The Role of Levofloxacin Versus Doxorubicin In Induced Aberrant Crypt Foci In Mice

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Abstruct

This study is conducted for investigation the role of levofloxacin versus doxorubicin in abolishing induced aberrant crypt foci (ACF) in mice . fourty adult Balb/C male mice used in this study, Thirty of them were intentionally induced aberrant crypt foci by azoxymethane and assigned as following . Group one G1 (control positive) without administration of any medicines, group two G2 administered levofloxacin 25 mg/kg. BW orally for four weeks, group three G3 administered doxorubicin 50 mg/m² once a week orally beginning one week after the last dose of AOM and the second dose repeated after 2 weeks ,continued for 4 weeks , the other ten mice neither induced aberrant crypt foci nor treated with any medicines as assigned as control negative , The results showed there were significant $P<0.05$ decrease in ACF of both treated groups(G2 and G3) in comparison with control positive (G1). While proliferating cell nuclear antigen (PCNA) scoring of colorectal tissue revealed significant $P<0.05$ decrease in PCNA labeling index of both group two and group three when compared to control positive.

Key words: levofloxacin , Doxorubicin , Aberrant ,crypt, foci , mice.

I. INTRODUCTION

Cancer is a type of diseases characterized by uncontrolled cell proliferation , There are over hundred different types of cancer , and each is classified by the type of cell initially affected (1).Cancer is often treated with some combination of radio therapy , surgery , chemotherapy and targeted therapy. The chance of survival depends on the type of cancer and extent of disease at the start of treatment(2).

Colorectal cancer (CRC) it is known as bowel cancer, colon cancer or rectal cancer, is any cancer of the colon and the rectum, the World Health Organization says it is the second most common cancer worldwide, Colorectal cancer often begins as a growth called a polyp, which may form on the inner wall of the colon or rectum. Some polyps become cancer over time (3).CRC is a preventable and treatable disease which depend on the type stage of the cancer, the age and health status. The treatment is included: surgery, Radiation and chemotherapy like:(Fluorouracil, Irinotecan , Oxaliplatin and Doxorubicin)(4). Early detection through screening is useful for cervical and colorectal cancer(5), Screening and awareness can reduce mortality of colorectal cancer by detecting and removing polyps before they become cancerous, or by discovering the cancer at an earlier stage, where treatment has a higher success rate(6). Incidence rates have also been increasing in various countries of the world wide (7), In Rodents Spontaneous gastrointestinal neoplasia is rare (8).

Crypt foci is a stem cell found in the intestine gland in the colon, Presently using a characters of reliable marker in the aim at root cause of cancer (9). In the colorectal cancer occur histological changes in crypt foci due to Aberrant crypt foci(ACF) which characterized is putative preneoplastic lesions originally described experimental models and have been proposed as intermediate cancer biomarkers (10).

(AOM) is a compound 1,2-dimethylhydrazine (DMH) and its metabolite, commonly used carcinogens to induce and promote colorectal cancer in rats and mice. DMH and AOM are alkylating agents that are typically injected intraperitoneally or subcutaneously over several weeks to induce development of tumors in the distal colon (11).

Levofloxacin is a broad-spectrum antibiotic of the fluoroquinolone. Its spectrum of activity includes most strains of bacterial Pathogens (12).The newly found that levofloxacin has a role in the treatment cancer when corrupts

the activities of prokaryotic type II topoisomerase, DNA gyrase, and induces them to kill cells by generating high levels of double-stranded DNA breaks (13).

Doxorubicin is a frontline drug (nonselective class-I anthracycline) has been used for treating cancer for over 30 years, and has shown great treatment potential, being regarded as one of the most potent of the Food and Drug Administration-approved chemotherapeutic drugs. The ability to combat rapidly dividing cells and slow disease progression has been widely acknowledged for several decades, Doxorubicin may be 2mg/ml Vial or 10mg ,20mg,50mg Powder injection, duration dosing 60-75mg/m² IV for 21days, 40-60mg/m² IV for 21days ,20mg/m² IV weekly (14).

Levofloxacin become of interest in treatment of cancer. So we are planning our study to investigate It's promise anti cancer role as new drug application.

II. Material AND METHODS

Chemicals and medicines:- AOM obtained from sigma-aldrich (merck) , levofloxacin raw material obtain from Tavipharma(Holland) , doxorubicin obtain from saba (turkey), PCNA kit as from abcam (Germany).

Animals, forty adult healthy male Balb-C mice purchased from Ministry of Science and Technology /Industrial Research Department, their ages were (8-12) weeks and weighing (28-42gm). They housed and bred in optimum condition at 25 ± 2˚, with14/10 hours light /dark cycle and left two weeks before study for acclimatization , water and food supplied add- libtum.

Experimental design, the experiment is conducted under the approval of scientific committee of department of Physiology, Biochemistry and Pharmacology, College of veterinary medicine, University of Baghdad,take in considerations the general ethic stander of animal welfare.

Induction of aberrant crypt foci in mice performed by administration of AOM according method(15), Immunohistochemical determination of proliferation cell nuclear antigen (PCNA) according for(16) and scored from (0-12), low levels of expression represented by scores of (0-4) and high levels of expression score was from (> 4-12) (17).

Experimental studying performed forty 40 Balb/C were selected and divided randomly into four equal groups and assigned as following ,group one G1 (positive control receiving AOM 10mg/kg. BW IP once weekly for 2 weeks), group two G2 (Injected with 10mg/kg. BW IP of AOM once weekly for 2 weeks and treated daily with levofloxacin 25mg/kg.BW orally for 4weeks) , group three G3(Injected with 10mg/kg. BW IP of AOM once weekly for 2 weeks and treated with doxorubacin 50mg/m² (once weekly) administered orally beginning 1 week after the last dose of AOM and repeated second dose after 2 weeks ,continued for 4 weeks) and group four G4 (negative control injected with normal saline for 6 weeks).

Statistical Analysis

Data analyzed by SPSS version 24.00, A one way Analysis of Variance (ANOVA) used for assessment the differences between mean at P-Value 0.05.Least significant difference (LSD) was depended for comparing between means.

III. Results

Aberrant Crypt Foci (ACF) Counting Grossly by Methylene Blue

The result of aberrant crypt foci (ACF) showed, there were significant P< 0.05 decrease in (ACF) of two treated groups, G2 (Azo. & levo.) and G3 (Azo. & Doxo.) in comparison with G1 (Control positive which received Azoxymethane only). Table (1), Figure (1).

Table (1) Aberrant Crypt Foci (ACF) of experimental group

	Groups	**ACF** **Mean ± S.E**
	(G1)+ve Control Azo.	9.66 ± 1.20 A
	(G2) Azo. & levo.	3.66 ± 0.33 B
	(G3) Azo. & Doxo.	1.66 ± 0.33 B
	(G4) -ve Control	0 C

*LSD=2.196

*Different letters denote significant P< 0.05 changes

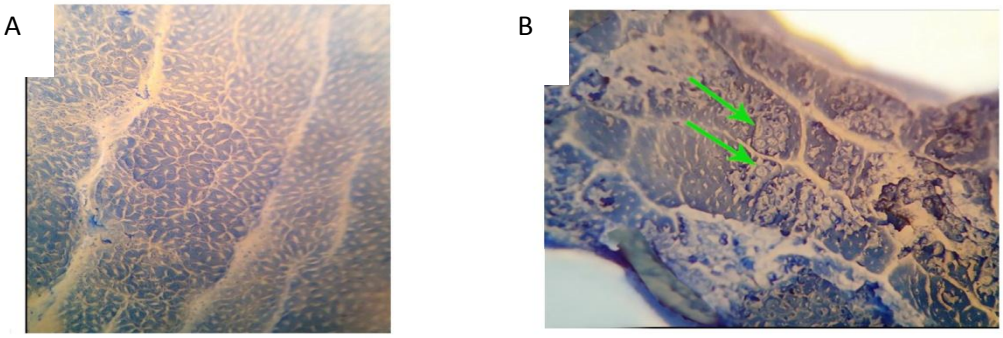


Figure (1) A: A crypt foci in the colon as seen in the figure observation under Inverted microscope x40 .
B: An aberrent crypt foci (ACF) as seen in the figure observation under Inverted microscope x40 .

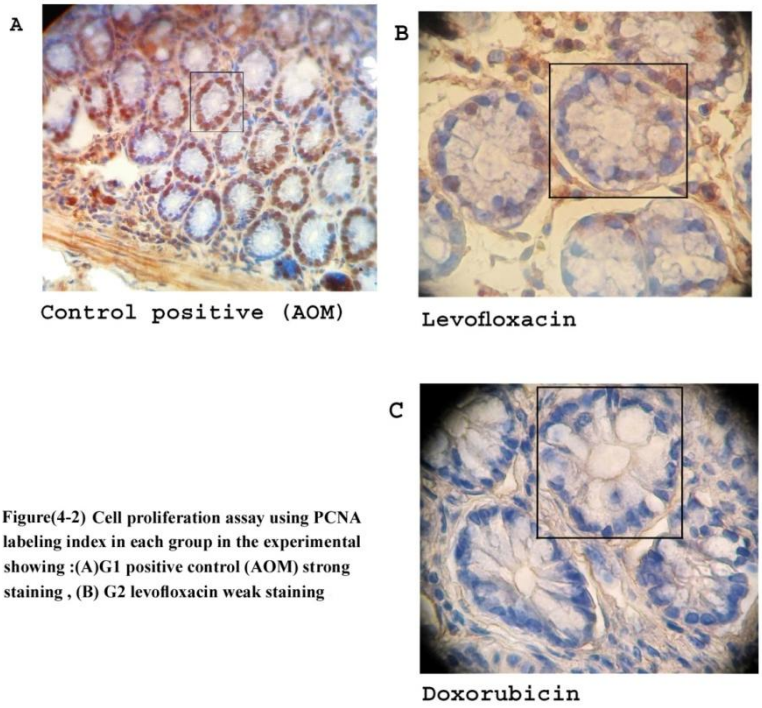
Proliferating cell nuclear antigen (PCNA) Labeling index and intensity staining

The results of PCNA scoring showed, various percent area of stained tissue and intensity of staining table (2) and figure (2). Statistically group G2 (Levofloxacin) and G3 (Doxorubacin) exhibited significant P< 0.05 decrease when compared to the G1(Control Positive) in descending manner. But fall into category of low level of expression according to the scale of scoring.

Table (2) Proliferating cell nuclear antigen (PCNA) of experimental groups

Groups	Percentage of cells stained %	Score	Intensity of stain	Score	Total Score	PCNALI% M±SE
(G1)+ve Control Azo.	80 80 70 75 75	4 4 4 4 4	Strong Strong Strong Strong Strong	3 3 3 3 3	12 12 12 12 12	12 ± 0 A
(G2) Azo. & levo.	90 85 75 75 85	4 4 4 4 4	Weak Weak Weak Weak Weak	1 1 1 1 1	4 4 4 4 4	4.0 ± 0 B
(G3) Azo. & Doxo.	5 5 10 10 10	1 1 1 1 1	Weak Weak Weak Weak No staining	1 1 1 1 0	1 1 1 1 0	0.80 ± 0.20 C

*LSD= 0.98



Figure(4-2) Cell proliferation assay using PCNA labeling index in each group in the experimental showing :(A)G1 positive control (AOM) strong staining , (B) G2 levofloxacin weak staining

IV. DISCUSSION

Formation of ACF has been used by many researchers as a biomarker of the early stage of colorectal carcinogenesis (18) and (19). Besid that ,(20) was evaluated the efficacy of 41 potential chemopreventive agents in CRC using inhibiton of AOM – induced ACF as measure of efficacy . That is why in our study we used this technique to evaluate the efficacy levofloxacin as a new chemopreventive agent in CRC and compare this efficacy with doxorubicin.

This study revealed that Levofloxacin can prevent the early stage of CRC by suppression the number and size of ACF induced by azoxymethane (AOM) in the colon and rectum of mice. In fact, there were significant $P<0.05$ decrease in ACF in two groups received medicines in question when compared to control positive group (AOM). Which also confirmed by histological involved restarted of goblet cells population decrease over all number and decrease number cell proliferation. Song and colleagues (21) found that levofloxacin (dose 50-200µg/ml) for lung cancer treatment that inhibiting proliferation and inducing apoptosis lung cancer cells through inducing mitochondrial dysfunction and oxidative damage this is in agreement with our results where we found that levofloxacin caused significant decrease in (ACF) in comparison with group G1 (control positive received azoxymethane only), levofloxacin is a third generation of fluoroquinilones commonely used to treat various bacterial infections (22),but recently found that levofloxacin used chemotherapy that cause mitochondrial damage which leads to oxidative stress and cell death , It interfere with the DNA replication process of mitochondria , they disrupt tubulin assembly (23) and that there is being investigated for their tumor killing abilities by which mechanism for action as topoisomerase interruptes (topoisomerases are necessary for proper DNA replication) are used as chemotherapy drug. Levofloxacin can kill cancerous tumor cells because in addition to killing bacterial cells, they also kill eukaryotic cells, levofloxacin is disrupted mitochondrial DNA (mtDNA) replication process and the disruption of that procees lead to mitochondrial damage, oxidative stress and cell death, Levofloxacin are effective cell killers(24) levofloxacin has also been shown to selectively target breast cancer cells compared to normal breast cells (25).

Bleiberg (26), considered that the PCNA immunostaining is a simple tool for prospective studies on pathological material aimed at evaluating the potential relevance of proliferative induces to clinical prognosis or prediction of colorectal cancer risk. Georgescu and colleagues (27), also said that the PCNA proliferating cell nuclear antigen is a 36-KDa DNA polymerase delta auxiliary protein that complexes with cyclin D and cyclin dependent kinases. It is involved in the proliferation of neoplastic as well as non- neoplastic cells and it is specifically expressed in proliferating cell nuclei. This specific antibody recognize PCNA protein, which is at the maximum level in the late G1 and S phase of proliferating cells. PCNA immunohistochemistry can be used as a reliable marker of the proliferative compartment in both normal and neoplastic colonic mucosa (28). It had been reported that PCNA-labeling index (PCNA-LI) is increased in the following order: normal mucosa of the large intestine, hyperplastic polyp, tubular adenoma with low- grade atypia , and tubular adenoma with high- grade atypia and adenocarcinoma . PCNA-LI of epithelial tumor cells is significantly increased in adenomas with high grade of dysplasia irrespective of the histological type or size of the tumor (29).Because of its direct relationship with cell proliferation, PCNA is considered to be an important factor in the prognosis of colorectal carcinoma (30). Previous studies showed that there was a significant relationship between PCNA-LI and grade of the tumor, vessel invasion, distant metastasis and prognosis (31) and (32).

Most studies have reported angiogenesis once the invasive carcinoma has been established. However, even in a premalignant stage, epithelial cells have increased proliferation (as a manifestation of the “field effect”) and therefore

would be expected to require increased blood supply. Angiogenesis has previously been shown as early as small adenomatous polyp or even the aberrant crypt foci (ACF) stage (33).

Ban and her colleagues (34) found a positive nuclear immunohistochemical staining for PCNA was all cases of colorectal carcinoma, adenoma, and control group. This can be illustrated by the fact that PCNA is expressed in all proliferating cells keeping in mind that intestinal mucosal cells are in continuous proliferation and shedding .Additionally, PCNA is also involved in the DNA repair and it is known that the PCNA immunostaining may appear in cases where DNA repairs occur. Moreover, unlike Ki-67, PCNA may continue to be expressed in cells that have left the cell cycle. In terms of staining intensity, they also found that carcinoma cases recorded significantly higher frequency of cases with strong intensity of staining followed by adenoma then control groups (54.54%, 33.33%, 15.15% respectively, $P < 0.001$) and when comparing the three digital parameters of Digimizer software (area "A," number of objects "N," and intensity "I"), In normal mucosa, PCNA-labeled cells were observed in the colonic crypts.

There are several studies in accordance with the present study (35) showed that the positive rate of PCNA expression increases in a sequence of normal mucosa–adenoma–carcinoma.(36) demonstrated that positive expression of PCNA is significantly increased during transformation from colorectal adenoma to carcinoma.(37) had measured the PCNA-LI by visual inspection and the PCNA area rate (PCNA-AR), determined with the newly developed image processor for analytical pathology (IPAP), in tissue samples obtained by endoscopy and concluded that the PCNA-AR and PCNA-LI increased significantly in the following order: normal mucosa of the large intestine, hyperplastic polyp, tubular adenoma with low-grade dysplasia, and tubular adenoma with high-grade dysplasia and adenocarcinoma(38). (39) and (40) reported that PCNA-LI was increased in proportion to the degree of dysplasia and size of the adenoma.(41)demonstrated that the mean PCNA-LI was significantly correlated with high-grade dysplasia irrespective of histologic type or size of adenoma, in accordance with the present data except for size of adenoma.(42) showed that carcinoma cells had a significantly higher PCNA index than adenomas or control specimens and PCNA was overexpressed in the villous, moderate, or severe dysplastic, and larger adenomas in keeping with this study. In addition, the transitional mucosa neighboring carcinoma showed an elevation of the mean PCNA index.

Debora and colleagues (43) found in study Immunohistochemical evaluation of e-cadherin, Ki-67 and PCNA in canine mammary neoplasias: correlation of prognostic factors and clinical outcome that The expression of these markers was related to the clinical-pathological characteristics of 73 surgically removed mammary tumors in female dogs by immunohistochemistry. There was no statistical correlation between these markers and death by neoplasm, survival time and disease-free interval. However, the loss of e-cadherin expression and marked Ki-67 expression were considered statistically significant for the diagnosis. When evaluated as independent factors, there was evidence of the relationship between the loss of e-cadherin expression and high PCNA expression with changes in the body status (divided into obese, normal and cachectic) of female dogs; there was also evidence of the relationship between pseudopregnancy and e-cadherin alone and for ulceration and PCNA alone. The significant correlation between the markers expression and these well known prognostic factors used individually or in combination suggests their prognostic value in canine mammary tumors and shows that the use of these antibodies combined to others which may establish a panel of markers, might contribute for a more accurate tumor prognosis, leaving aside a complex and inconclusive classification as a single alternative in the pursuit for a better and longer survival of cancer patients. The immunohistochemical diagnosis of tumors by markers combined with clinical-pathological parameters allow a better assessment of the prognosis, leading to a better clinical outcome of the patient.

Kyoung and colleagues (44) observed despite significant advancements in osteosarcoma research, the overall survival of canine and human osteosarcoma patients has remained essentially static over the past 2 decades. Post-operative limb-spare infection has been associated with improved survival in both species, yet a mechanism for improved survival has not been clearly established. Given that the majority of canine osteosarcoma patients experiencing post-operative infections were treated with fluoroquinolone antibiotics, the thought that fluoroquinolone antibiotics might directly inhibit the survival and proliferation of canine osteosarcoma cells. Ciprofloxacin or enrofloxacin were found to inhibit p21WAF1 expression resulting in decreased proliferation and increased S-G2/M accumulation. Furthermore, fluoroquinolone exposure induced apoptosis of canine osteosarcoma cells as demonstrated by cleavage of caspase-3 and PARP, and activation of caspase-3/7. That results support further studies examining the potential impact of quinolones on survival and proliferation of osteosarcoma. These finding is supporting the efficiency of levofloxacin in prevention progressing of precancerous tissue (ACF) in mice of groupG2.

In conclusion, PCNA plays an important role in colorectal neoplastic progression and can be utilized as ancillary marker for the risk of malignant transformation in colorectal adenomas being highly correlated with high-grade dysplasia and increasing size. So regarding to our investigations, levofloxacin has a potential role to counteract the colorectal preneoplastic tissue in mice.

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