Amlodipine (P-glycoprotein) inhibitor modulation effect on the Hematological, Oxidative and Inflammatory effects of Doxorubicin in Colorectal cancer induced in Mice

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Abstract

An experiment was performed to assess the effect of P-gp inhibitor Amlodipine (AML) when given alone or combined with Doxorubicin (DXO) therapeutically against Colorectal cancer (CRC) induced by Azoxymethane (AOX) in mice. Forty-eight adult Albino mice equal were divided into six groups consisting of C-ve given NS and five groups treated after CRC induction according to dosing regimen (C+ve (AOX 10 mg/kg and NS), T1 (AML 1.8 mg/kg), T2 (DOX 5mg/kg), T3 (AML1.8 mg/kg with DOX 2.5mg/kg/) and T4 (AML 1.8 mg/kg with DOX 5 mg/kg). Dosing continued for four weeks followed by two weeks recovery through which blood were collected to evaluate their hematological oxidative and inflammatory effects. Hematological result recorded significant reduction in the blood RBC, PLT count and HCT percentage mainly in T1 (DOX) and T4 groups in comparison with the other experimental groups that showed nearly normal level both at end of treatment and recovery periods. While Differential (WBC) count showed significance increase in C+ve group and significance decrease in (T1, T4 and T3). Plasma GSH Concentration recorded significantly less reduction in T2 and T3 groups than other treated groups compared with control one at both periods while IL-6 concentration showed in all treated group significant increase with less effect recorded in T2 and T4 compared with control one at both end of treatment and recovery periods.

Keywords: *Doxorubicin, Amlodipine, P-glycoprotein inhibitor, Hematological, Oxidative, Inflammatory, Colorectal cancer, mice.*

Introduction

P-glycoprotein (P-gp) a crucial participant in the development of resistance to anticancer medicines, a phenomenon known as multidrug resistance (MDR). P-glycoprotein (P-gp) acts as a transmembrane efflux pump that has high selectivity for several different anticancer drugs, reducing intracellular drug concentrations and cytotoxicity, hence affecting circulatory concentrations.[1]. Anthracyclines and other anticancer drugs with similar chemical properties and mechanism of action share some degree of cross-resistance, this play a crucial role in most curative therapy regimens because they are substrates of P-gp [2].

Several non-cytotoxic pharmacologically competitive inhibitors have been demonstrated to decrease the transport function of P-gp,

restore the defects of cytotoxin accumulation, and reversing the MDR in vivo and in vitro. [3], [4] Amlodipine is just one example of a very diverse group of medicines that fall under this category. [5]. Amlodipine 1,4-dihydropyridine calcium antagonist is lipid-bilayer-incorporated[6]. Amlodipine's modulation of P-glycoprotein efflux activity suggests an inhibitory role also alters the lipid bilayer's organization and thermodynamics in the plasma membrane. [7]. Independent of calcium channel antagonists, dihydropyridine diminishes intracellular ROS generation and antioxidant activity [8].

The anticancer antibiotic doxorubicin (DOX) is effective and useful for treating many human malignancies, but severe cardiotoxicity or leukopenia limits its usage in humans and animals at dosages sufficient for effective treatment. [9]. Doxorubicin and other anthracyclines are cytotoxic due to damage caused by oxygen free radicals to membrane lipids and other cellular structures. [10], [11]. It has been demonstrated that doxorubicin produces hydroxyl radicals. [12], [13], superoxide anions, and hydrogen peroxide. [14]. NADPH-cytochrome P-450 is responsible for the conversion of doxorubicin into a free radical. This process results in the production of superoxide anion and hydroxyl radicals, which in turn causes membrane lipid peroxidation. [15].

Azoxymethane (AOM) is often used to study colon cancer in animals [16]. This intermediary metabolite of dimethylhydrazine produces methyl diazonium and methyl carbonium, which damage biomolecules and may cause colon cancer. [16], [17]

Materials and Methods

Ethics

The Scientific Committee of the Department of Physiology, Biochemistry, and Pharmacology in the College of Veterinary Medicine at the University of Baghdad, as well as the Ethics Committee, reviewed and approved all of the procedures that were to be used in this study to ensure that they adhered to ethical standards regarding animal welfare.

Animals and drugs:

Forty-eight adult healthy Albino mice at 12 weeks old at an average of (25-30 gm) body weight, were housed in the College of veterinary medicine\ Baghdad University's animal house. and water and standard pellets have been provided ad libitum. The animals were housed in special cages with optimal conditions three weeks before the experiment and maintained with the standard condition at 12 hour light-dark cycle, (20- 25 °C) in an air-conditioned room. The bed was wood shaves that continuously changed, and the cages were cleaned twice per week.

The C-ve (N=8) administered N.S. IP for seven weeks, the C+ve Group (N=8) administered AOM at 10 mg/kg/wk IP for three weeks and two weeks waiting to induce (CRC) then treated with N.S IP for four weeks, The T1 (N=8) CRC-induced mice treated by Normal Saline IP for two weeks then treated with DOX at 5mg/kg/wk IP. for two weeks, The T2 (N=8) CRC induced mice treated by AML at 1.8 mg/ kg/ day P.O for one month, the T3 (N=8) CRC induced mice treated with AML 1.8 mg/ kg /day P.O for two weeks followed by a combined dose of AML 1.8 mg/ kg/ day P.O and DOX 2.5mg/kg/wk. IP for two weeks, The T4 (N=8) CRC induced mice treated with AML 1.8 mg/ kg/ day P.O for two weeks followed by a combined dose of AML 1.8 mg/ kg /day P.O and DOX 5 mg/ kg/ wk. IP for two weeks. The experimental period in each group include five

weeks CRC induction followed by four weeks treatment and two weeks recovery period.

Induction of Colorectal Cancer

A frequent model and probable carcinogen for inducing colon cancer in albino mice is azoxymethane (AOM) [18]. CRC induction was done by given each mouse AOM at a dose of 10mg/kg/wk intraperitoneally for three weeks, the optimal amount for inducing aberrant crypt foci (ACF), and then waiting two weeks for the appearance of ACF as markers of CRC [19]

Materials

AOM was purchased from the Sigma-Aldrich (Germany), Amlodipine from Pfizer (USA) and Doxorubicin from Medac Gmbh (Germany).

Hematological Examination:

After exposing the mice to an overdose of diethyl ether, a cardiac puncture was performed in order to obtain blood samples for hematological examination. These blood samples were then collected in a test tube that contained 20 mg/mL of ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Using an automated blood analyzer (Diagon, Hungary), the red blood cells (RBC), white blood cells (WBC), differential leucocyte count, hematocrit%, and other parameters were tallied from one milliliter of each sample. This counting was done for each sample.

Biochemical assay:

The Reduced Glutathione test was carried out in accordance with the directions provided by the manufacturer of the plasma GSH ELISA kit (Elabscience, China) and according to the manufacturer's instructions of mice IL-6 ELISA kit (Elabscience, China) the Interleukin-6 test was performed.

Statistical Analysis

Analyzing the DATA is done by SPSS 26 by using a variance analysis (ANOVA) two-way test to evaluate whether there were significant differences between and within the groups at ($P \leqslant 0.05$), and using the less significant difference (LSD) test to comparison the mean values. It is presented as mean ± standard deviation [20]

RESULTS

The results of the plasma reduced GSH in CRC induced groups recorded at the end of treatment a significantly higher decrease ($p \leqslant 0.05$) by 2.9, 2.43-fold recorded in T1 and T4 respectively, when compared with C-ve group, while the significantly lowest decrease by 1.27, 1.50-fold in T2 and T3 respectively. While at the end of recovery (T1, T2, T3, T4) and C+ve groups recorded a significant decrease when compared with C-ve group by 2.65, 1.22, 1.29, 2.32 and 2.03-fold respectively. The significant increase ($p \leqslant 0.05$) recorded when compared within the groups between periods in GSH concentration. (Table 1), (fig. 1).

Table (1): Plasma GSH and Interleukin-6 Concentration in CRC induced and treated mice group with Doxorubicin and Amlodipine at different experimental periods.

Groups	End of treatment				Two-week recovery			
	Plasma GSH		Plasma Interleukin-6		Plasma GSH		Plasma Interleukin-6	
N=4	Mean ± S.D.(µg/ml)	Fold change	Mean ± S.D. (pg/ml)	Fold change	Mean ± S.D.(µg/ml)	Fold change	Mean ± S.D. (pg/ml)	Fold change
C-Ve	45.68 ± 3.31 *A a	1	43.31 ± 1.67 *E a	1	45.77 ± 1.97 A a	1	42.91 ± 1.88 E a	1

G+ve	21.40 ± 1.91 D b	2.13	91.59 ± 1.25 B a	2.11	22.50 ± 2.19 D a	2.03	80.74 ± 1.29 B b	1.88
T1 (DXO)	15.67 ± 1.46 F b	2.92	96.63 ± 3.07 A a	2.23	17.21 ± 2.37 F a	2.66	93.05 ± 3.03 A b	2.17
T2 (Aml)	36.07 ± 0.93 B b	1.27	76.06 ± 1.82 D a	1.76	37.47 ± 1.76 B a	1.22	72.95 ± 1.30 D b	1.7
T3 (Combined-1)	30.49 ± 1.85 C b	1.5	77.17 ± 1.49 D a	1.78	35.59 ± 1.75 C a	1.29	76.22 ± 1.14 C a	1.78
T4 (Combined-2)	18.78 ± 1.17 E b	2.43	81.46 ± 1.71 C a	1.88	19.72 ± 1.43 E a	2.32	79.86 ± 1.05 B b	1.86

Different capitalization letters are used When there are statistically significant differences between groups at ($p \leqslant 0.05$), Different Small letters are used When there are statistically differences within groups at ($p \leqslant 0.05$). **GSH LSD = 1.961, Interleukin-6 LSD = 1.867

Figure (1) Plasma GSH Concentration of experimental groups

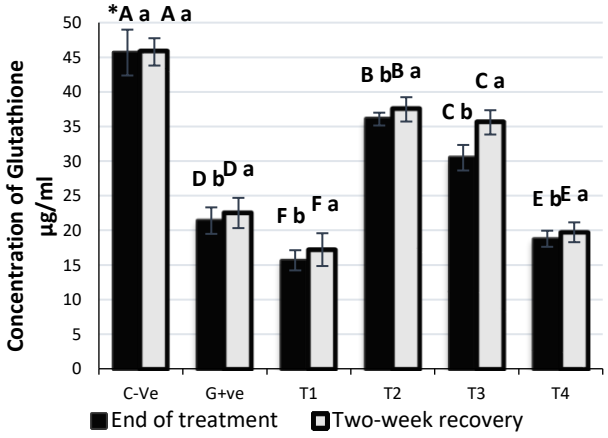
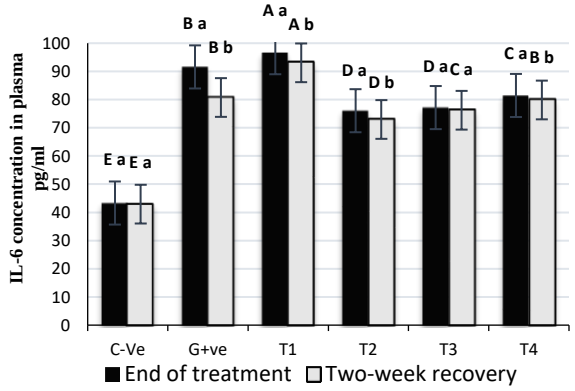


Figure (2) Interleukin-6 concentration of experimental groups



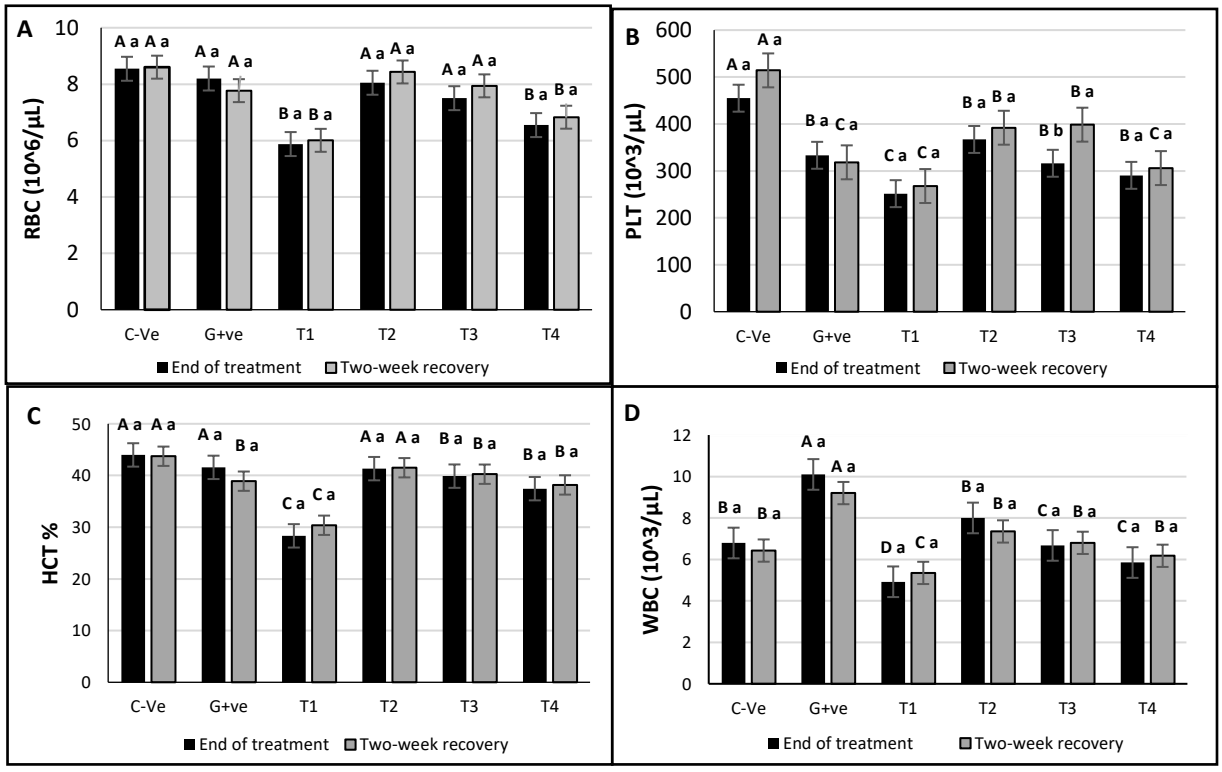
Plasma Interleukin-6 (IL-6) results showed at end of treatment a significant increase at ($p \leqslant 0.05$) its concentration in T1 group by 2.23-Fold over C-Ve group. And the significantly lowest increase by 1.76-fold in T2 and 1.78-fold in T3 when there are compared with other groups, also there was significantly differences between groups and significant increase of concentration when compared with C-ve group. The results at recovery period recorded a significant increase in T1 group by 2.17-Fold over C-Ve group. while other treated groups recorded significantly less increase by (1.88, 1.86, 1.78 and 1.70) Fold over C-Ve group in C+ve, T4, T3 and T2 respectively. within-group comparisons recorded significantly decrease ($p \leqslant 0.05$) at recovery period in all groups except in control negative and T3 groups. Table (1) Fig. (2)

Hematological Parameters

At the end of treatment, the Red blood cell count results displayed significance decrease ($p \leqslant 0.05$) in T1 and T4 when compared with other groups, also same result displayed in the second sample after recovery with slightly increase in RBC count when compared with first sample. Fig (3). The platelet count (PLT) test results showed significant decrease ($p \leqslant 0.05$) in CRC induced groups when compared with control negative group, with significantly highest decrease in T1 within each period of sampling while there were nonsignificant change in PLT count between the two periods with exception of T3 group there was significant increase after recovery.

The Hematocrit (HCT)% results showed significant decrease at ($p \leqslant 0.05$) in T1, T4, and T3 groups respectively when they are compared with the control negative group, with significantly highest decrease in T1 within each period of sampling and there was nonsignificant change in HCT% between the two periods with exception of C+ve group there was significant decrease when compared with C-ve in the second sample after recovery. Hematological result record significant reduction ($p \leqslant 0.05$) in the blood RBC, PLT count, and HCT percentage mainly in T1 (DOX) and T4 groups in comparison to the other experimental groups that showed nearly normal levels both at the end of treatment and recovery periods. The white blood cell (WBC) count results showed significant ($p \leqslant 0.05$) increase in the C+ve group when compared with other treated groups, also this result showed significance decrease at ($p \leqslant 0.05$) in the (T1, T4, and T3) groups respectively when they compared with control Positive, control negative, and T2 groups. and there was nonsignificant change in WBC count results between two periods (fig. 3. D).

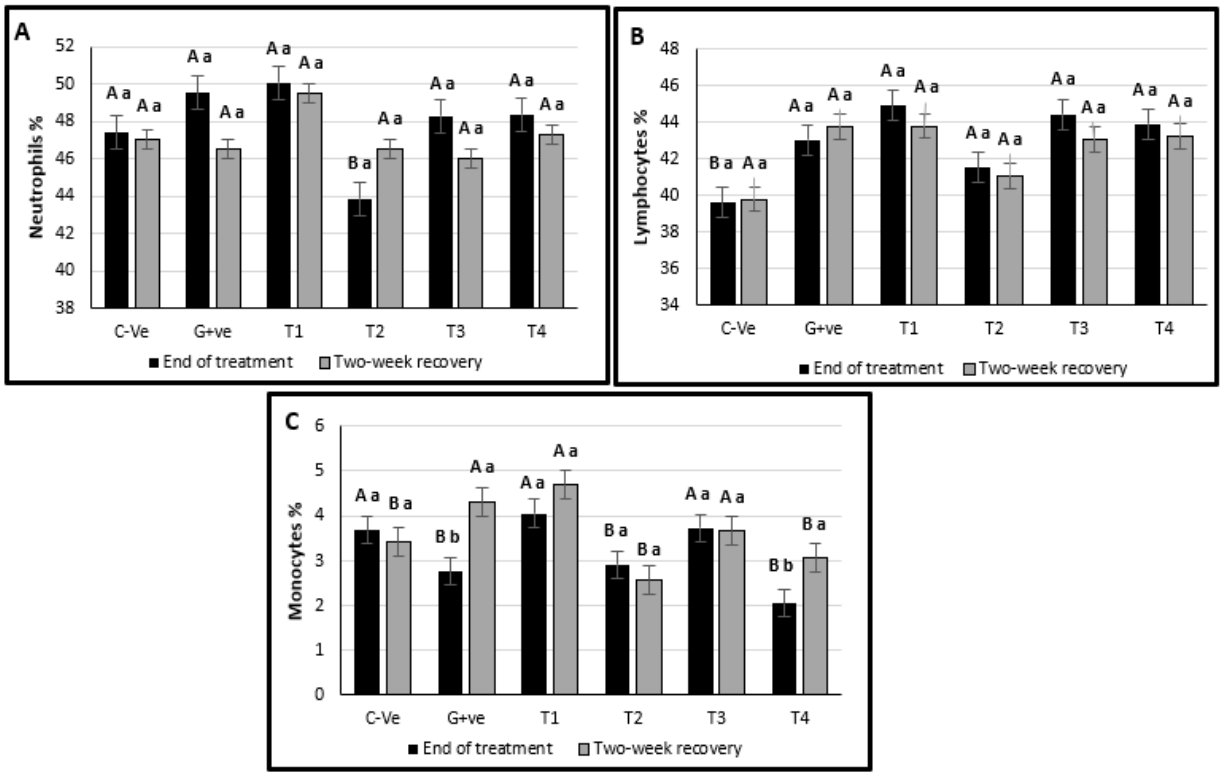
Figure (3) RBC, WBC, PLT count and HCT% in CRC induced and treated groups at different experimental periods. * Different capitalization letters are used When there are statistically significant differences between groups at ($p \leqslant 0.05$), Different Small letters are used When there are statistically differences within groups at ($p \leqslant 0.05$). ** A- RBC LSD = 1.124, B- PLT LSD= 81.681, C- HCT LSD = 2.814, D- WBC LSD= 1.316



The Differential leukocyte results of the first sample at the end of treatment showed in fig (4) a non-significant increase in Neutrophils percent in C+ve, T1, T3 and T4 groups, while significant decreased in T2 group at the end of treatment when compared with C-ve group. (fig. 4 A). Lymphocyte percentage results significantly increased in C+ve, T1, T3 and T4

groups at both experimental periods, while mainly increased in T1 group after recovery period. (fig. 4 B). Monocytes percentage results showed significantly decrease in C+ve, T2 and T4 groups at the end of treatment period, while after recovery period significantly increased in C+ve, T1 and T3 groups when compared with C-ve group. (fig. 4 C).

Figure (4) Differential leukocyte count in CRC induced and treated groups at different experimental periods *Different capitalization letters are used When there are statistically significant differences between groups at (p≤0.05), Different Small letters are used When there are statistically differences within groups at (p≤0.05). A- Neutrophils LSD = 5.286, B- Lymphocytes LSD= 5.242, C- Monocytes LSD = 1.053



DISCUSSION

Hypertensive patients used Amlodipine chronically may experience an interaction between the P-gp substrate and its inhibitor when they undergo chemotherapy with DOX used for the treatment of CRC.

P-gp as efflux membrane transporter that reduce the efficacy of its anticancer drug substrate leading to MDR [21], [22]

We hypothesized that Amlodipine (P-gp inhibitor) might modulate the efficacy and cytotoxicity of DOX substrate when used for treatment of induced CRC in mice by Azoxymethane.

It has been found that the development of noncommunicable diseases such as cancer is associated with increases in oxidative stress and/or decreases in antioxidant capacity. [23]. In point of fact, AOM is a procarcinogen that goes through oxidative metabolism in the liver.

This results in the generation of active electrophiles carcinogen (diazonium ion), which are then released into blood circulation. These electrophiles eventually cause peroxidation of RBC membranes[24], [25]

Doxorubicin is known to have extremely harmful toxic effects as well as oxidative damage that impact the cardiovascular system, which prevents the medicine from being used in certain situations. It is generally agreed that the damage to membrane lipids that is caused by the impact of oxygen radicals is the single most essential element in the progression of doxorubicin-induced toxicity [26]. Doxorubicin metabolism induces in vivo penetrating generation of oxygen centered free radicals [27], [28].

In the present study, the development of tissue toxicity induced by doxorubicin was recognized by a significant decrease in GSH. The following decrease order (T1, T4, T3) with lesser decrease in T2 that used AOM and AML ware attributed to DOX induced oxidative injuries effect by their induced free radical [29], The highest significant decrease in plasma GSH in T1 was possibly attributed to the additive or potentiated oxidative toxic effect of both drugs DOX and AOM.

Amlodipine have been demonstrated to have antioxidant activity that reducing the intracellular generation of ROS, irrespective of the calcium channel's modulation. Amlodipine significantly lowered malondialdehyde, nitric oxide levels, and nitric oxide synthase activity considerably. Although there are evidence suggesting the antioxidative mechanisms of amlodipine belong to the group of chain breaking antioxidants. [30]

It seems from our result that the combined-1 group with half therapeutic dose of DOX have less oxidative effect (high plasma GSH) than combined-2 group with full therapeutic dose.

This might be attributed to P-gp inhibition effect of AML that decrease DOX circulatory concentration and increase DOX intracellular concentration to the level that give lesser oxidative effect in T3 than T4 which may have higher increase in DOX concentration intracellularly causing more cytotoxic and oxidative effects[31]

The present results of a mice Interleukin-6 (IL-6) in plasma of AOM treated mice revealed a significant increase in the circulating levels of proinflammatory cytokines in comparison with control negative group this finding is consistent with prior findings showing that AOM-treated mice have a significantly increased level of interleukins, especially IL-6, compared to saline-treated control mice [32] .

Furthermore, cells injury induced by Doxorubicin oxidative stress and induction of inflammatory response [33]. which might explain initiation of inflammatory cytokines IL-6 one of a well-known critical mediator of inflammatory disorders [34] And this fact explains our result of the significantly higher increase in proinflammatory cytokines IL-6 in T1 group by 2.23-Fold increased over C-Ve group and other treated groups indicating that both have proinflammatory effect as reported by other researcher [35], [36]

Our result show that doxorubicin treated groups had elevated pro-inflammatory parameters compared with combined treatment groups that can possibly generated by the P- glycoprotein inhibitory effect of amlodipine on DOX circulatory concentration that effect the inflammatory stress parameters in circulation [7].

Amlodipine induced anti-inflammatory effects and these potential properties of amlodipine as reported by Navarro-Gonzalez, et al., [37], reporting that the valuable effects of amlodipine therapy have been attributed not only to amlodipine antihypertensive effects but also to its direct vasculo-protective effects through modulating nitric oxide (NO) generating capacity and decreases in oxidative stress and inflammation.

Amlodipine has been studied in vitro for its possible anti-inflammatory actions, and results have revealed that it can block the generation of cytokines in vascular smooth muscle cells and peripheral blood mononuclear cells. [38]. In addition, in vivo studies have showed that amlodipine reduces the concentrations of proinflammatory cytokines in serum in different animal species [39], [40]

The hematological values of DOX-treated mice showed decrease in the number of red blood cells and HCT which representing the proportion of cells and fluids in blood value [41]. compared with the controls. Although the white blood cells counts were slightly less than those of the control group. These findings supported the exacerbation of bone marrow toxicity from chemotherapeutics due to the considerable pharmacokinetic effects seen, although regulation of low-level P-gp expression in hematopoietic stem cells might have played a role[42]–[44]

As in a previous study, the number of white blood cells (WBC) in 16–18-week-old adult mice given DOX was much significantly less than in control mice given saline. [45]

All this evidence from the blood parameters provides further information for toxic side effects associated with DOX administration that was apparent over and above any effects of the AOM. By comparison, following injection the mice with AOM.

Conclusion

The difference between combined groups and T1 group results were attributed to the P-gp inhibition by AML that decrease the blood concentration of DOX and increase its intracellular concentration, so increase its hematological, inflammatory and oxidative effect especially inT1 with DOX alone and T4 that used double dose DOX than T3 group.

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