Amlodipine (P-glycoprotein inhibitor) modulation Doxorubicin Immunohistochemical effect in induced CRC in Mice

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Abstract

A trial was conducted to assess the P-gp inhibitor Amlodipine (AML) in induced colorectal cancer (CRC) in mice by Azoxymethane (AOX) treated with Doxorubicin (DXO) alone or combined with AML. Forty-eight adult Albino mice were equally divided to six groups consisting of C-ve given NS and five groups treated after CRC induction according to the dosing regimen: C-ve (AOX 10 mg/kg and NS), T1 (AML 1.8 mg/kg), T2 (DOX 5 mg/kg), T3 (AML 1.8 mg/kg with DOX 2.5 mg/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 clinical, physiological and proliferating cell nuclear antigen (PCNA) parameters were evaluated. Clinical observation showed slightly weakness in all treated animal groups with abnormal clinical symptoms and recorded a noticeable appearance of swelling masses in the tail and partial alopecia especially in the C+ve group. Also, the results showed there was a significant decrease in animal body weight after CRC induction by AOM, while at the second week of recovery body weight recorded a significant increase in the T3 and T4 groups while a significant decrease in animal body weight in the T2 and control positive groups at the end of the experiment. The findings score of PCNA showed a therapeutic effect on CRC-induced mice within the two periods of experiment in the treated groups T1, T3, and T4 except the T2 group in comparison with the C+ve group. The results recorded a significance decrease in PCNA-LI score in T3, T4, and T1 respectively in comparison with the C+ve group with an improved anti-colorectal cancer effect recorded in T3 combined therapy according to the result of least PCNA level than other treated groups.

Keywords: CRC, Doxorubicin, Amlodipine, P-glycoprotein inhibitor, PCNA, Immunohistochemical.

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INTRODUCTION

The importance profiles of the third most common cancer diagnosed and rank second in cancer-related deaths worldwide recorded in Colorectal cancer. [1] The risk of CRC increases with multidrug resistance (MDR) through P-glycoprotein (P-gp) a crucial player in the development of resistance to anticancer medicines. P-gp acts as an efflux transmembrane pump with broad selectivity for a number of anticancer medicines, reducing intracellular drug concentrations and cytotoxicity, hence affecting circulatory concentrations. [2]. 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 [3]

Several noncytotoxic pharmacologic competitive inhibitors have been shown to decrease P-gp transport function [4], restore cytotoxin accumulation defects, and reverse MDR in vitro and in vivo. [5]. Amlodipine is just one example of a very diverse group of medicines that fall under this category. [6]. Amlodipine, a 1,4-dihydropyridine calcium antagonist, is lipid-bilayer-incorporated. Amlodipine's modulation of P-gp 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 diminish intracellular ROS generation and antioxidant activity [8];[9].

The anticancer antibiotic doxorubicin (DOX) is effective and useful for treating many human malignancies [10], [11], but severe cardiotoxicity or leukopenia limits its usage in humans and animals at dosages sufficient for effective treatment. [12]. Doxorubicin and other anthracyclines are cytotoxic due to oxidative damage to membrane lipids and other cell components. [13]. Doxorubicin has been shown to generate hydroxyl radicals [14], hydrogen peroxide, and superoxide anions[15]. Doxorubicin is converted to a free radical by NADPH-cytochrome P-450, which produces superoxide anion and hydroxyl radicals, resulting in membrane lipid peroxidation. [16].

Azoxymethane (AOM) is often used to study colon cancer in animals [17]. This intermediary metabolite of dimethylhydrazine produces methyl diazonium and methyl carbonium, which damage biomolecules and may cause colon cancer. [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 fed standard pellets and water ad libitum. The animals were kept 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 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 includes five weeks of 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 I.P 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). Anti-PCNA Antibody purchased from the Elabscience (China). Immunohistochemistry kit purchased from the Dako (Envision FLEX, Dako, K8000, Denmark).

Clinical and physiological parameters:

Animal's weight were measured weekly and the clinical observation done daily throughout all experimental periods for all groups.

At the end of experiment half of the animal groups euthanized by exposing to overdose Diethyl ether for examining the Immunohistochemical determination of proliferation (PCNA) Parameters and the other half left for recovery for 14 days and repeating determination the same Parameter

Immunohistochemical determination of proliferation (PCNA)

Cell proliferation in colonic epithelium was examined by the PCNA labeling index (PCNA-LI). The specifics of the experiment were carried out as previously stated [20]. For each stained segment, the Image J software [21]. was used for image analysis to grade each one as the number of positive cells /100 colonic mucosal epithelial cells was counted and was considered to be the proliferation index [22]. To get average positive level of each case, six microscopic fields of 400x magnification were selected which included two representative fields each of considerable, medium and a few positive cells. PCNA-LI is the percentage of immunohistochemical staining positive cells in 1000 Colorectal cells counted. [23]. The immunohistochemistry was performed as per manufacturer's instruction.

Statistical Analysis

Analyzing the DATA is done by SPSS 26 by using a variance analysis (ANOVA) two-way test to determine whether there were significant differences between the groups at ($P \leq 0.05$), and using the less significant difference LSD test to compare the mean values. It is presented as mean ± standard deviation [24]

RESULTS

Clinical observation and physiological study:

During the experiment period, the results of Clinical observation showed a slightly weakness nearly in all treated animal groups, Corner aggregation with less food intake as compared with the same groups at zero time and control negative group. The results of clinical observation at the end of experiment showed in the CRC induced groups especially in C+ve group there were a noticeable appearance of swelling masses in their tail with partial alopecia (fig. 1). with some abnormal clinical symptom like decrease in movement, apathy and incoordination movement as compared with zero time that showed normal behavior and symptoms.

Also, at the end of treatment there were a noticeable grossly change in distal colon as thickened masses noticed in Control Positive and T2 groups when compared with C-ve, T1, T3 and T4 groups. (fig.2).

Figure 1. Clinical observation (A) possible tumor masses and (B) partial alopecia).

Figure 2. Distal colon grossly changes (possible tumor masses)

Body weight change:

The results of current study showed there were a significant decrease ($p \leq 0.05$) in animals body weight at the 5th week after CRC induction by AOM for treated and C+ve groups as compared with the zero time and C-ve group, also there were significant decrease ($p \leq 0.05$) in body weight of groups T1, T2, T3, T4 and C+ve at the treatment weeks of experiment.

While slightly non-significant increase in body weight of T1 group recorded after the second week of recovery in comparison with significant increase in body weight of T3 and T4 groups, while at the same time there were significant decrease in animals body weight in T2 and C+ve groups at the end of experiment. (fig. 3).

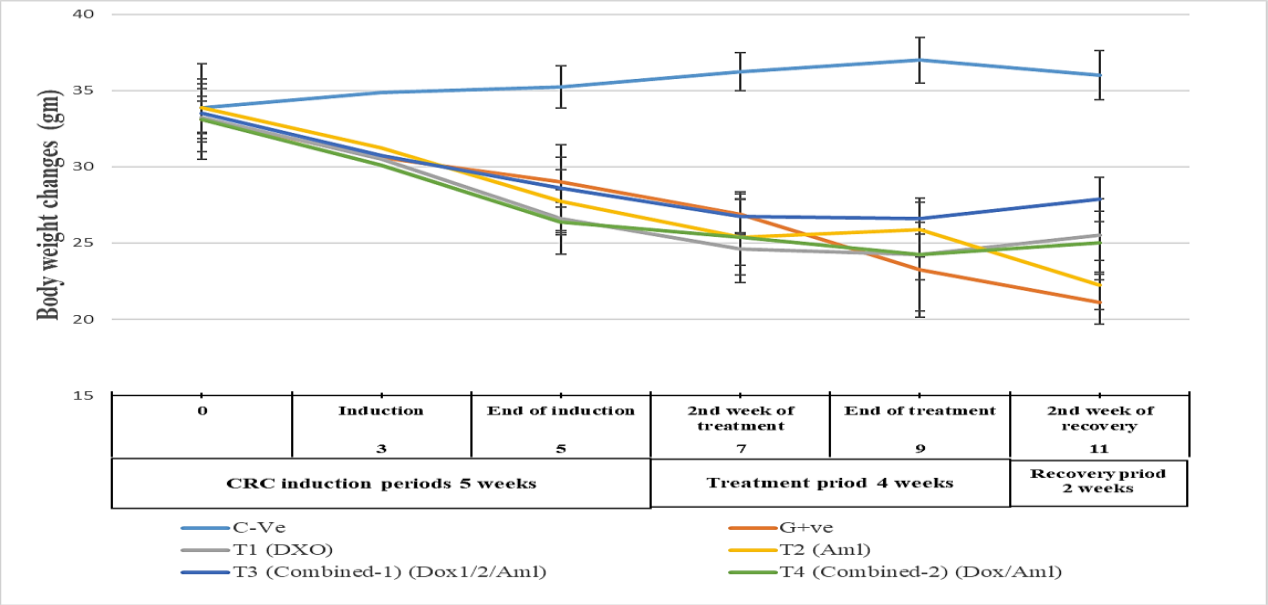

Figure (3) Body weight changes (gm) in mice

Immunohistochemical determination of proliferation (PCNA)

The findings score of Proliferating cell nuclear antigen labeling index (PCNA-LI) showed various percent areas of positive stained tissue and intensity showed in table (2) and figure (4). The treated groups showed a therapeutic effect on experimental AOM-induced CRC in mice within the two periods of experiment except T2 group with comparison to the C+ve group. T3 group treated with combined dose AOM and half dose DOX (2.5mg/kg/wk) and T4 Group treated with combined dose of AOM and therapeutic dose DOX, and T1 Group treated with DOX only exhibited respectively significance decrease($P \leq 0.05$) in comparison with control positive. The T3 recorded more powerful anti-colorectal cancer effect than other treated groups, according to the result of PCNA since it showed significantly the least PCNA- LI level than T4 and other treated groups.

The Photomicrograph of C-ve mice at the both period of experiment showed low expression in PCNA within the crypt epithelial cells of large intestine (fig. 4 C-ve A,B), while C+ve group showed overexpression of PCNA in neoplastic epithelial cells that observed in adenocarcinoma masses within the affected villi (fig. 4 C+ve A,B). The T1 groups Photomicrograph showed PCNA overexpression observed in neoplastic epithelial cells with a small mass of adenocarcinoma separated in affected villi (fig. 4 T1). also, the T2 group showed overexpression of PCNA in neoplastic epithelial cells but recorded formation of massive mass of adenocarcinoma above the muscularis layer, and extended toward the lumen center also, the affected area showed absence of villi (fig. 4 T2). The Combined treatment groups T3 and T4 Photomicrograph showed less overexpression of PCNA from C+ve and other treated groups, observed in epithelial cells with possible small mass of adenoma within Lamina propria of affected villi, while T3 showed lesser epithelial PCNA score than T4. (fig. T3, T4).

Table (4-7): Expression of Proliferating cell nuclear antigen (PCNA) in CRC induced and treated mice group with Doxorubicin and Amlodipine at different experimental periods.

Groups N=4	End of treatment						Two-week recovery					
	Percentage of cells stained %	Quantity score	Intensity Score	Staining Intensity Score	Total score	PCNA-LI M±SD	Percentage of cells stained %	Quantity score	Intensity Score	Staining Intensity Score	Total score	PCNA-LI M±SD
C-Ve	0.395	0	2	moderate	0	1.50 ± 1.00 D a	0.395	0	2	moderate	0	1.50 ± 1.00 D a
	2.27	1	2	moderate	2		1.37	1	2	moderate	2	
	1.29	1	2	moderate	2		1.29	1	2	moderate	2	
	1.206	1	2	moderate	2		2.106	1	2	moderate	2	
G+ve	20.848	2	2	moderate	4	5.00 ± 1.15 A a	20.848	2	2	moderate	4	5.50 ± 1.00 A a
	40.288	2	2	moderate	4		50.288	3	2	moderate	6	
	27.693	2	3	strong	6		27.693	2	3	strong	6	
	14.068	2	3	strong	6		24.068	2	3	strong	6	
T1	14.534	2	2	moderate	4	4.00 ± 0.00 B a	14.534	2	2	moderate	4	4.00 ± 0.00 B a
	22.051	2	2	moderate	4		20.051	2	2	moderate	4	
	14.163	2	2	moderate	4		10.163	2	2	moderate	4	
	16.07	2	2	moderate	4		14.07	2	2	moderate	4	
T2	20.576	2	2	moderate	4	5.00 ± 1.15 A a	21.576	2	3	strong	6	5.50 ± 1.00 A a
	19.015	2	2	moderate	4		24.015	2	2	moderate	4	
	24.494	2	3	strong	6		24.494	2	3	strong	6	
	15.132	2	3	strong	6		25.132	2	3	strong	6	
T3	9.239	1	2	moderate	2	2.50 ± 1.00 C a	9.139	1	2	moderate	2	2.00 ± 1.00 D a
	9.237	1	2	moderate	2		9.227	1	2	moderate	2	
	11.099	2	2	moderate	4		9.099	1	2	moderate	2	
	9.514	1	2	moderate	2		9.314	1	2	moderate	2	
T4	9.913	1	2	moderate	2	3.50 ± 1.91 B a	9.413	1	2	moderate	2	3.00 ± 1.15 C a
	9.811	1	2	moderate	2		9.211	1	2	moderate	2	
	12.983	2	3	strong	6		11.183	2	2	moderate	4	
	10.055	2	2	moderate	4		10.055	2	2	moderate	4	
LSD	1.008											1.008

* PCNA-LI Proliferating cell nuclear antigen labeling index score according to (Yousef, et al., 2014), Capital letters donate significant differences between groups at ($p \leq 0.05$), Small letters donate differences within groups at ($p \leq 0.05$).

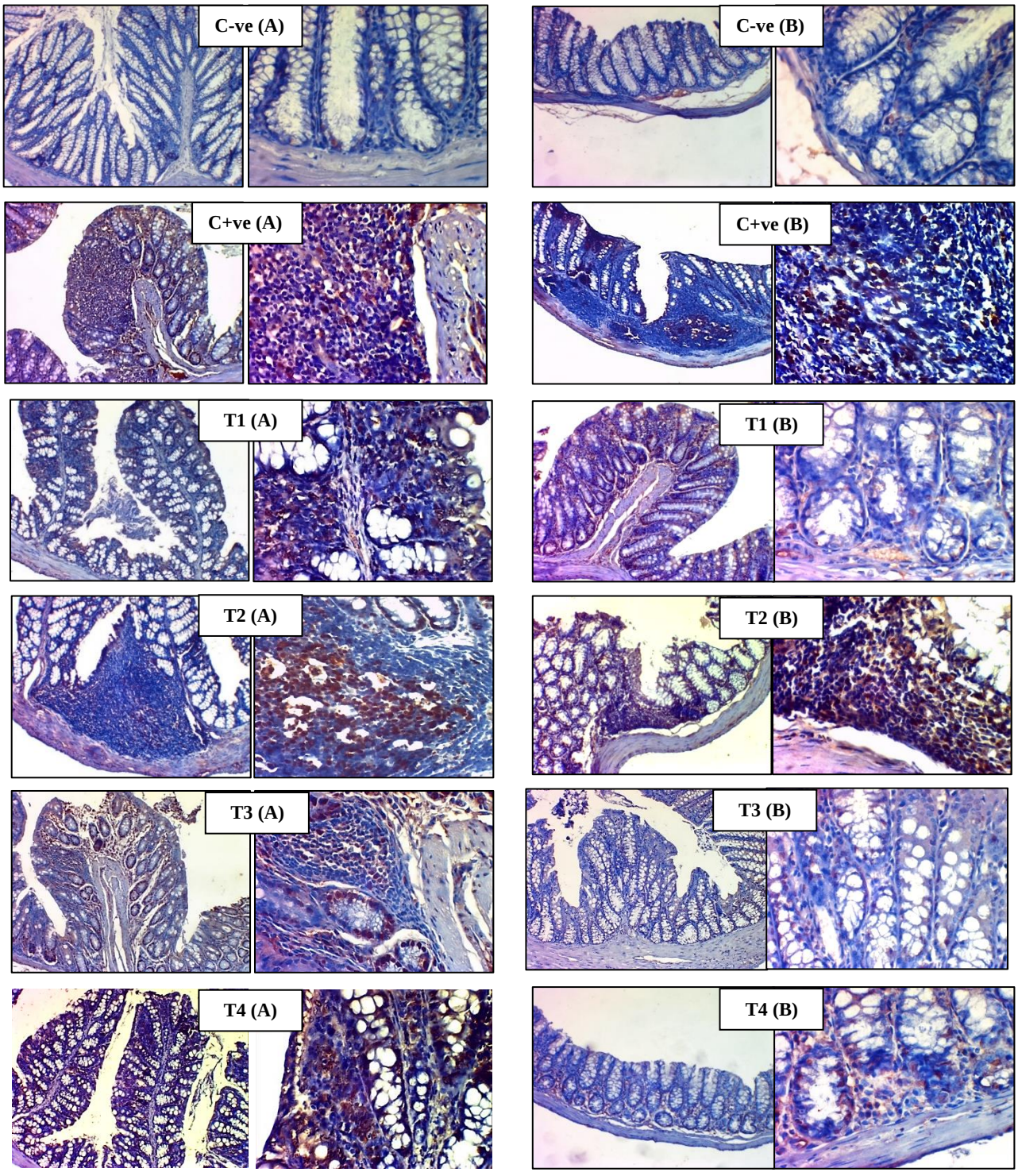

Figure (4): Photomicrograph of PCNA of large intestine of experimental mice groups (A) at End of treatment, (B) at Two-week recovery. IHC: DAB and Mayer hematoxylin. Left X100 and right X400.

DISCUSSION

Multidrug Resistance is a significant impediment to clinical chemotherapy efficacy, particularly in the CRC treatment. Among the various MDR mechanisms, P-gp-mediated MDR plays a significant pathological role [25]. The relatively brief mechanism of P-gp-mediated MDR is association with substrate efflux to the extracellular medium.[26] Notably, most drugs used in CRC chemotherapy, including DOX, are P-gp substrates and thus have lower intracellular accumulation. [27];[28];[29]

We hypothesized that Amlodipine (P-gp inhibitor) beside that it has antioxidative effect [30] that might modulate the efficacy and cytotoxicity of DOX substrate when used for treatment of induced CRC in mice by Azoxymethane.

The significant body weight loss of CRC induced groups and the groups treated with DOX which could be explained by development of cancer or treatment side effects that cause appetite loss attributed to irritation, effect on immune system and metabolic rate[31]. Also Dox. cause Nausea, vomiting, constipation, chewing and swallowing difficulties, and loss of taste, desquamation, and damage of glandular tissue in the mucosa enhance loss of food absorption and metabolism [32]. It was reported by [32], [33] that AOM treated mice group exhibited body weight loss following its administration due to inflammation and induced colon crypt pathology with clinical signs of colitis such as inflammation, and swelling, diarrhea. Although the DOX have contributed to weight loss, caused by colitis, and a slight alteration in the stomach and intestinal epithelium and adjusted glands.[34]; [35].

Result of body weight loss in all treated groups in comparison with the control negative group mice that treated with normal saline refer to the effect of AOM and adverse effects of DOX that cause epithelial cell atypia within intestinal mucosa and other adverse effects reported by histopathological effects [36].

All CRC induced treated groups as well as C+ve showed significant decline in body weight throughout experimental period in comparison with C-ve group the exception were the combined groups T3 and T4 that showed significant increase in body weight at the end of recovery period.

The PCNA immunostaining, was used as sensitive method for evaluating the possible relevance of cell-proliferation that might cause development colorectal neoplasm and give priduction of the cancer risk. In adenomatous polyps the PCNA-LI of epithelial tumors cell is significantly elevated, regardless of histological form or tumor volume. [37].

The result of our experiment recorded significantly increase in PCNA-LI value in all CRC induced groups by AOM and this result in compatible with the results of positive CRC-carcinoma patient that founded to have a positive nuclear IHC-staining PCNA [38]. PCNA is expressed as proof in almost all constantly proliferating and shedding cells including gastrointestinal mucosal cells.

Results showed that Proliferating-cell Nuclear Antigen expression was significantly higher in a progression from normal mucosa to adenoma and then carcinoma. [38].

Several studies support our findings result. Yan-Fang and colleagues found in 2006 [39] that PCNA expression rises in a (“normal mucosa–adenoma-carcinoma”) sequence [38]. In CRC, Expression in PCNA is related to the degree of differentiation and metastasizing into the lymphatic system. Furthermore, overexpression of PCNA was also linked to metastasizes to the lymphatic system, [39]; [38]; [40].

According to [41]; [42], the PCNA-LI increased in tandem with the adenoma's size and dysplasia grade.

Moreover, Colorectal tissue samples from azoxymethane-induced colorectal damage in mice showed an increase in cell proliferation activity with formation of massive mass of adenoma in C+ve and T2 response to azoxymethane's destructive effect, implying early damage or ongoing injury. The azoxymethane-induced mitogenic effect resulted in increased turnover of the epithelial cells lining the crypts with the highest PCNA-LI, likely representing a regenerative phenomenon [43] In addition, mice treated with Doxorubicin after receiving AOM exhibited reduced mitotic activity by recording less overexpression of PCNA from C+ve with possible small mass of adenoma indicating the presence of a cytotoxic effect. By examining histopathological characteristics and

immunohistochemistry PCNA figures We observed a significant inhibition of mitotic activity in CRC treated groups that were treated with Doxorubicin (T1, T3 and T4), in comparison with C+ve that received only AOM and T2 that have been treated with AOM and AML without any anticancer drugs. Analyzing and discussing the PCNA results of all experimental groups, as shown in table (4) as well as in related figures, showed the following events; the presence of various percent of stained area (PCNA-LI) tissue with intensity of staining, which indicates the total score of PCNA-LI. In descending manner, groups T1, T4, and T3 showed a significant decrease of the PCNA-Labeling index in comparison with the C+ve and T2.

The superiority of T3 over T4 group might attributed to the use of half therapeutic dose of DOX in T3 than full dose, the matter that increase the anti-PCNA effect in T3 than T4 effect possibly due to increase of DOX concentration in T3 to the level of the best therapeutic effect possibly due to the inhibition of P-gp efflux by Amlodipine while in T4 there was increase DOX cytotoxic side effect accompanied with its therapeutic effect.

Our findings suggest the modulatory effect of Amlodipine on DOX concentration through the effect of AML as P-glycoprotein (P-gp) inhibitor that possibly effect the DOX potency and adverse effects. [36]

Conclusion

The difference between combined groups and T1 group results were attributed to the reverse MDR by using P-gp inhibitor by AML that decrease the blood concentration of DOX and increase its intracellular concentration, so increase its anti-CRC efficacy especially in combined T3 with DOX half therapeutic dose + AML and T4 that used double dose DOX than T3 group.

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