes or cancer as well as DOX treatment raises OS in mice **[41]**, with a decrease in the activities of SOD and CAT and an increase in the MDA levels **[3, 48, 49]**. In all treatment groups, the OS was decreased relative to the EAC group, reflecting the curative impact of the treatments. A previous study reported that COL had antioxidant properties **[50]**. This was further emphasized by our findings that show the co-treatment of DOX^{high} and COL decreased the OS when compared to EAC mice that only received DOX^{high}. CRP is an unspecific inflammatory marker **[51]**, while IL-1β is a cytokine produced, by one mean, as a result of the NLRP3 inflammasome pathway **[10]**. Tumor inoculation increased CRP and IL-1β levels, as also previously shown by **[52]**. Administration of DOX^{high}, further, raised the CRP and IL-1β levels

when contrasted with untreated EAC-harboring mice. This may be due to the action of DOX on the NLRP3 inflammasome pathway **[9]**. DOX^{low} only slightly increased CRP and IL-1β levels in EAC mice indicating a dose-dependent inflammatory response. In contrast, COL-treated EAC-bearing mice exhibited lower CRP and IL-1β levels when compared to EAC-bearing mice, which can be explained by the NLRP3 inflammasome pathway-inhibitory capacity of COL **[15]**, but platelets activation **[53]** or neutrophils infiltration **[54]** effect via myeloperoxidase enzyme and prostaglandin E2 affection, by either tumor stress/injury or DOX treatment as toxic effect whether linked to NLRP3 inflammasome pathway or not, should be further explored. This is in line with our findings that revealed that co-treatment of EAC-harboring mice with COL and DOX mitigated the inflammation burden when contrasted with EAC-harboring mice that received DOX only.

Conclusion

The present study displays that tumor co-treatment by either DOX^{high} or DOX^{low} with COL enhanced the antitumor effect of DOX and ameliorated the DOX-mediated adverse effects.

Study Strength and Recommendation

These data are useful to explore more the benefits of COL as such in addition to other chemotherapeutics to enhance the latter efficacy, decrease DOX dose, decrease multi-drug resistance to cancer as well as decrease DOX side effects. However, a previous case report showed that a patient with FMF and Hodgkin's lymphoma who received COL with prednisone, DOX as well as vincristine, and etoposide, suffered from severe side effects and had to stop COL during chemotherapy **[55]** which would be considered a **limitation** its use in the future clinical trials.

Future prospective

More COL toxicological studies and further clinical examination(s), however, based on our previously published recommendation [56] to use selective target inhibitor chemotherapeutic drugs (via nano-formulation) as targeting 20S proteasomes **[57]** may be a potential strategy for overcoming clinical drug resistance, as well as if we would add vitamin E or D to ameliorate any cardiovascular side effects **[58, 59]**.

In summary

Although Doxorubicin is widely used in chemotherapeutic regimens, it has drastic side effects with declining efficacy. Therefore, we thought to use the anti-inflammatory medication Colchicine, commonly prescribed for the treatment of gout and FMF, to reduce Doxorubicin-associated toxicity and enhance its antitumor activity/efficacy.

Investigation of apoptosis, cell cycle, survival, tumor count and tumor volume, and inflammatory and biochemical markers were done in an Ehrlich Ascites Carcinoma bearing mice model, which demonstrated potential promising effect(s). However, further investigations should be done to enable their combined use in clinical settings.

List of Abbreviations

ALT, Alanine transaminase; ANOVA, Analysis of variance; ASC, Apoptosis associated Speck-like protein containing a CARD; AST, Aspartate transaminase; Bax, B-cell lymphoma-2-associated X; Bcl-2, B-cell lymphoma-2; BW, Body weight; % BW, The percentage of body weight change; CARD, Caspase Activation and Recruitment Domains, CAT, Catalase; COL, Colchicine; CRP, C-reactive protein; DOX, Doxorubicin; DOX^{high}, High dose of doxorubicin; DOX^{low}, Low dose of doxorubicin; EAC, Ehrlich Ascitic Carcinoma; ELISA, Enzyme-linked immuno-sorbent assay; FMF, Familial Mediterranean Fever; G1, First Growth/Gap

phase; G2M Second Growth/Gap phaseto Mitotic phase of cell cycle; GSDMD, Gasdermin D; Hb, Hemoglobin; Hct%, Hematocrit; HDL-C, High-density Lipoprotein-Cholesterol; IL-1β, Interleukin-1 beta; IL-18, Interleukin-18; i.p., Intraperitoneal; LDL-C, Low density Lipoprotein Cholesterol; MDA, Malondialdehyde; NLRP3, Nucleotide-binding domain-like receptor protein 3; OS Oxidative stress; PBS, Phosphate Buffer Saline; PI, Propidium iodide; P2X7, Well known mentioned in papers by abb. (a protein coded by the P2X7 gene); RBCs, Red Blood Cells; ROS, Reactive Oxygen Species; SOD Superoxide dismutase; S, Synthesis phase, TAG, Triacyl glycerol; TNF-α, Tumor Necrosis Factor alpha; WBCs, White Blood Cells.

Declarations

Ethical approval and consent to participate

The research ethics committee (REC) for experimental studies at the Faculty of Pharmacy, Ain Shams University, Cairo, Egypt approved the protocol (ENREC-ASU-2020-7). The research was conducted with minimal harm to animals following the ARRIVE guidelines for animal handling and welfare.

Consent for publication

Not applicable

Availability of data and material

The data of the current study are available within tables and figures and any further details will be provided by the corresponding author.

Competing interest

The authors declare no competing interests.

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Authors' contributions

R.El-T., E.F.W., N.M.H., and M.L.S. drafted, rewrote, and revised the manuscript. R.El-T., N.M.H., and M.L.S. contributed to the experimental work. N.M.H. and M.L.S. contributed to study design, data acquisition, data analysis, data interpretation, and intellectual content and shared the last authorship. S.V.L. conceived the study and revised the manuscript. All authors revised the manuscript and gave their final approval for publication and authorship.

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